GENETICALLY DETERMINED EPILEPSIES: PERSPECTIVES IN THE ERA OF PRECISION MEDICINE

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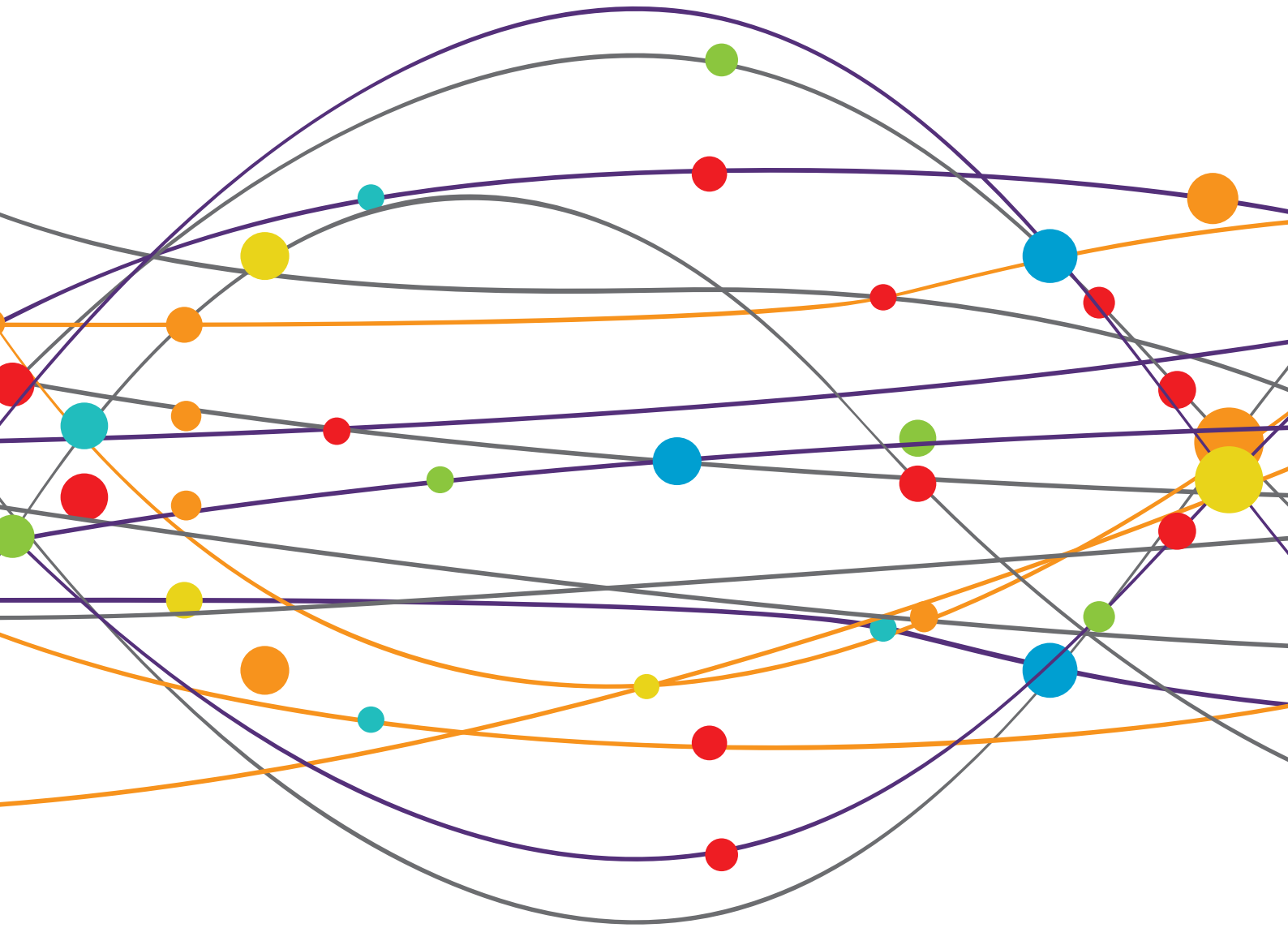

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GENETICALLY DETERMINED EPILEPSIES: PERSPECTIVES IN THE ERA OF PRECISION MEDICINE

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Editorial: Genetically determined epilepsies: Perspectives in the era of precision medicine

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Editorial on the Research Topic
Genetically determined epilepsies: Perspectives in the era of precision medicine

The complex landscape of genetic epilepsies was largely expanded in the last decade. To date, a specific genetic etiology can be identified for up to 40% of severe early onset drug-resistant developmental and epileptic encephalopathies, but also for milder seizure phenotypes with no significant comorbidities (1). Around 7,000 single genes and more than 400 different chromosomal imbalances with a presumed pathogenic role were identified and reported in the literature (1–3). More than 210 genes were directly implicated in peculiar epilepsy phenotypes, even though eight genes accounted for most of cases in a recent epidemiological survey in United Kingdom (1–3). These numbers mirror a huge progress since the first association between *CHRNA4* and nocturnal frontal lobe epilepsy in 1995 (4).

The aim of this Research Topic is to offer a real-world panorama on the field of genetic epileptology. Several experts, from different countries all over the world, provided new updated data and interesting analysis of the correlated clinical and therapeutic perspectives (https://www.frontiersin.org/research-topics/19762).

The ease of obtaining next generation sequencing (NGS) in clinical practice has resulted in significant improvement in the diagnostic yield with a remarkable increase of etiological diagnosis. This led to a reduction of diagnostic timing and costs, while also allowing for more precise genetic counseling and an ongoing discovery of new disease-causing genes with novel genotype-phenotypes associations (5). The possibility of concurrently analyzing a wider group of disease-causing genes and the faster gene-sequencing raises other clinical questions that must be considered including (but not

limited to): (i) the availability of a large amount of data that often complicate genotype-phenotype correlations; (ii) the frequent detection of variants of uncertain significance; (iii) the frequent need for functional studies to assess the real pathogenic effect of the detected variants; (iv) a limited epidemiological impact (most of the known disease-causing genes associated with epilepsy accounts for a limited quote of cases).

As more genetic etiologies are going to be discovered, this requires parallel changes in the clinical approach to patients with epilepsy as careful phenotyping is critical for the correct interpretation of molecular genetic data. Further practical implications include heterogeneity with regard to different clinical phenotypes resulting from variants of the same genes (e.g., *SCN2A* causes both familial benign neonatal infantile epilepsy and a severe epileptic encephalopathy; *KCNQ2* was initially associated with familial benign neonatal seizures and, subsequently, with an early onset epileptic encephalopathy) or similar clinical syndromes caused by different genes (e.g., Dravet syndrome can be caused by pathogenic variants in *SCN1A*, *PCDH19*, *STXBP1* or *GABRA1*). The additional role of several genetic modifiers in defining increased susceptibility to epileptogenic processes added complexity to the polygenic etiological basis of different epilepsy phenotypes (5).

The impact of a molecular genetic diagnosis on treatment choices has resulted in different possible approaches (e.g., avoiding, stopping, or initiating specific antiseizure medications or ketogenic diet; addressing or avoiding surgery) in 12 and 80% of the cases significant (6). Unlike other neurological diseases, gene therapy for genetic epilepsies still has a number of obstacles to overcome (e.g., excessive size of some epilepsy genes, such as *SCN1A*, for common viral vectors; immunogenicity of CRISPR-Cas-9 system; difficulties to control gene dosing) and the ambitious target to alter the natural history and improve upon other non-seizure related comorbidities (7).

The advances in making a genetic diagnosis are likely to lead to changes in how were approached patient diagnoses and management such as:

a) a gradual shift from classical syndromic classifications of epilepsies to a nosology based on molecular genetic etiology (e.g., *SCN2A*-encephalopathy, KCNQ2 encephalopathy etc.);
b) increasing knowledge regarding the natural history and longer-term outcomes of monogenic and copy-number variation-related epilepsy disorders by collecting more data on adult patients (both in terms of prolonged follow-up since infancy and as novel diagnosis in adult neurology settings).
c) an increasing number of treatments tailored to the molecular genetic defects (e.g., sodium channel blockers for *SCN2A* and *SCN8A* encephalopathy; ezogabine for KCNQ2/3 defects; quinidine for *KCNT1* variants; everolimus for m-TORopathies; ketogenic diet for GLUT-1 deficiency; etc.).

The understanding of these advances is pivotal to more defined phenotyping and timely diagnosis (e.g., next generation sequencing as gold standard for early genetic diagnosis, links between international clinical reference networks, online databases or *ad-hoc* registries) and increasing collaboration between basic scientists and clinical researchers (e.g., early availability of functional studies and advanced tools for designing oriented therapeutic trials) to improve upon our treatment approaches and outcomes that extends beyond treating just seizures and rather treats the entire genetic epilepsy condition.

Author contributions

MM projected the Research Topic, coordinated the process, and wrote the first draft of the editorial. VS and JS coordinated the revision process of the papers and revised the draft of the editorial. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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Cortical and Subcortical Network Dysfunction in a Female Patient With *NEXMIF* Encephalopathy

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The developmental and epileptic encephalopathies (DEE) are the most severe group of epilepsies. Recently, *NEXMIF* mutations have been shown to cause a DEE in females, characterized by myoclonic–atonic epilepsy and recurrent nonconvulsive status. Here we used advanced neuroimaging techniques in a patient with a novel *NEXMIF de novo* mutation presenting with recurrent absence status with eyelid myoclonia, to reveal brain structural and functional changes that can bring the clinical phenotype to alteration within specific brain networks. Indeed, the alterations found in the patient involved the visual pericalcarine cortex and the middle frontal gyrus, regions that have been demonstrated to be a core feature in epilepsy phenotypes with visual sensitivity and eyelid myoclonia with absences.

Keywords: *NEXMIF*, non convulsive status epilepticus, developmental and epileptic encephalopathy, epilepsy, eyelid myoclonia with absences, fMRI

INTRODUCTION

The developmental and epileptic encephalopathies (DEEs) are the most severe group of epilepsies in which frequent epileptic activity, in addition to the underlying etiology, contributes to developmental impairment, with onset typically in infancy or childhood (1). At least 50% of the DEEs have a genetic cause (2), and there is significant etiological overlap with other neurodevelopmental disorders such as intellectual disability (ID) and autism spectrum disorder (3).

The Neurite EXtension and MIgration Factor gene (*NEXMIF*), previously called *KIAA2022* (OMIM 300524), is an X-linked gene thought to play an important role in early brain development (4–7). Pathogenic *NEXMIF* variants were first identified in males with non-syndromic X-linked ID with poor or absent speech, subtle dysmorphic features, and sometimes epilepsy (8, 9). Subsequently, affected females have been described (10–14) and recently a large multicentric study outlined the epilepsy phenotype of affected females (15) which is consistent with a generalized DEE characterized by myoclonic–atonic epilepsy overlapping with eyelid myoclonia with absence. Notably a considerable proportion of affected females present prolonged seizures characterized by

absence status with eyelid myoclonia (15, 16). We report a female patient with a *de novo NEXMIF* pathogenic variant and recurrent prolonged episodes of absence status with eyelid myoclonia. In order to evaluate the consequences of *NEXMIF* mutation at brain structural and functional MRI level (17) two different studies were carried out comparing the patient with respect to patients with genetic generalized epilepsy (GGE; formerly idiopathic) and healthy controls (HC).

PATIENT AND METHODS

The patient is a 28-year-old woman, with a drug-resistant epilepsy starting at the age of 9 years with recurrent episodes of prolonged non-convulsive status epilepticus (NCSE) characterized by mydriasis, eyelid myoclonia and reduced responsiveness to environmental stimuli with a frequency of 1–2 episodes/month. She was born pre-term (8th gestational month) from a dichorionic diamniotic twin pregnancy, from unrelated parents. Family history is unremarkable for epilepsy, febrile seizures, and any other neurological condition. Her developmental milestones were slightly delayed (she started walking at 16 months and talking at 24 months). She afterwards achieved borderline intellectual functioning (full-scale IQ 75 at the age of 24 years; WAIS) with difficulties in visuo-spatial information processing and dyspraxia. The patient also presented night terrors and enuresis until late adolescence. Her neurological examination is unremarkable.

During NCSE, which may last up to 48 h, the patient first complains of an epigastric discomfort, followed by headache and subsequent clouding of consciousness when she becomes progressively more unaware and detached from the environment. During this phase, the patient has mydriasis and presents subcontinuous eye-blinking. EEGs recorded during NCSE show a continuous, diffuse, spike- and poly-spike and wave-discharge, worsened by eye closure, hyperventilation and by intermittent photic stimulation (**Supplementary Figure 1**). Characteristically eye closure induces the paroxysmal discharges while these tend to reduce or disappear when the patient is asked to open her eyes/stare or to perform a mental task. Beyond these episodes the patient's EEG background activity appears normal, with only occasional diffuse sharp waves, mainly elicited by eye closure.

Various anti-seizure medications (ASM) in numerous combinations were tried without achieving substantial benefit. She was first started on valproic acid, with clonazepam, and then switched to topiramate because of lack of efficacy. Lamotrigine, lacosamide, zonisamide, acetazolamide and ethosuximide were ineffective or even detrimental. A vagal nerve stimulator was implanted at the age of 25 years. At the time of this report, the patient is on valproate (1,000 mg), lamotrigine (150 mg) and brivaracetam (150 mg) but continues to have recurrent NCSE even if more rarely than in the past.

Extensive metabolic and endocrinological screening showed no significant abnormalities except for secondary amenorrhea. Liver and renal functions are normal, and no alterations were found on abdominal ultrasound and echocardiography. Brain structural MRI shows no abnormality.

Genetic Testing

A NGS exome sequencing was performed on genomic DNA from the patient and her parents by using the Nextera Rapid Capture Exomes kit and massively parallel sequencing (Illumina, PE 2 × 150). Sequence mapping and variant calling were performed using GATK software. Variants with certain or probable pathogenic significance based on ACMG guidelines were validated by Sanger sequencing.

The analysis revealed the presence of the chrX-73962221-C- (GRCh37/hg19 assembly) variant in the *NEXMIF* gene (c.2171delG, NM_001008537, p.S724MfsTer5) in the heterozygous state. This variant is not reported in the international registry of mutations published in the literature (ClinVar) and is not present in the human polymorphism databases (GnomAD, dbSNP147). The segregation analysis showed the variant was *de novo*. The variant is predicted to have pathogenic consequences according to ACMG criteria. Additionally, at the protein level, the p.S574MfsTer5 variant affects an evolutionarily highly conserved amino acid according to *in-silico* tool GERP++ score 5.73. The variant is predicted to cause a non-sense mediated decay and a premature truncation with effect on protein function according to *in-silico* tools (Sift score 0.858, Provean score −5.16 and Mutation taster).

Structural MRI Study

For comparison, 20 matched females with GGE and 20 matched healthy females (HC) were recruited. The mean age of the GGE group was 22.74 years old; that of the HC group was 28.45 ($p > 0.05$). All GGE patients had normal structural brain MRI on conventional diagnostic protocol at 3 Tesla and no intellectual disability (full-scale $IQ > 80$) or psychiatric comorbidities. Since the patient was under treatment with valproate at the time of the study, and valproate has been demonstrated to be associated with subcortical atrophy and posterior cortical thinning (18, 19), we included in the GGE control group only females (selected from our MRI research database) that were already on treatment with valproate at the time of the MR imaging study. The HC had no history of neurological diseases or past valproate use, or family history of epilepsy, and had normal structural neuroimaging. Moreover, all controls had a normal EEG, since they were recruited for previous EEG-fMRI co-registration study protocols by our group. The details of MRI acquisition and post-processing analyses are reported in **Supplementary Material**.

MRI Cortical Thickness and Subcortical Volume Analyses

Scans were analyzed using a standardized image toolbox (Freesurfer, version 5.0) (20), quality assurance (outlier detection based on inter quartile of 1.5 standard deviations along with visual inspection of segmentations), and statistical methods. Visual inspections of subcortical and cortical segmentations were conducted following standardized ENIGMA protocols (http://enigma.usc.edu), used in prior genetic studies of brain structure (21, 22), large-scale case-control studies of epilepsy (23) and neuropsychiatric illnesses (24, 25).

Statistical analyses were performed using SPSS software 26.0 (IBM, Chicago, IL). To compare cortical measures

between the proband and each group, we conducted the Crawford's modified independent sample *t*-test using the program singlims.exe (https://homepages.abdn.ac.uk/j.crawford/pages/dept): this tests whether a patient's score is significantly below controls, thus providing a point estimate of the abnormality of the patient's score (i.e., it estimates the percentage of the control population exhibiting a lower score), accompanying confidence limits on this quantity, and results with point and interval estimates of effect sizes (26). Percentile calculation for each variable was performed to inspect case's distribution values in comparison to HC and GGE.

TABLE 1 | Significant differences in subcortical volumes and cortical thickness in the patient compared to GGE sample.

	GGE			Significance test		Estimated percentage of the GGE population obtaining a lower score than the case			Estimated effect size		
	Mean	Standard deviation	Proband	*t*	*p*	Point	95% CI		Point	95% CI	
Subcortical structures volumes (mm^3)											
R thalamus	6479.69	704.78	5236.7	−1.721	**0.05**	5.07	0.68	14.77	−1.764	−2.463	−1.046
Cortical thickness (mm)											
L caudal middle frontal	2.50	0.22	1.938	−2.528	**0.02**	1.02	0.02	4.88	−2.564	−3.51	−1.656
L lingual	2.02	0.13	1.77	−1.952	**0.05**	3.29	0.28	11.06	−2	−2.759	−1.223
R caudal middle frontal	2.47	0.24	1.847	−2.673	**0.01**	0.75	0.01	3.89	−2.739	−3.7	−1.763
R pars triangularis	2.58	0.26	1.992	−2.215	**0.03**	1.96	0.09	7.75	−2.269	−3.1	−1.421
R precentral	2.31	0.15	1.926	−2.537	**0.02**	1	0.02	4.82	−2.6	−3.522	−1.662
R superior temporal	2.89	0.20	2.431	−2.196	**0.04**	2.03	0.1	7.96	−2.25	−3.076	−1.407

p-values are written in bold.

TABLE 2 | Significant difference in subcortical volumes and cortical thickness in proband compared to healthy controls.

	Healthy controls sample			Significance test		Estimated percentage of the HC population obtaining a lower score than the case			Estimated effect size		
	Mean	Standard deviation	Proband	*t*	*p*	Point	95% CI		Point	95% CI	
Subcortical structures volumes (mm^3)											
R thalamus	6785.84	624.26	5,237	−2.423	**0.02**	1.27	0.03	5.74	-2.482	−3.372	−1.577
L caudal	3624.45	448.02	2,614	−2.2	**0.04**	2.01	0.1	7.91	-2.254	−3.081	−1.411
R amygdala	2028.33	216.60	1,545	−2.187	**0.04**	2.07	0.1	8.06	-2.241	−3.064	−1.401
Cortical thickness (mm)											
L caudal middle frontal	2.50	0.17	1.938	−3.11	**0.005**	0.28	0	1.85	-3.187	−4.277	−2.084
L fusiform	2.71	0.13	2.398	−2.327	**0.03**	1.55	0.05	6.6	-2.385	−3.247	−1.506
L inferior parietal	2.36	0.13	1.923	−3.228	**0.004**	0.22	0	1.5	-3.308	−4.432	−2.169
L lateral occipital	2.22	0.15	1.849	−2.649	**0.01**	0.79	0.01	4.05	-2.714	−3.668	−1.745
L lingual	2.02	0.11	1.77	−2.342	**0.03**	1.51	0.05	6.46	-2.4	−3.266	−1.517
L middle temporal	2.88	0.17	2.397	−2.813	**0.01**	0.55	0	3.1	-2.882	−3.884	−1.866
L pars opercularis	2.47	0.17	2.104	−2.067	**0.05**	2.63	0.18	9.5	-2.118	−2.908	−1.31
L postcentral	2.01	0.13	1.697	−2.402	**0.02**	1.33	0.04	5.91	-2.462	−3.345	−1.562
L superior temporal	2.90	0.20	2.391	−2.62	**0.01**	0.84	0.01	4.24	-2.684	−3.63	−1.723
L supramarginal	2.46	0.16	2.116	−2.135	**0.04**	2.3	0.13	8.66	-2.188	−2.996	−1.361
R caudal middle frontal	2.52	0.18	1.847	−3.904	**<0.001**	0.04	0	0.39	-4	−5.329	−2.659
R lateral occipital	2.18	0.11	1.92	−2.307	**0.03**	1.62	0.06	6.8	-2.36	−3.22	−1.491
R pars triangularis	2.49	0.19	1.992	−2.711	**0.01**	0.69	0.008	3.66	-2.778	−3.75	−1.792
R peri calcarine	1.64	0.12	1.356	−2.484	**0.02**	1.12	0.02	5.23	-2.545	−3.452	−1.623
R precentral	2.39	0.15	1.926	−3.058	**0.006**	0.32	0.001	2.041	-3.133	−4.207	−2.045
R superior temporal	2.99	0.16	2.431	−3.416	**0.002**	0.14	0	1.05	-3.5	−4.681	−2.306

p-values are written in bold.

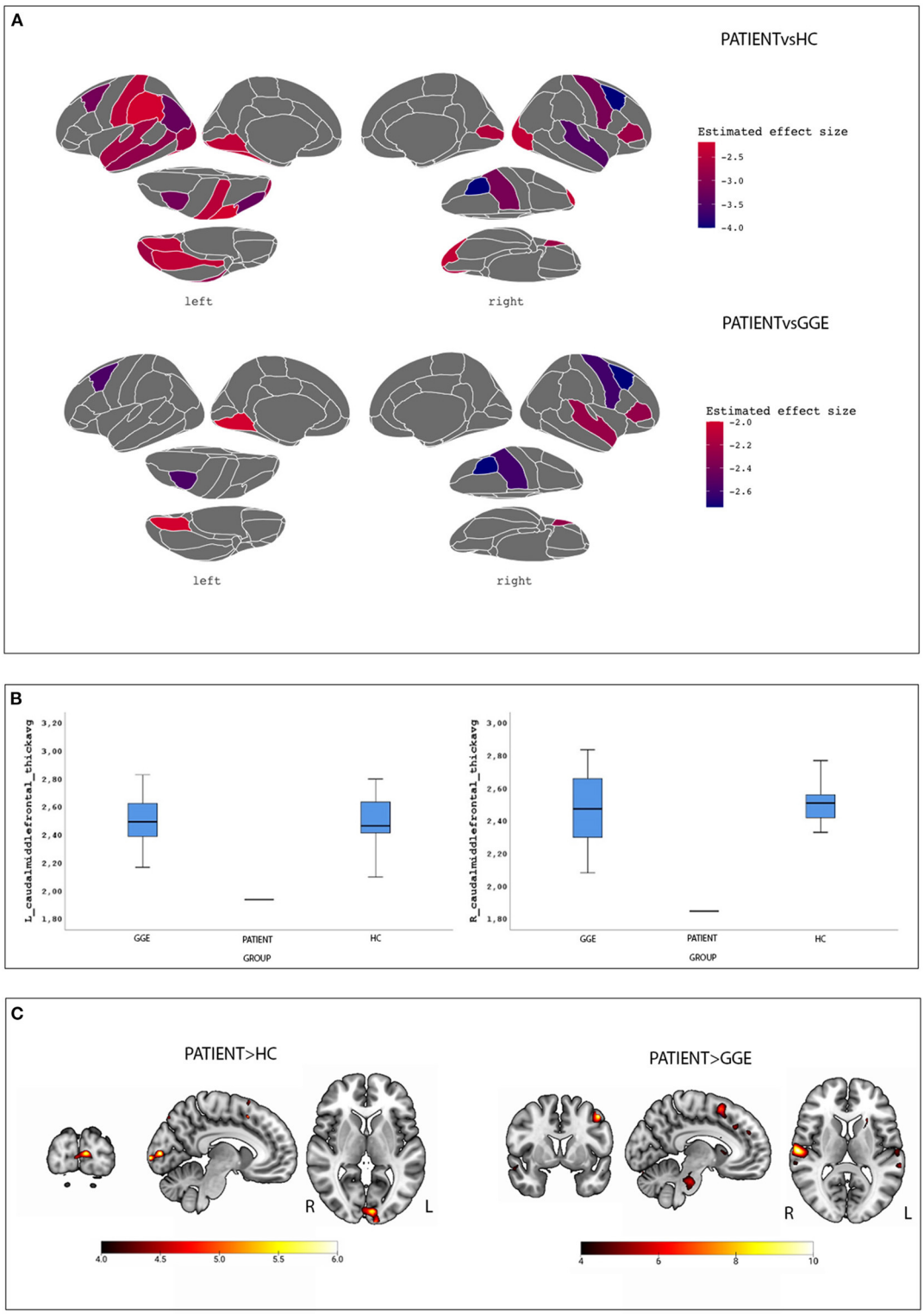

FIGURE 1 | Morphometric and Functional results. **(A)** surface brain templates depicting regions of cortical thinning in the proband compared to HC (top images) and GGE (bottom images). Brain statistics were displayed using the ggseg and ggseg3d packages integrated into the software R environment using the Desikan-Killiany cortical atlas (28). **(B)** Percentile distribution of cortical thickness for the left and caudal middle frontal gyri in the case compared to controls and GGE; see text for details. **(C)** BOLD maps related to the eye closure conditions in the patient compared to controls (left images) and GGE (right images). Results are overlaid into the MNI152 template as provided by the MRIcroGL toolbox.

Functional MRI Study

Patients (the proband and GGE) and controls were investigated by means of a task-related EEG-fMRI protocol in order to elucidate brain activity related to eye-closure condition. In this second study the GGE population consisted of 14 patients (13 females, mean age = 24.9 years, mean age of epilepsy onset= 12.6 years). The healthy control group consisted of 16 subjects (12 females, mean age = 28 years). The experimental protocol and EEG-fMRI data pre-processing and analysis have been extensively described previously by our group (see **Supplementary Materials**) (27).

RESULTS

Cortical Thickness and Subcortical Volumes

Subcortical structural comparison between the proband and HC group showed volume reduction in the right thalamus ($p = 0.02$), right amygdala ($p = 0.04$), and left caudate ($p = 0.04$). Cortical thickness analyses showed that the patient had reduced cortical thickness in several brain regions in comparison to HC, including left ($p = 0.005$) and right caudal middle frontal gyrus ($p < 0.001$), left fusiform ($p = 0.03$) and left inferior parietal gyrus ($p = 0.004$), left ($p = 0.01$), and right ($p = 0.03$) lateral occipital gyrus, and left lingual gyrus ($p = 0.03$; **Table 1**).

Subcortical structural comparison between proband and GGE group showed that the case had volume reduction in the right thalamus ($p = 0.05$). Cortical thickness analyses showed that the patient had reduced cortical thickness in several brain regions in comparison to GGE, including left ($p = 0.02$) and right caudal middle frontal gyrus ($p = 0.01$), and the left lingual gyrus ($p = 0.05$; **Table 2**).

Figure 1A shows surface brain template depicting regions of cortical thinning in the proband compared to HC and GGE. Percentile distribution confirmed that in the patient, right and left caudal middle frontal gyri cortical thickness had values below IQR and extreme values in comparison to both HC and GGE (**Figure 1B**). **Supplementary Table 1** show percentile distribution for the patient in comparison to all HC and GGE.

Brain Correlates of Eye-Closure

A total of 13 voluntary eyes closure conditions were recorded. Compared to HC, the patient demonstrated increased BOLD signal changes at the left cuneus. When compared to GGE, a diffuse network appeared encompassing the left precentral gyrus, the left basal ganglia, the bilateral superior temporal gyrus, the right inferior frontal gyrus and the pons (**Figure 1C**; **Supplementary Table 2**). No significant BOLD changes were observed in the opposite comparisons (i.e., HC and GGE vs. the proband).

DISCUSSION

NEXMIF plays an important role in neural circuit formation during development (4–8). Knockdown of *NEXMIF* leads to dramatic impairment in neurite outgrowth, with a particular impact on the lengths of dendrites and axons (9). To our knowledge, this is the first study to attempt to identify whether pathogenic *NEXMIF* variants induce alterations in brain structure or in functional networks in humans. In summary, compared to other populations, the studied patient shows a thinning of the prefrontal cortex and in particular of the middle frontal gyrus, of the temporal lobe cortex (including the fusiform gyrus) and of pericalcarine visual cortex. Consistently, these areas have shown functional alterations (increase of BOLD signal compared to controls) in the condition of eye closure: this finding is of interest because this pattern of functional activation was previously documented in patients with a clinical phenotype characterized by eyelid myoclonia and absences (27). In fact, the patient, although not having the clinical phenotype typical of Jeavons syndrome, demonstrated prolonged NCSE episodes characterized by absences with eyelid myoclonia.

Here the use of advanced neuroimaging techniques in a specific genetic phenotype revealed brain structural and functional changes that can bring the clinical phenotype to alteration within specific brain networks, and especially in networks physiologically involved in several visuomotor function, including the motor control of eye-closure and eye-movements, and attention to visual targets. Notably, it is not possible to determine whether the observed morphometric alterations are ascribable to the dysfunction of the NEXMIF gene primarily, or what role has the repetition of prolonged NCSE on these regions. Indeed, this study needs replication in both males and females carrying pathogenic gene mutations in NEXMIF gene to come to the conclusion that the observed network alterations are gene specific, or mutation mediated effects, or a feature of the association of epileptic seizure phenotype of eyelid myoclonia with absences. To note, it is unlikely that the observed structural/functional MRI changes are result of single gene defect but may be the consequence of effects mediated by more than one gene involved in the development of visuomotor networks. Of course, the results obtained should be considered with caution and reflect patient-specific brain changes. That said, the study has identified consistent alterations in cortical/subcortical morphometry and functional imaging thus providing a link between the genetic alteration and *in vivo* brain functioning/morphology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Dryad [https://doi.org/10.5061/dryad.kwh70rz49].

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Provinciale, Azienda Ospedaliero-Universitaria di Modena (study no. 80/10 and 268/15). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s) for the publication

of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MC, AC, AV, GG, and SM contributed to the conception of the subject of the manuscript. MC, AC, and SM searched the patient files, interpreted literature, and wrote the manuscript. AC, MI, FZ, SK, and SS contributed to the genetic analysis and genetic interpretation of data. MT, AV, and SM performed the fMRI data analysis. AC, GG, SS, and SM revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.722664/full#supplementary-material

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ORIGINAL RESEARCH
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CNNM2-Related Disorders: Phenotype and Its Severity Were Associated With the Mode of Inheritance

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CNNM2 (Cystathionine-β-synthase-pair Domain Divalent Metal Cation Transport Mediator 2) pathogenic variants have been reported to cause hypomagnesemia, epilepsy, and intellectual disability/developmental delay (ID/DD). We identified two new cases with *CNNM2* novel *de novo* pathogenic variants, c.814T>C and c.976G>C. They both presented with infantile-onset epilepsy with DD and hypomagnesemia refractory to magnesium supplementation. To date, 21 cases with *CNNM2*-related disorders have been reported. We combined all 23 cases to analyze the features of *CNNM2*-related disorders. The phenotypes can be classified into three types: type 1, autosomal dominant (AD) inherited simple hypomagnesemia; type 2, AD inherited hypomagnesemia with epilepsy and ID/DD; and type 3, autosomal recessive (AR) inherited hypomagnesemia with epilepsy and ID/DD. All five type 1 cases had no epilepsy or ID/DD; they all had hypomagnesemia, and three of them presented with symptoms secondary to hypomagnesemia. Fifteen type 2 patients could have ID/DD and seizures, which can be controlled with antiseizure medications (ASMs); their variations clustered in the DUF21 domain of CNNM2. All three type 3 patients had seizures from 1 to 6 days after birth; the seizures were refractory, and 1/3 had status epilepticus; ID/DD in these AR-inherited cases was more severe than that of AD-inherited cases; they all had abnormalities of brain magnetic resonance imaging (MRI). Except for one patient whose serum magnesium was the lower limit of normal, others had definite hypomagnesemia. Hypomagnesemia could be improved after magnesium supplement but could not return to the normal level. Variations in the CBS2 domain may be related to lower serum magnesium. However, there was no significant difference in the level of serum magnesium among the patients with three different types of *CNNM2*-related disorders. The severity of different phenotypes was therefore not explained by decreased serum magnesium. We expanded the spectrum of *CNNM2* variants and classified the phenotypes of *CNNM2*-related disorders into three types. We found that DUF21 domain variations were most associated with *CNNM2*-related central nervous system phenotypes, whereas hypomagnesemia was more pronounced in patients with CBS2 domain variations, and AR-inherited *CNNM2*-related disorders had the most severe phenotype. These results provide important clues for further functional studies of *CNNM2* and provide basic foundations for more accurate genetic counseling.

Keywords: *CNNM2*, epilepsy, intellectual disability/developmental delay, hypomagnesemia, DUF21

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INTRODUCTION

Magnesium is an important cation in the human body. It is involved in the function of a variety of enzymes in cells and associated with the excitability of nerve cells and muscles. Magnesium transporter-associated genes include *MMGT1*, *MAGT1*, *NIPAL1*, *NIPAL4*, *MRS2*, *CNNM2*, and *CNNM4*. *CNNM2*, previously known as *ACDP2* (Ancient Conserved Domain Protein 2), has been demonstrated to be related to magnesium homeostasis in humans (1). In 2011, Stuiver et al. reported that *CNNM2* pathogenic variants could cause hereditary hypomagnesemia (2). Subsequently, epilepsy and intellectual disability/developmental delay (ID/DD) had been added to the spectrum of phenotypes of *CNNM2* variants (3). Up to now, a total of five articles have reported 21 cases of *CNNM2*-related disorders (2–6). But the relationship between genotypes and phenotypes of *CNNM2*-related disorders remains unknown. In this study, we reported two new Chinese cases of hypomagnesemia with epilepsy and DD caused by novel identified *de novo* heterozygous variants. We also investigated the relationship between the phenotypes and the variant sites/mode of inheritance in all 23 cases related to *CNNM2* mutations including two cases we found and 21 cases previously reported.

MATERIALS AND METHODS

Participants

Two Chinese cases with infantile-onset epilepsy and global DD had been identified to have novel *CNNM2* pathogenic variants. These two cases and previously reported 21 *CNNM2* mutation-related cases were collected for all available data, including clinical manifestations, electroencephalogram (EEG), brain MRI, serum electrolytes, gene variants information, and mode of inheritance.

Variation Analysis

Written informed consent was obtained from the parents of both patients. This study was approved by the institutional review boards of Peking University First Hospital. We collected 5 ml of peripheral venous blood from patients and their parents. Genomic DNA was extracted for trio-whole exome sequencing (WES). The pathogenicity of the variants was predicted by SIFT, PolyPhen-2, CADD, and MutationTaster. Variants were evaluated against the overall population in 1000 Genomes, gnomAD, and ExAC databases. The pathogenicity was classified according to variant classification standards based on the American College of Medical Genetics and Genomics (ACMG) guidelines, 2015.

RESULTS

Clinical Features

Case 1

Case 1 was a 2-year-old boy who carried a novel identified *de novo* missense heterozygous variant c.814T>C [p.Phe272Leu]. He still could not raise his head steadily at 6 months of age. His seizures started at 10 months after birth, mainly characterized by eyes gazing to the left side and perioral cyanosis, sometimes accompanied by jaw tremor, right lower limb raising, and hand tremor. EEG showed focal frontal epileptic discharge. Although ictal EEG of this patient was not available, the electrical-clinical features indicate that his seizures were focal seizures. The seizures were clustered, up to eight attacks per day, only 2–3 days per month. He had been seizure-free from 12 months of age by levetiracetam monotherapy. He had DD. When he was 1 year old, he could sit and crawl without others' help. He could walk without support from 1 year 3 months of age, but he still could not walk steadily now. He began to vocalize at 1 year old, but he could not make the sound of "babamama" till now. He had no apparent malformations. Brain MRI was normal. His initial serum magnesium was 0.56 mmol/L. After his seizures were completely controlled, he was diagnosed with causative gene and began to receive magnesium supplement at 1 year old. But diarrhea occurred after oral administration of magnesium, which was improved after suspension of magnesium supplementation. It was considered that diarrhea was related to gastrointestinal side effects of oral magnesium, so the treatment with magnesium supplementation was interrupted, and his parents did not monitor serum magnesium level for him regularly. During magnesium supplementation treatment, his highest serum magnesium was 0.62 mmol/L and he had no seizures, but no improvement in psychomotor development was observed.

Case 2

Case 2 was a 4-year-old girl who carried a novel identified *de novo* missense heterozygous variant c.976G>C [p.Val326Leu]. Her seizures started at 8 months after birth and always occurred during sleep. Seizures involved eye opening and fearful expression, eye and head turning left, and then tonic and clonic jerks of the four limbs. Seizures were accompanied by perioral cyanosis. Ictal EEG showed the seizure onset from the right hemisphere (**Figure 1**). She was treated with sodium valproate at first, and the frequency of seizures decreased. Levetiracetam and lamotrigine adjunctive therapy had not shown any effect. Treatment with oxcarbazepine was discontinued due to allergic rash. Lacosamide was started 1 month before the last follow-up. Now she is treated with sodium valproate and lacosamide, and no seizure was seen in the recent 1 month. Gesell's developmental scale revealed severe DD. When she was 4 years old, she could recognize animal pictures, but she could not speak their names. Now she still cannot say anything but "baba" and "mama". Physical examination revealed no apparent malformations. Brain MRI was normal. Her initial serum magnesium was 0.44 mmol/L. She tolerates oral magnesium supplementation at a dose of 0.1–0.2 mmol/kg/day, and her serum magnesium elevated to 0.52 mmol/L. But her serum magnesium could not return to the normal level.

Whole Exome Sequencing Results

Both cases were identified *de novo* heterozygous variants in *CNNM2* by trio-WES, which were c.814T>C [p.Phe272Leu] and c.976G>C [p.Val326Leu]. We used multiple *in silico* tools to analyze these variants' conservation, pathogenicity,

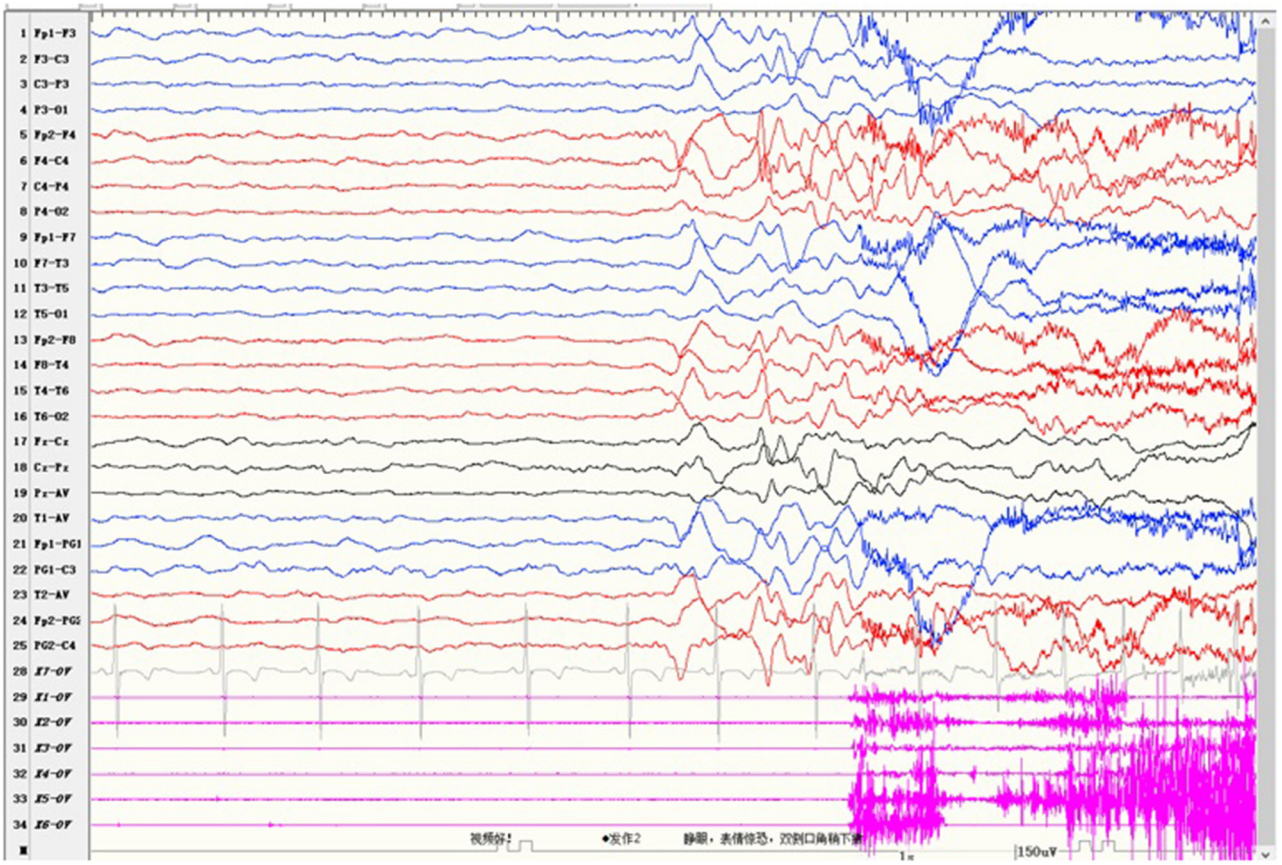

FIGURE 1 | Ictal electroencephalogram of case 2 showed the seizure onset from the right hemisphere.

TABLE 1 | Analysis of pathogenicity of the variants in *CNNM2*.

Case	Gene	Variant	Variant origin	MAF			PolyPhen-2	SIFT	Mutation Taster	CADD	Evidence	Category
				1000 Genomes	ExAC	gnomAD						
1	*CNNM2*	c.814T>C [p.Phe272Leu]	*De novo*	NE	NE	NE	0.81	0.13	DC	25.3	PS2+PM2+PP3	LP
2	*CNNM2*	c.976G>C [p.Val326Leu]	*De novo*	NE	NE	NE	0.18	0.01	DC	24.2	PS2+PM2+PP3	LP

Transcript: NM_017649. NE, not existing; DC, disease causing; LP, likely pathogenic.

and minor allele frequency (MAF). Finally, according to the ACMG guidelines, both variants were clarified as likely pathogenic (LP) (**Table 1**). Other pathogenic variants associated with hypomagnesemia, epilepsy, and ID/DD were not found by trio-WES.

Phenotype and Genotype Relation Analysis

We used "CNNM2" as the keyword to search on PubMed. Excluding variants of unknown significance or non-pathogenic, there are totally 23 cases with CNNM2-related disorders, including the two cases we reported here (2–6) (**Table 2**). The features of these cases can be classified into three types: type 1, autosomal dominant (AD)-inherited simple hypomagnesemia; type 2, AD-inherited hypomagnesemia with epilepsy and ID/DD; and type 3, autosomal recessive (AR)-inherited hypomagnesemia with epilepsy and ID/DD. Type 1, AD-inherited simple hypomagnesemia, was the first phenotype to be discovered. The onset of hypomagnesemia was insidious. The age at which hypomagnesemia was found varied from 1 to 16 years. Two of five cases of type 1 were asymptomatic, and their hypomagnesemia was detected in serum electrolyte tests; the other cases had atypical clinical symptoms associated with hypomagnesemia, including muscle spasms, headache, fatigue, and vertigo. Type 1 patients had no epilepsy or ID/DD. Their initial serum

TABLE 2 | The variants and clinical phenotypes of patients with *CNNM2*-related disorders.

Case	Variant	Amino acid changes	Homo/ het	Mode of inheritance	Gender	Epilepsy				ID/DD	Hypomagnesemia			Physical development			Others
						With/ without	Onset age	Refractory seizures	Effective ASM		Initial serum Mg (mmol/L)	Serum Mg after supplementation (mmol/L)	Symptoms	Microcephaly	Structural abnormalities of brain	Malnutrition	
1 (2)	c.117delG	p.Ile40SerfsX15	Het	AD	M	–				–	0.46	NA	Muscle spasms, headache	–	–	–	
2 (2)	c.117delG	p.Ile40SerfsX15	Het	AD	F	–				–	0.51	0.64	Muscle spasms, stuttering LOC	–	–	–	
3 (2)	c.1703C>T	p.Thr568Ile	Het	AD	F	–				–	0.52	NA	-	–	–	–	
4 (2)	c.1703C>T	p.Thr568Ile	Het	AD	M	–				–	0.36	0.61	Weakness, vertigo, headache	–	–	–	
5 (3)	c.364G>A	p.Glu122Lys	Homo	AR	M	+	1 day	+	VPA, LTG	Severe	0.5	0.66	–	+	Myelination defects, opercularization defect, widened outer cerebrospinal liquor spaces, calcification of basal ganglia	–	
6 (3)	c.364G>A	p.Glu122Lys	Homo	AR	F	+	6 days	+	VPA, LEV	Severe	0.5	0.54	–	+	Calcification of basal ganglia	–	
7 (3)	c.1069G>A	p.Glu357Lys	Het	AD	F	+	7 months	–	PB	Moderate	0.56	0.56	–	NA	–	–	
8 (3)	c.806C>G	p.Ser269Trp	Het	AD	F	+	1 year	–	VPA	Moderate	0.44	0.53	–	NA	–	–	
9 (3)	c.1069G>A	p.Glu357Lys	Het	AD	M	+	4 months	–	CLB	Moderate	0.5	0.68	–	NA	–	–	
10 (3)	c.988C>T	p.Leu330Phe	Het	AD	F	+	16 years	–	Unknown	Mild	0.66	Unknown	–	NA	–	–	
11 (4)	c.1642G>A	p.Val548Met	Homo	AR	M	+	1 day	+	TPM	Severe	0.38	0.49	-	NA	Cerebral cortical atrophy, global reduction of white matter	Normal at birth; height, weight and head circumference all less than P3 at 15 years	Hypotonia, swallowing difficulties, recurrent aspiration pneumonias; bilateral optic disc pallor; abnormal bone metabolism; facial abnormalities
12 (5)	c.2384C>A	p.Ser795*	Het	AD	M	–				–	0.575	NA	–	NA	NA	NA	
13 (7)	g.(?_104678237)_(104816721_?)del		Het	AD	F	+	NA	NA	NA	+	0.63	0.65	NA		–	NA	

(Continued)

TABLE 2 | Continued

Case	Variant	Amino acid changes	Homo/ het	Mode of inheritance	Gender	Epilepsy				ID/DD	Hypomagnesemia			Physical development			Others
						With/ without	Onset age	Refractory seizures	Effective ASM		Initial serum Mg (mmol/L)	Serum Mg after supplementation (mmol/L)	Symptoms	Microcephaly	Structural abnormalities of brain	Malnutrition	
14 (7)	g.104814162_ 104814164 del		Het	AD	M	+	NA	NA	NA	+	0.57	NA	NA	NA	–	NA	
15 (7)	c.143T>C	p.Leu48Pro	Het	AD	M	+	NA	NA	NA	–	0.45	0.53–0.66	NA	NA	–	NA	
16 (7)	c.942C>G	p.Tyr314*	Het	AD	M	–				+	0.48	NA	NA	NA	–	NA	
17 (7)	c.961_963del	p.Leu321del	Het	AD	M	+	NA	NA	NA	+	0.5	0.51	NA	NA	–	NA	
18 (7)	c.970G>A	p.Val324Met	Het	AD	M	+	NA	NA	NA	+	0.54	0.52	NA	NA	–	NA	
19 (7)	c.1253T>C	p.Leu418Pro	Het	AD	F	+	NA	NA	NA	+	0.49	0.58	NA	NA	–	NA	
20 (7)	c.2384C>T	p.Ser795Leu	Het	AD	F	–				+	0.72	0.7	NA	NA	–	NA	
21 (7)	c.2389C>T	p.Arg797*	Het	AD	F	+	NA	NA	NA	+	0.57	0.69	NA	NA	Corona radiata and centrum semiovale white matter T2 slightly hyperintense	NA	
22	c.814T>C	p.Phe272Leu	Het	AD	M	+	6 months	–	LEV	+	0.56	/	–	–	–	–	
23	c.976G>C	p.Val326Leu	Het	AD	F	+	8 months	Unknown	VPA, LCM?	Severe	0.44	0.52	–	–	–	–	

Case 1 is the father of case 2. Case 3 is the mother of case 4. Case 5 and case 6 were the two siblings of same suspected consanguineous parents. Case 11 was the sibling of consanguineous parents. Case 12 had two family members carrying the same variant, both with insidious hypomagnesemia. Case 15 had six family members carrying the same variant, they all presented with hypomagnesemia, but only three of them had defects in motor skills or speech, and none of them had seizures. ID/DD, intellectual disability/developmental delay; ASM, antiseizure medication; AD, autosomal dominant; AR, autosomal recessive; VPA, valproic acid; LEV, levetiracetam; LOC, locus of control; LTG, lamotrigine; PB, phenobarbital; CLB, clobazam; TPM, topiramate; LCM, lacosamide.

magnesium was 0.36–0.575 mmol/L; it was reported that the serum magnesium could be increased to 0.61–0.64 mmol/L in two cases by magnesium supplementation but could not return to the normal level. Type 2, AD-inherited hypomagnesemia with epilepsy and ID/DD, is the most common phenotype in cases with *CNNM2* variant (15/23). Most of the cases are with seizures at onset. In type 2 cases, 86.7% (13/15) had epileptic seizures, and 93.3% (14/15) had ID/DD in the course of the disease. Based on limited available details, the age at seizure onset was 4 months to 1 year in most cases, but 16 years of age in one case. Their seizures showed good response to antiseizure medications (ASMs); multiple kinds of ASMs had been reported to be effective, including phenobarbital, valproic acid, clobazam, levetiracetam, and lacosamide. All type 2 patients had ID/DD, characterized by language expression dysfunction. Physical development was normal in type 2 cases. Physical examination revealed no apparent malformations. Brain MRI was normal except for corona radiata and centrum semiovale white matter T2 slightly hyperintense in one case. Of the cases, 93.3% had definite hypomagnesemia; their initial serum magnesium was 0.44–0.66 mmol/L. However, borderline hypomagnesemia was found in one case whose initial serum magnesium was 0.72 mmol/L. Hypomagnesemia could be improved after magnesium supplement but could not return to the normal level. Type 3, AR-inherited hypomagnesemia with epilepsy and ID/DD, was relatively rare. Only three cases had been reported. But this is the most severe type of *CNNM2*-related disorders. Their seizures onset from 1 to 6 days after birth, much earlier than that of type 2. They could have myoclonic seizures, generalized tonic–clonic seizures, and status epilepticus. In type 3 cases, multiple ASMs were used, but seizures were refractory; valproic acid, levetiracetam, lamotrigine, and topiramate may decrease the frequency of seizures. All type 3 cases had severe ID/DD, and language development was most severely delayed. They all had brain MRI abnormalities, including dysmyelination and progressive cerebral cortical atrophy. The patient who carried c.1642G>A homozygous variant had more severe clinical manifestations, including swallowing difficulties, recurrent aspiration pneumonias, bilateral optic disc pallor, bone metabolism disorder, and facial abnormalities, which were not seen in the two patients who carried c.364G>A homozygous variants. In contrast, hypomagnesemia was no more severe in this severe type than in the other two types. Initial serum magnesium was 0.38–0.5 mmol/L. Serum magnesium could be increased to 0.49–0.66 mmol/L after magnesium supplement but could not be corrected normal. **Table 3** demonstrates the features of these three types of *CNNM2*-related disorders. We used one-way ANOVA to compare the initial serum magnesium in patients with three types of *CNNM2*-related disorders, and no significant difference was found (**Figure 2**). And we used the Mann–Whitney test to compare the initial serum magnesium in patients with epilepsy and ID/DD (type 2 and type 3) and without epilepsy and ID/DD (type 1); no significant difference was found either (**Figure 3**).

CNNM2 is located on chromosome 10q24.32. CNNM2 protein is a transmembrane protein on the cell membrane and is composed of 875 amino acids. CNNM2 contains one DUF21 domain, two CBS domains, and one CNBH (Cyclic Nucleotide-Binding Homology) domain (8). Variants caused *CNNM2*-related disorders type 1 were frameshift variant,

TABLE 3 | Three types of *CNNM2*-related disorders and their features.

Type	1	2	3
Phenotype	AD-inherited simple hypomagnesemia	AD-inherited hypomagnesemia with epilepsy and ID/DD	AR-inherited hypomagnesemia with epilepsy and ID/DD
Number of cases	5/23	15/23	3/23
Age of onset	1–16 years	5/6 4 months−1 year, 1/6 16 years	1–6 days
Epilepsy	-	13/15 Focal seizures, easy to control with ASMs	3/3 Multiple forms, refractory seizures, may have status epilepticus
Psychomotor development	Normal	14/15 Mild to severe ID/DD, language expression inability	3/3 Severe ID/DD, nonverbal
Apparent malformations	–	–	May have facial abnormalities
Brain MRI abnormalities	–	–	+
Hypomagnesemia	+	+	+
Initial serum magnesium (mmol/L)	0.36–0.575	0.44–0.72	0.38–0.5
Others			Swallowing difficulties, recurrent aspiration pneumonias; bilateral optic disc pallor; abnormal bone metabolism
Mode of inheritance	AD	AD	AR

AD, autosomal dominant; ID/DD, intellectual disability/developmental delay; AR, autosomal recessive; ASM, antiseizure medication.

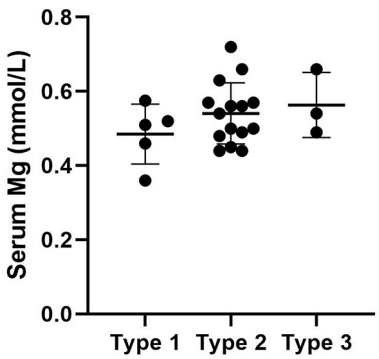

FIGURE 2 | Initial serum magnesium in patients with three types of *CNNM2*-related disorders. No significant difference was found. $p = 0.3496$.

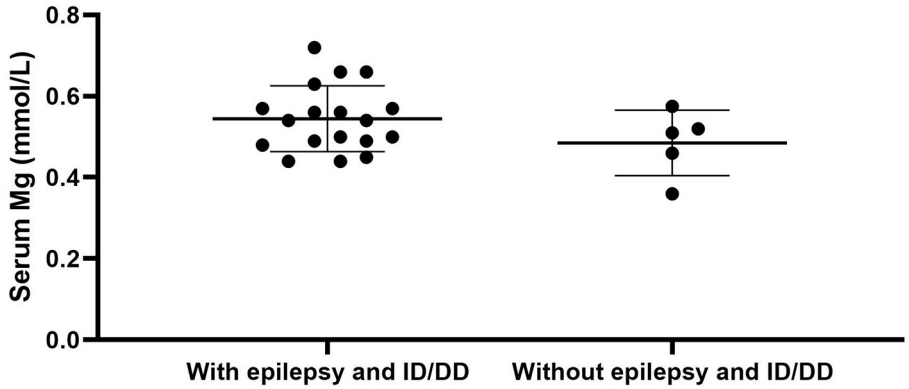

FIGURE 3 | Initial serum magnesium in patients with epilepsy and ID/DD (type 2 and type 3) and without epilepsy and ID/DD (type 1). No significant difference was found. $p = 0.3905$. ID/DD, intellectual disability/developmental delay.

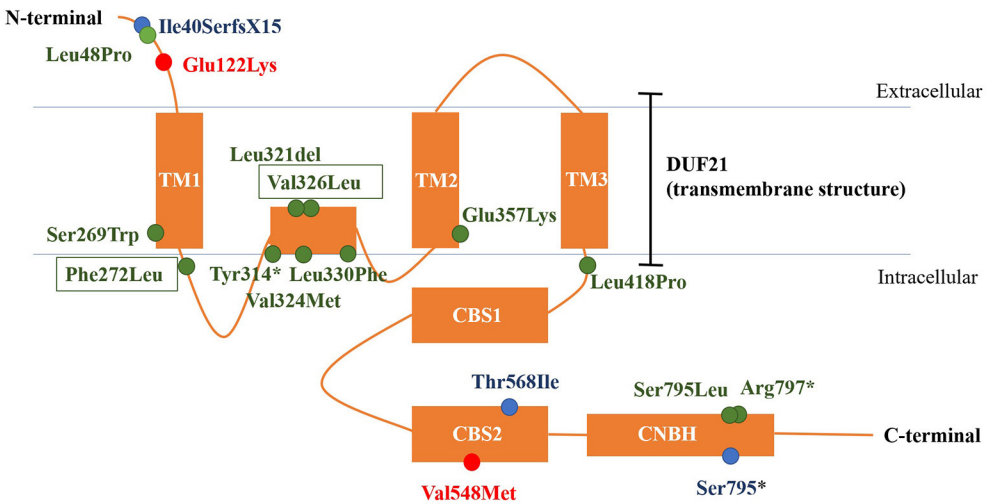

FIGURE 4 | The structure of CNNM2 and the sites of variations. Blue, AD-inherited simple hypomagnesemia; red, AR-inherited hypomagnesemia with epilepsy and ID/DD; green: AD-inherited hypomagnesemia with epilepsy and ID/DD; black box, our cases. AD, autosomal dominant; AR, autosomal recessive; ID/DD, intellectual disability/developmental delay.

missense variant, and stop-gain variant. The changed amino acids were located on various domains and include extracellular segment near the N-terminal, the second CBS domain, and the CNBH domain, but were not within the DUF21 domain (**Figure 4**). Variants caused *CNNM2*-related disorders type 2 included missense variants, stop-gain variants, deletion variant, and two deletion copy number variants (CNVs). Of the variant site, 75% is located in or immediately adjacent to the DUF21 domain. All variants that caused *CNNM2*-related disorders type 3 were missense variants. One variant was located on the extracellular segment near the N-terminal, and another was located on the second CBS domain, whereas the latter variants cause a more severe phenotype. It was noteworthy that all variants located in DUF21 domain caused type 2 of *CNNM2*-related disorders. Among all 23 cases, patients with variants on CBS domain had lower serum magnesium, but the difference was not significant (**Figure 5**).

DISCUSSION

Magnesium plays an important role in the human body. It is the fourth most abundant cation and is a constituent of a variety of enzymes in the body that are involved in various important metabolic processes including DNA synthesis, protein synthesis, and oxidative phosphorylation (9). Besides, magnesium ions (Mg^{2+}) have an inhibitory effect on the excitability of nerve cells in the central nervous system, skeletal muscle, and myocardium. Normal serum magnesium is 0.7–1.1 mmol/L. Neuromuscular irritability, tremor, hypokalemia, and hypocalcemia present when serum magnesium is less than 0.7 mmol/L. If serum magnesium is less than 0.4 mmol/L, hypomagnesemia can cause more severe clinical symptoms, including tetany, nystagmus, seizures, mental disorders, and arrhythmias (10). *CNNM2* has been demonstrated to play a role in magnesium homeostasis in humans (1).

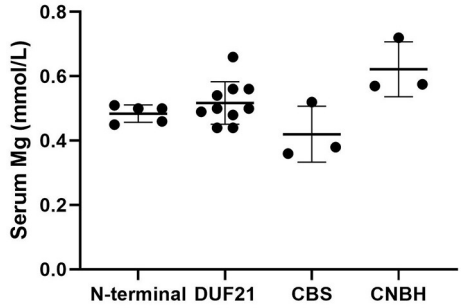

FIGURE 5 | Initial serum magnesium of patients with variants of different domains. We used one-way ANOVA to compare the difference. $p = 0.20$.

We summarized and clarified phenotypes of *CNNM2*-related disorders into three types: AD-inherited simple hypomagnesemia; AD-inherited hypomagnesemia with epilepsy and ID/DD; and AR-inherited hypomagnesemia with epilepsy and ID/DD. Type 1, AD-inherited simple hypomagnesemia, has an insidious onset without epilepsy and ID/DD. Some type 1 cases had symptoms associated with hypomagnesemia, including muscle spasms, headache, fatigue, and vertigo; however, the others were asymptomatic. Type 2, AD-inherited hypomagnesemia with epilepsy and ID/DD, mostly presented with ASM-effective seizures at onset at the age of 4 months to 1 year, and ID/DD is characterized by language expression inability. Type 3, AR-inherited hypomagnesemia with epilepsy and ID/DD, presented with more severe phenotypes. Multiple forms of seizures present at 1–6 days after birth, including status epilepticus. The seizures were refractory. ID/DD of type 3 is more severe than type 2. Patients with type 3 could have brain and facial abnormalities. Therefore, *CNNM2*-related disorders should be considered if a patient presented with any one of the three phenotypes.

All patients who carried pathogenic variants in *CNNM2* had hypomagnesemia. Some of them were asymptomatic. However, serum magnesium could not be corrected to the normal level by magnesium supplement. There was no significant difference in serum magnesium levels between different types of cases. Hypomagnesemia could not be correlated to seizures directly in these cases, because the seizures could not be controlled by magnesium supplement. Differences in the severity could not be explained by differences in the degree of decreased serum magnesium. The phenotype of AR-inherited hypomagnesemia with epilepsy and ID/DD was more severe than that of AD-inherited, which suggests that the causative variant of *CNNM2* may be loss of function (LOF). Some pathogenic missense variants have been proved as LOF variants (6). However, this conclusion still needs further functional studies to be confirmed. In AD-inherited *CNNM2*-related disorders, the basis of abnormal protein function caused by pathogenic variants may be haploinsufficiency.

CNNM2 is highly expressed in the brain and kidney, while it is widely expressed in various organs including the digestive tract, cardiovascular system, lungs, endocrine glands, and blood cells (11). *CNNM2* variants may lead to serum magnesium reduction by attenuating the reabsorption of magnesium at the distal convoluted tubule in the kidney (2). However, the exact function of CNNM2 protein and its mechanism are still unknown. CNNM2 contains one DUF21 domain, two CBS domains, and one CNBH domain (1). The DUF21 domain contains three transmembrane structures and one intramembrane structure, and the specific function is currently unknown. However, this domain is present in all proteins of the CNNM family and is highly conserved from prokaryotic to eukaryotic cells, suggesting that it may have an important biological function. The two CBS domains (also known as Bateman modules) are demonstrated to be closely related to the function of the protein (12). They may dimerize by binding with Mg^{2+}-ATP and alter the conformation of the CNNM2 protein (8). Inactivation of the CNBH domain can cause loss of CNNM2 function (13); however, the function of this domain is unknown. It is thought that its dimerization can assist the CBS domain to function. The mechanisms by which CNNM2 regulates magnesium homeostasis are still under discussion.

Most investigators suggest that CNNM2 itself is a transporter for Mg^{2+} (8). Immunohistochemical staining of human kidney sections confirmed that *CNNM2* is expressed in the distal convoluted tubule, which is the last site of Mg^{2+} reabsorption (2). Low magnesium caused high expression of *Cnnm2* on the lateral side of the basement membrane of the distal convoluted tubule in the rat kidney, suggesting that CNNM2 may be related to the transport of magnesium from within renal tubular epithelial cells into capillaries. Thereafter, in HEK293 cell line-based experiments, it was found that the influx of sodium ions was decreased in cells transfected with the *CNNM2* p.Thr568Ile mutation (a mutation causing hereditary hypomagnesemia) compared with wild type. Therefore, it is thought that CNNM2 protein is involved in the reverse transport of magnesium and sodium ions in the distal convoluted tubule of the kidney, transporting magnesium ions from within renal tubular epithelial cells to capillaries (14). Besides, Tremblay's (15) and Miki's (16) team found that the interaction between CNNMs and PRL (phosphatases of the regenerating liver) was associated with tumorigenesis in 2014. PRL is a molecule highly expressed in solid and hematologic tumor cells (17, 18). PRLs can form complexes with CNNMs, thereby inhibiting the activity of CNNM to transport Mg^{2+} extracellularly, increasing the intracellular concentration of Mg^{2+}, and promoting the growth of tumor cells. In breast cancer cells, when the intracellular Mg^{2+} concentration is low, the expression of PRL-1 can be increased. PRL-1 can anchor the CBS domain, after which the charge interaction between two adjacent proteins alters the conformation of the CBS domain, which causes changes in the structure of the transmembrane region of the protein, allowing magnesium ion influx. Also, Mg^{2+}-ATP can bind to the CBS domain as the intracellular Mg^{2+} concentration increases, to maintain the state of transporter opening. However, this mechanism cannot explain the mechanism of CNNM2 in the distal convoluted tubule of the kidney. And the function and role of CNNM2 in the central nervous system are still unknown. Some investigators have also suggested that CNNM2 is not a Mg^{2+} transporter *per se* but a factor affecting Mg^{2+} transporters (7).

Different phenotypes of *CNNM2*-related disorders are associated with different modes of inheritance and different domains in which variation occurs. Variation of AD-inherited simple hypomagnesemia (type 1) contains a frameshift variant located near the N-terminal (p.Ile40SerfsX15), a missense variant in the CBS domain (p.Thr568Ile), and a stop-gain variant located in the CNBH domain, where p.Ile40SerfsX15 and p.Ser795* can cause changes in mRNA and protein length, and the structurally abnormal mRNA and proteins may be degraded, causing a decrease in intracellular *CNNM2* expression, which affects the transmembrane transport of magnesium. p.Thr568Ile is located in the CBS2 domain in the core region of the CNNM2, which is highly conserved, and the variation may cause a loss in CNNM2 function. Variation of AD-inherited hypomagnesemia with epilepsy and ID/DD (type 2) included missense variants, stop-gain variants, deletion variant, and two heterozygous deletion CNVs. Most of them (p.Ser269Trp, p.Phe272 Leu, p.Tyr314*, p.Leu321del, p.Val324Met, p.Val326Leu, p.Leu330Phe, p.Glu357Lys, and p.Leu418Pro) clustered in the DUF21 domain, the transmembrane structure of CNNM2 with unknown function. And five of the nine variant sites are located in the intramembrane structure. These patients had manifestations of central nervous system involvement independent of hypomagnesemia, including ID/DD and epilepsy, suggesting that functional abnormalities in DUF21 domain may be more closely related to neurological function. The pathogenic mechanism may be abnormal transmembrane structure caused by mutations, rather than a decreased expression of *CNNM2*, causing abnormal neuron excitability. This suggests that in addition to affecting magnesium reabsorption in the kidney, the function of CNNM2 may also include participating in the regulation of neural cell excitability in the central nervous system, and the important core of regulatory function may be the transmembrane region DUF21. p.Leu48Pro is located near the N-terminal, and this site was located in a region that crosses the endoplasmic reticulum (ER) membrane during protein transport (6). This suggested that p.Leu48Pro may affect the transport of CNNM2 protein to the cell membrane, which in turn reduced the amount of CNNM2 protein on the cell membrane. However, p.Ser795Leu and p.Arg 797* are located in the CNBH domain, and another pathogenic variant at Ser795 (p.Ser795*) was related to type 1 *CNNM2*-related disorders. This may suggest that the region near Ser795 has a relatively important effect on the function of the CNBH domain. But the pathogenic mechanisms of these three variants could not yet be well-explained. Variations of AR-inherited hypomagnesemia with epilepsy and ID/DD (type 3) are missense variants, including p.Glu122Lys located between the N-terminal and DUF21 domain and p.Val548Met located in CBS domain. p.Glu122Lys is adjacent to the DUF21 domain, and this variant causes a change in the charge of the amino acid residue, suggesting that some regions of the N-terminal of CNNM2 may form some interaction with the DUF21 domain electrostatically, which, like the variants of type 2, alters the membrane structure of DUF21 domain, causing changes in neuron membrane excitability, resulting in similar central nervous system manifestations. p.Val548Met is located in the CBS domain of CNNM2, and this variant causes the most significant decrease in serum magnesium and the most severe neurological phenotype, suggesting that this variant site may be the core position of the CBS domain, and the amino acid changes caused by this variant significantly affect the function of the CNNM2. The patient carried p.Val548Met had initial serum magnesium of 0.38 mmol/L; however, considering that p.Thr568Ile carriers had equally severe hypomagnesemia but did not develop any central nervous system phenotype, this indicated that the remarkable hypomagnesemia *per se* cannot explain the severe phenotype of the central nervous system in this patient. Therefore, the mechanism by which p.Val548Met leads to severe CNS phenotypes may be at least partially related to the DUF21 domain, as in type 2 cases.

Although all patients with *CNNM2*-related disorders had hypomagnesemia, the serum magnesium was different in each case, which may be related to the protein domain where the variant was located. Among the 23 reported cases, those with variants in the CBS domain had lower serum magnesium levels, and the lowest two had variants both located in the CBS2 domain (p.Val548Met and p.Thr568Ile). This suggests an important role for the CBS domain in the Mg^{2+} transport function of CNNM2.

In summary, we reported two cases first in the Chinese population with hypomagnesemia, epilepsy, and DD caused by novel *de novo* heterozygous variants in *CNNM2* (c.814T>C [p.Phe272Leu] and c.976G>C [p.Val326Leu]). We summarized and classified the phenotypes of *CNNM2*-related disorders into three types. We found that *CNNM2* related central nervous system phenotypes were most associated with DUF21 domain variations, whereas hypomagnesemia was more pronounced in patients with CBS2 domain variations, and AR-inherited *CNNM2*-related disorders had the most severe phenotype. The limitation of our study is the relatively small sample size. The features summarized from 23 patients might not be the real characteristics for such a complicated disease. More cases and further biological functional studies are needed to confirm, modify, and interpret our findings. However, our findings provide important clues for mechanism studies of *CNNM2*-related disorder and provide the possibility for accurate genetic counseling.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YJ: study design and revision of the manuscript. YW and YJ: collection of clinical and WES data. HZ: follow up the patients' information and analyses and draft preparation.

All authors contributed to the article and approved the submitted version.

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De Novo Variants in the *DYNC1H1* Gene Associated With Infantile Spasms

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Objective: The *DYNC1H1* gene is related to a variety of diseases, including spinal muscular atrophy with lower extremity–predominant 1, Charcot–Marie–Tooth disease type 2O, and mental retardation, autosomal dominant13 (MRD13). Some patients with *DYNC1H1* variant also had epilepsy. This study aimed to detect *DYNC1H1* variants in Chinese patients with infantile spasms (ISs).

Methods: We reviewed clinical information, video electroencephalogram (V-EEG), and neuroimaging of a newly identified cohort of five patients with *de novo DYNC1H1*gene variants.

Results: Five patients with four *DYNC1H1*variants from four families were included. All patients had epileptic spasms (ESs), the median age at seizure onset was 7.5 months (range from 5 months to 2 years 7 months), and the interictal V-EEG results were hypsarrhythmia. Four of five patients had brain magnetic resonance imaging (MRI) abnormalities. Four *de novo DYNC1H1* variants were identified, including two novel variants (p.N1117K, p.M3405L) and two reported variants (p.R1962C, p.F1093S). As for the variant site, two variants are located in the tail domain, one variant is located in the motor domain, and one variant is located in the stalk domain. All patients had tried more than five kinds of antiepileptic drugs. One patient has been controlled well by vigabatrin (VGB) for 4 years, and another patient by VGB and steroids for 1.5 years. The other three patients still had frequent ESs. All patients had severe intellectual disability and development delays.

Significance: IS was one of the phenotypes of *DYNC1H1* variants. Most patients had non-specific brain MRI abnormality. Two of four *DYNC1H1* variants were novel, expanding the variant spectrum. The IS phenotype was related to the variant's domains of *DYNC1H1* variant sites. All patients were drug-refractory and showed development delays.

Keywords: infantile spasms, epilepsy, malformations of cortical development, *DYNC1H1* gene, intellectual disability

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INTRODUCTION

The *DYNC1H1* gene located in 14q32.31 encodes for dynein cytoplasmic one heavy chain 1. It is a large protein of 530 kDa and 4,646 amino acids (aa), which is highly conserved and has a few housekeeping roles (1). *DYNC1H1*comprises four major protein regions (**Figure 1**), that is, tail domains (aa residues 1–1,373 and 4,222–4,646), linker domain (aa 1,374–1,867), motor domains with AAA domains (ATPases associated with a variety of cellular activities, aa 1,868–3,168 and 3,553–4,221), and the stalk or microtubule-binding domain (MTBD, aa 3,169–3,552) (2).

DYNC1H1 is highly intolerant to missense change (3). Heterozygous variants in the *DYNC1H1* gene have been associated with a variety of diseases. In 2010, Harms et al. first described dominant spinal muscular atrophy (SMA) with lower extremity with *DYNC1H1* variant (4). Weedon et al. identified a *DYNC1H1* variant in a large pedigree with autosomal dominant axonal Charcot–Marie–Tooth disease in 2011 (5). Willemsen et al. and Poirier et al. reported *DYNC1H1* variants caused severe intellectual disability with neuronal migration defects and malformations of cortical development (MCDs) (6, 7). Different *DYNC1H1* gene variant sites were related to different phenotypes. However, patients with the same variant might also have different phenotypes; for example, p.Arg598Cys variant was found in one patient diagnosed as having SMA, and the other patient diagnosed with myopathy (8, 9).

Some patients with *DYNC1H1* variants manifest epilepsy. In 2013, Poirier et al. reported eight patients with *DYNC1H1* gene variant when studying the mutated genes of patients with MCD and microcephaly, among whom seven patients developed epileptic seizures, and one was diagnosed with Lennox-Gastaut syndrome (LGS) syndrome (7). In 2020, Amabile et al. summarized 103 *DYNC1H1* variants in 200 patients with neurological developmental phenotypes across 143 unique families (10). Seizures were found in 18.5% (37/200) of patients. Here, we report five infantile spasm (IS) patients with *DYNC1H1*variants and characterize in detail the clinical phenotype, brain magnetic resonance imaging (MRI) features, and response to treatment and outcome.

METHODS

Patients

Five patients in four families with *DYNC1H1* variants were retrospectively recruited from the Department of Pediatrics, Peking University First Hospital, from June 2017 to October 2020. This study was approved by the Peking University First Hospital Medical Ethics Committee. Information about the age at epileptic spasm (ES) onset, developmental milestones, neurological status, family history, video electroencephalogram (V-EEG), brain MRI results, treatment, and outcomes was collected in the clinic. Brain MRI and V-EEG were reviewed by a neuroradiologist and neurophysiologist, respectively. Patients were followed up at the pediatric neurology clinic or by telephone.

DNAs (3 μg) extracted from peripheral blood from probands and their parents were analyzed using whole-exome sequencing. Variants were checked with population databases gnomAD (http://gnomad.broadinstitute.org/) and evaluated using Polyphen2, SIFT, and Variant Taster. Variant pathogenicity was interpreted according to the American College of Medical Genetics (ACMG) guidelines (11). The variants were further confirmed by Sanger sequencing.

Literature Review

A systematic research of articles published in PubMed registered from 2012 to 2021, was performed. All the researched articles are based on the following terms "DYNC1H1." The relevance of each result was determined, and references were reviewed to identify missing studies. All the epilepsy phenotype, epilepsy onset age, treatment, and prognosis are summarized in **Supplementary Table 1.**

RESULTS

Clinical Features

Clinical features of affected individuals with *DYNC1H1* variants are summarized in **Table 1**.

Seizures, EEG, and Brain MRI Information

All five patients were from full-term birth. Patients 3 and 4 were dizygotic twins. Patient 5 had two febrile convulsions at 1½ and 2 years old. All patients had ES. For patients 1–4, the minimum seizure onset age was 5 months, and the maximum onset age was 7.5 months. As for patient 5, the first seizures occurred at the age of 2 years 7 months. All patients have tried at least five kinds of antiepileptic drugs (AEDs), including adrenocorticotropic hormone (ACTH), valproate (VPA), zonisamide (ZNS), topiramate (TPM), levetiracetam (LEV), vigabatrin (VGB), steroids, and a ketogenic diet. The

FIGURE 1 | The protein regions of DYNC1H1.

TABLE 1 | The clinical information and gene variants of five patients.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Sex	Female	Female	Female	Female	Female
Current age	5 y	2 y 9 m	3 y	3 y	3 y
Gestation	At term	At term	At term	At term	At term
Birth history	Normal	Normal	ABO hemolytic disease of newborn	ABO hemolytic disease of newborn	Normal
Age at seizure onset	10 m	5 m	7.5 m	7.5 m	2 y 7 m
Seizure type	Spasms	Spasms	Spasms	Spasms	Spasms
AEDs	ACTH, VPA, ketogenic diet, ZNS, TPM, LEV	LEV, TPM, ACTH, VGB, steroids	LEV, TPM, VPA, CLB, VGB, LTG	LEV, TPM, VPA, CLB, VGB, LTG	VPA, TPM, CAP, LTG, VGB, steroids, ketogenic diet
Treatment	VGB controlled 4 y	VGB+ steroids controlled 1.5 y	Uncontrolled	Uncontrolled	Uncontrolled
EEG	Hypsarrhythmia	Hypsarrhythmia	Hypsarrhythmia	Hypsarrhythmia	Hypsarrhythmia
Brain MRI	White matter dysplasia	Enlarged bilateral frontal gyrus, thickened cortex, and reduced subcortical white matter	Enlarged bilateral hemispheric gyrus, ventricles, and corpus callosum dysplasia	Enlarged bilateral hemispheric gyrus, ventricles, and corpus callosum dysplasia	Normal
Genetic test	DYNC1H1, *de novo*, c.3351C>G (p.N1117K) (novel)	DYNC1H1 *de novo*, c.5884C>T (p.R1962C) (reported)	DYNC1H1 *de novo*, c.10213A>C (p.M3405L) (novel)	DYNC1H1 *de novo*, c.10213A>C (p.M3405L) (novel)	DYNC1H1 *de novo*, c.3278T>C (p.F1093S) (reported)
Psychomotor development	Severe delay	Severe delay	Severe delay	Severe delay	Severe delay
Sitting	1 y 5 m	No	No	No	8 m
Walking	5 y	No	No	No	1 y 8 m
Speech	No	No	No	No	No

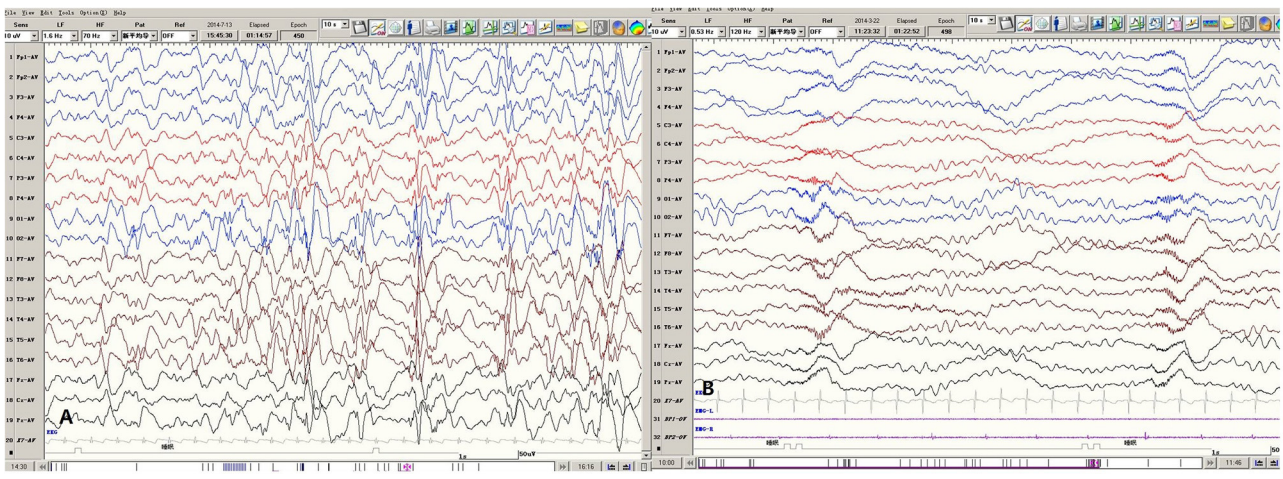

FIGURE 2 | Representative EEGs of Patient 1. **(A)** The Interictal hypsarrhythmia EEG pattern. **(B)** The ES ictal EEG pattern.

ES of patient 1 was controlled by VGB at the age of 1.5 years for more than 4 years, albeit multifocal discharges on EEG were still observed until the last follow-up. For patient 2, ES was controlled by VGB and steroids at the age of 1 year 5 months for 1.5 years. The ES was not controlled for patients 3, 4, and 5.

EEG data for five patients all showed hypsarrhythmia (**Figure 2**). Brain MRI was abnormal in patients 1–4 and normal in patient 5. Brain MRI showed white matter dysplasia and developmental regression in patient 1; enlarged bilateral frontal gyrus, thickened cortex, and reduced subcortical white matter in patient 2; and enlarged bilateral hemispheric gyrus,

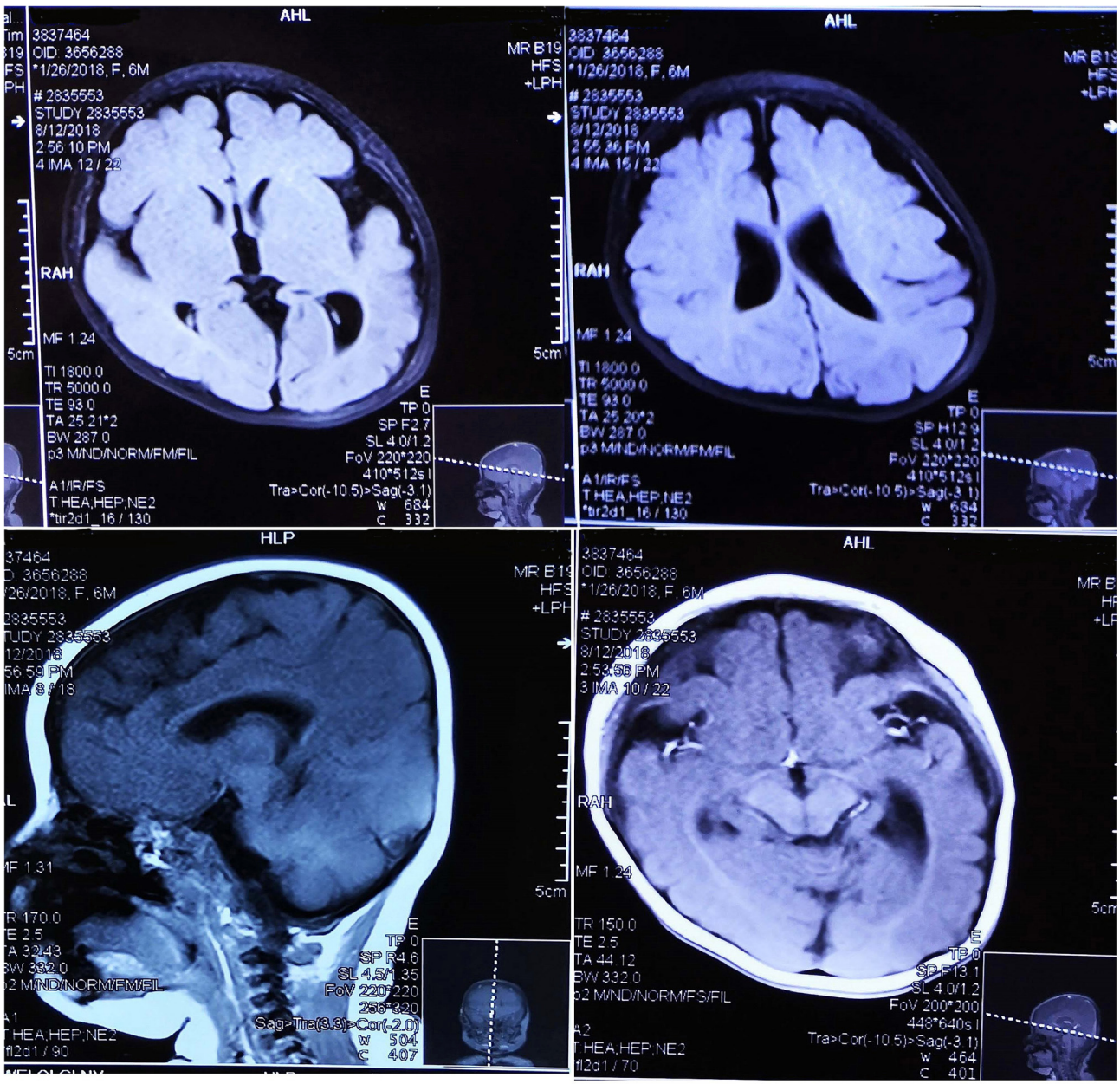

FIGURE 3 | The brain MRI of patient 3 showed MCD.

ventricles, and corpus callosum dysplasia in patients 3 and 4 (**Figure 3**).

Genetic Analysis

Four *de novo* variants were identified in five patients from four families. Two variants (p.N1117K, p.M3405L) were novel, and two (p.R1962C, p.F1093S) were previously reported (12, 13). All four variants located in different domains, including p.N1117K in the distal dimerization domain of the protein (tail domains), p.R1962C in theAAA1 of the protein (motor domain), p.M3405L in the AAA4 of the protein (stalk or MTBD, motor domain), and p.F1093S in the AAA1 of the protein (tail domains). All the detected variants were pathogenic according to the ACMG criteria.

Neurodevelopment

All patients had severe developmental delays and intellectual disabilities, but no developmental scales were available. Among them, only two patients could walk independently at the last follow-up. The motor development of patients 3 and 4 underwent a retrograde process. Patients 3 and 4 could control their necks at the age of 5 months. After seizure onset, they could not hold

their head well and could not sit until 3 years old. At the last follow-up, all patients still had language backward. Only patient 5 could understand simple instructions and speak a few words, but he had from significant language regression to unable to speak after ES onset. The other four patients could not speak any words.

Results on Literature Search

A total of 18 articles found *DYNC1H*1 gene associated with epilepsy. Forty-three patients were reported to have epilepsy. In these 43 patients, the epilepsy phenotype was described in 44.2% (19/43), including focal seizure, myoclonic seizures, tonic seizures, atonic seizures, generalized tonic–clonic seizures, and IS. EEG results were reported in 11.6% of patients (5/43), including multifocal epileptiform discharges; interictal EEG showed waxing and waning of waves in the frontal, temporal, and occipital areas, and high-amplitude rhythmic waves were frequently observed; generalized spike-and-wave complexes and irregular polyspikes and slow waves, predominantly in the left frontal area; an attenuated background of mixed theta and delta frequencies; and semiperiodic spike and slow wave activity in the right temporal occipital region and generalized slowing. Treatment was described in 48.8% of patients (21/43). Nine patients were seizure-free or had controlled epilepsy. Ten patients had uncontrolled epilepsy or were therapy-refractory. The other three patients did not have their therapy result.

DISCUSSION

We reported five patients with IS from four families with *de novo DYNC1H1* variants, including two novel and two previously reported variants. We also reviewed previous literature about *DYNC1H1* gene–associated epilepsy.

In 2018, Palmer et al. reported one patient with p.R1962C variant had IS (12). His EEG revealed an abnormal background and multifocal epileptiform activity. His brain MRI showed pachygyria (12). This patient had severe developmental delay and intellectual disability, and he was diagnosed with an autism spectrum disorder (12). Patient 2, carrying the same p.R1962C variant, manifested ES, MCD, severe developmental delay, and intellectual disability, similar to the reported patient (12). However, Poirier et al. reported a 19-year-old patient with p.R1962C variant who had a focal seizure, severe developmental delay, intellectual disability, and the predominant postpachygyria (7). Therefore, patients with the same *DYNC1H1* variant might have different seizure types. In 2016, Helbig et al. reported a patient diagnosed as having IS with the variant site p.F1093S. The same variant was found in patient 5 (13). Besides IS, several epileptic phenotypes have been reported in patients with *DYNC1H1* variants, such as focal onset epilepsy, myoclonic epilepsy, and atonic seizures (2, 6, 7, 12–17). All the reported epileptic phenotypes are summarized in **Supplementary Table 1**.

Patients with pachygyria carrying *DYNC1H1* variants manifested epilepsy (10, 14). Abnormal brain MRI was observed in our four patients, including three MCD and one white matter dysplasia. *DYNC1H1* is known to bind both bicd cargo adaptor (BICD2), a microtubule motor adaptor associated with SMA lower extremity–predominant 2, and lissencephaly 1 (LIS1), a dynein regulator associated with MCD/lissencephaly (18), which might explain why the variant of *DYNC1H1* could lead to pachygyria or MCD. For those patients with MCD, posterior predominant lesions were most common (7). In 2020, Amabile et al. found 37 patients had epileptic seizures, among whom 28 patients showed MCD (10). For our patients, MCD was mainly observed at the frontal lobe in one patient and bilateral hemispheres in two, which were different from the previous reports (7). Epileptogenic mechanisms linked to pathogenic variants of *DYNC1H1* gene were not clear; MCD may be the reason; however, patients from previous and our studies without MCD could also have epilepsy (16). In 2017, Lin et al. reported an epileptic encephalopathy patient who had *DYNC1H1* mutation through analysis of the interaction network of DYNC1H1, which showed that DYNC1H1 interacts with many epilepsy genes, such as *TBC1D24*, *ALDH7A1*, *MECP2*, *DEPDC5*, *SGCE*, *GRIN2B*, *ATP1A2*, *MYO5A*, *NBEA*, *CLCN4*, *IFT172*, *UBE3A*, *PCDH19*, *KCNQ3*, and so on. Thus, DYNC1H1 may cause epilepsy by affecting other epilepsy-related gene function, such as interaction with other mutations present in their genomes or environmental factor. In addition to the above speculation, the mutation of *DYNC1H1* gene itself may also cause epilepsy, because DYNC1H1 is highly conserved and takes part in a variety of intracellular functions (17).

Several studies have reported the relationship between domain location of the variants and clinical phenotype (2, 10). A previous study reported that *DYNC1H1* variants in eight patients with MCD were located in the stalk domain, AAA1, the linker region, and the tail domain (7). The study also reported that four unrelated patients with MCD disorder had *de novo* variants in the stalk domain who exhibited obvious clinical symptoms of early-onset epilepsy encephalopathy (7). Amabile et al. summarized 103 *DYNC1H1* variants and concluded that among 26 neuromuscular patients with obvious central nervous system involvement (intellectual disability, MCD, or other brain MRI abnormalities), 23 had variants located in the stem or neck domains, and only three had variants located in the motor domain (10). In contrast, of the 59 patients classified as having a primarily intellectual disability, MCD, and autism, 18 had variants located within the stem domain, five in the neck/linker, and 36 in the motor domain (10). Beecroft et al. summarized that a majority of patients with MCD had variants in the stalk of the motor domain (9). Some studies reported that variants associated with central nervous system manifestations such as intellectual disability and MCD had clustered in the motor domains (4, 18). Variants from the patients with seizures were mostly reported in the motor domain, whereas variants from the patients with behavioral abnormalities were largely reported in the beginning tail, linker, and motor domains (2). Variants from the patients with MRI abnormalities, specifically, pachygyria, were largely reported in the motor domain (2). In our study, the patient numbers were too small to conclude the relationship between the variant domain and the clinical phenotype. The *de novo* p.N1117K and p.F1093S variant of *DYNC1H1* identified in patients 1 and 5 were located in the tail domain and the stem domain of the protein. Both patients had obvious intellectual

disability and development delay and severe epilepsy. The brain MRI revealed white matter dysplasia in patient 1, whereas it was normal in patient 5. As indicated earlier, the variants in patients with epilepsy were usually located in the motor domain of the protein; thus, these two patients were not consistent with the previous study (7, 10). The p.R1962C variant identified in patient 2 was located in the motor domain. She had ES and MCD, which was consistent with the previous reports (7, 19). The p.M3405L variant identified in patients 3 and 4 was in the stalk domain (MTBD), which belonged to the motor domains. Both patients had early-onset epilepsy and MCD, consistent with the previous report (2, 7, 19–21).

Until now, few cases have described the epilepsy treatment of patients with *DYNC1H1* variant in detail. In 2020, Becker et al. reported four patients with *DYNC1H1* variants had epilepsy and found that most patients remained seizure-free with single or combined anticonvulsive medication, whereas one patient had a therapy-refractory course (2). Di Donato et al. reported 13 patients with *DYNC1H1* variants complicated with epilepsy, among which seven cases had seizures controlled by drugs (14). Matsumoto et al. reported two patients with *DYNC1H1* variants who had refractory epilepsy. The patients were treated with more than five kinds of AEDs, but their epilepsy was not controlled successfully (16). In our study, all five patients tried at least five AEDs, and two patients were controlled by VGB and steroids.

In our study, all five patients had severe developmental delay and intellectual disability; in addition, three patients had development or language retrograde after seizure onset. In the previous studies, patients with intellectual disability were usually diagnosed with MRD13, who had the intellectual disability and cortical malformations (6). Four of our patients had abnormal brain MRI, which was consistent with the previous report (6). Some studies indicated that variants involved in central nervous system manifestations were clustered in the motor domains (4, 19). As for patient 5, the *DYNC1H1* variant was located in the tail domain, but her brain MRI was normal, which was not consistent with the previous reports (4, 19). Therefore, the mechanism of intellectual disability in patients with *DYNC1H1* variants still needs further research.

CONCLUSION

Here we summarized the clinical features, treatment, and outcomes of five patients with *DYNC1H1* variants. IS was one of the phenotypes of *DYNC1H1* variants. Most patients had nonspecific brain MRI abnormalities. *DYNC1H1*-associated diseases had heterogeneity of gene variants and clinical phenotypes. All patients were drug-refractory, although some could be controlled lately. All patients had severe developmental delay and intellectual disability. For the present study, the data were too small to conclude the relationship between the clinical phenotypes and the variants' domain location; further study may be needed to draw a more accurate conclusion.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: BankIt2488557 Seq1 MZ733295; BankIt2489627 Seq1 MZ736872; BankIt2489989 Seq1 MZ754976, and BankIt2490423 Seq1 MZ781301.

ETHICS STATEMENT

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HY and ZY designed the study, drafted the initial manuscript, and revised the manuscript. PG, XJ, QZ, YN, and YZ helped to collect and summarize data and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.733178/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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New Trends and Most Promising Therapeutic Strategies for Epilepsy Treatment

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Background: Despite the wide availability of novel anti-seizure medications (ASMs), 30% of patients with epilepsy retain persistent seizures with a significant burden in comorbidity and an increased risk of premature death. This review aims to discuss the therapeutic strategies, both pharmacological and non-, which are currently in the pipeline.

Methods: PubMed, Scopus, and EMBASE databases were screened for experimental and clinical studies, meta-analysis, and structured reviews published between January 2018 and September 2021. The terms "epilepsy," "treatment" or "therapy," and "novel" were used to filter the results.

Conclusions: The common feature linking all the novel therapeutic approaches is the spasmodic rush toward precision medicine, aiming at holistically evaluating patients, and treating them accordingly as a whole. Toward this goal, different forms of intervention may be embraced, starting from the choice of the most suitable drug according to the type of epilepsy of an individual or expected adverse effects, to the outstanding field of gene therapy. Moreover, innovative insights come from *in-vitro* and *in-vivo* studies on the role of inflammation and stem cells in the brain. Further studies on both efficacy and safety are needed, with the challenge to mature evidence into reliable assets, ameliorating the symptoms of patients, and answering the challenges of this disease.

Keywords: anti-seizure medications, epilepsy, genetics, inflammation, precision medicine

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INTRODUCTION

Epilepsy is the enduring predisposition of the brain to generate seizures, a condition that carries neurobiological, cognitive, psychological, and social consequences (1). Over 50 million people worldwide are affected by epilepsy and its causes remain partially elusive, leaving physicians, and patients an unclear insight into the etiology of the disease and the best treatment approach (2). Over than 30% of individuals do not respond to common anti-seizure medications (ASMs) and are addressed to as "drug-resistant," a term which the International League Against Epilepsy (ILAE) applies to those patients who do not respond to the combination of two appropriately chosen and administered ASMs (3, 4). Hence, a great deal of responsibility laid upon the research and

development of innovative pharmacological and non-pharmacological approaches given a targeted approach, aiming at improving the symptoms of patients and their quality of life (QoL), together with that of the caregivers.

As several investigations are currently in progress, this review aimed to discuss the novel therapeutic insights, with the hope they may establish as turning points in the treatment of patients in the next few years.

METHODS

A search on PubMed, Scopus, and EMBASE databases using the terms "epilepsy," "treatment" or "therapy," and "novel" was conducted. The search covered the period between January 2018 and September 2021. Existing literature was reviewed, including both experimental and clinical studies, meta-analysis, and topic reviews summarizing the most up-to-date researches. Only studies published in English were reviewed.

PRECISION MEDICINE

Precision medicine (PM) is an outstanding approach tended to use the genetics, environment, and lifestyle of individuals to help determine the best "way" to prevent or treat disease (5). It embeds a holistic evaluation, assessing not only the effect of an own condition but also that of treatment (6). Precision medicine is endorsed in epilepsy management for many decades, as in the clinical practice ASMs are selected after a careful and pointful evaluation of seizure types of patients, their epilepsy syndrome, comorbidities, concomitant drugs, and expected vulnerability to specific adverse events (AEs) (7). Discoveries and progress in genetics have provided the strongest basis for PM: as more and more genes are being identified as disease-causing, hope has grown on possible targeted approaches (6). An "ideal" therapy would be able to both relieve symptoms and reverse the functional alterations caused by specific genetic mutations. This firstly implies identifying putative disease-causing genes and, secondly, the specific functional alterations caused by the pathogenic variants. Lastly, it should have been demonstrated that therapeutic intervention may modify the effect caused by the mutation.

The ketogenic diet (KD) used to treat glucose transporter 1 (GLUT1) deficiency syndrome is probably the best example of PM applied to epilepsy. In GLUT1 patients the uptake of glucose into the brain is impaired because of the *SLC2A1* mutation, hence, the KD provides neurons with an alternative source of energy, compensating for the consequences of the metabolic defect (8). Another clear application of a PM-based approach is the avoidance of those drugs which may cause worsening of seizures by exasperating the underlying molecular defect, i.e., sodium channel blockers must be avoided in patients with Dravet syndrome (DS) carrying loss-of-function mutations in the sodium voltage-gated channel alpha subunit 1 (*SCN1A*). Another one is memantine for the treatment of GRIN-related disorders due to gain-of-function mutations in the NMDA receptor (8–11) or quinidine and retigabine for epilepsies caused by potassium channels genes mutations (*KCNT1* and *KCNQ2*) (6, 12). In epileptic encephalopathies (EE), it would be also of interest to investigate the effect of a PM treatment on cognitive function, to that targeting a specific gene mutation and abolishing related epileptic activity may result in improved cognitive functions (10).

Precision medicine may prove complex, as the same mutation may cause quite different clinical phenotypes; moreover, additional genetic variants may contribute to modifying a phenotype. Again, wide-genome variations or even the epigenome may influence the resulting expression of pathogenic variants (5).

Nowadays, evidence indicates PM may be applied to individuals with both rare and common forms of epilepsy, and, consequently, drug development is increasingly being influenced by PM approaches. Although extensive research focuses on genome-guided therapies, important opportunities also derive from immunosuppressive therapies and neuroinflammation-targeting treatments (2, 13). The identification of cellular and molecular biomarkers would possibly allow clinicians to have early prediction markers of a disease and its progression. Additionally, it could lead to the development of unique models to cost-effectively screen treatments and also decrease the costs of clinical trials through better patient selection (14).

NOVEL MECHANISMS OF ANTI-SEIZURE MEDICATIONS

Many medications are currently under study in clinical practice, ranging from those with a mechanism similar to that of well-known ASMs, like the GABA-A receptor agonists, to those with novel mechanisms such as the stimulation of melatonin receptors. Moreover, some drugs are yet known medications, previously used for other indications; while a large group remains orphan of a well-comprised mechanism of action (6). It is in this perspective, that the wider term ASMs should be addressed, aiming at referring to the large heterogeneity of action mechanisms nowadays available to counteract seizures.

Cannabidiol

In 2018, the Food and Drug Administration (FDA) approved the first-in-class drug derived from the cannabis plant. Although the precise mechanism by which the cannabidiol (CBD) exerts its anti-seizure effects is still poorly known, it seems not to act through interaction with known cannabinoid receptors (15), but holds an affinity for multiple targets, resulting in the reduction of neuronal excitability which is relevant for the pathophysiology of the disease (16, 17).

Cannabidiol is approved for the treatment of seizures in children with DS or Lennox-Gastaut syndrome (LGS) aged 2 years or older, based on three pivotal phases 3 trials (12). In 2019 CBD gained approval in Europe in conjunction with clobazam (CLB), based on clinical trial data showing that the combination of both CBD and CLB resulted in greater efficacy outcomes (16).

The first clinical trial (17) included 120 patients with DS aged between 2 and 18 years. The median frequency of convulsive seizures decreased from 12.4 to 5.9 per month, as compared

with a decrease from 14.9 to 14.1 per month with the placebo. Furthermore, 43% of patients in the active arm and 27% in the placebo group showed at least a 50% reduction in the convulsive-seizure frequency. Overall, 62% of patients under CBD did gain at least one category at the seven-category Caregiver Global Impression of Change scale, as compared to 34% in the placebo group. Five percent of patients under CBD became seizure-free, while none in the placebo group did.

Another randomized, double-blind, placebo-controlled, trial (18) included 171 LGS patients aged between 2 and 55 years and measured the reduction in drop-seizures. The median percentage reduction was 43.9% in the CBD group and 21.8% in the placebo group. In 2018, Devinsky et al. (19) compared a lower 10 mg/kg/day dose of CBD with the full 20 mg/kg/day in LGS patients. A median 41.9% reduction in drop-seizure frequency was observed in the 20-mg CBD group, while the median reduction was 37.2% in the 10-mg group and 17.2% in the placebo group. Although this study demonstrated patients may gain benefit in seizure reduction by increasing the dose to 20 mg/kg/day, it also displayed an increased risk in AEs. It is generally recommended to begin at 5 mg/kg divided into two intakes a day, then increase to 10 mg/kg/day. If the 10 mg/kg/day dose is well-tolerated and the anti-seizure effect continues, dosing can be increased to the maximum of 20 mg/kg/day (15).

Cannabidiol also proved to effectively reduce seizure frequency at long-term follow-up (20), retaining a consistent reduction (between 42.9 and 44.3%) in seizure frequency at 48 weeks of follow-up. Moreover, 5 out of 104 patients (4.8%) were convulsive seizure-free at 12 weeks of treatment, with more than 40% having a reduction of convulsive seizure frequency $\geq$50% at each programmed visit of follow-up (18). In terms of median percentage reduction in convulsive seizures, rates of responders, reduction in total seizures, and CGIC-assed improvements, CBD proved greater in the subset of patients concomitantly treated with CLB. Moreover, the combination CBD+CLB showed a benefit in the number of convulsive seizure-free days (16). However, a drug-to-drug interaction increasing levels of active metabolites of both compounds must be assessed and hence CLB dose reduction is recommended if patients experience somnolence or sedation (15, 16).

In conclusion, RCTs settle CBD as a well-tolerated drug, with patients primarily experiencing somnolence, diarrhea, and decreased appetite. The elevation of liver transaminases may be observed mostly in patients on concomitant valproate, and the dose reduction of valproate or CBD is often decisive. The efficacy of CBD on both convulsive and drop seizures is established, with retained efficacy at long-term follow-up. New RCTs in other syndromic or isolated epilepsies populations may widen the field of use of CBD in the next few years.

Fenfluramine

Fenfluramine (FFA), formerly used at 10 times higher dosage (up to 120 mg/day) as a weight-loss drug, exerts its anti-seizure effect both through the release of serotonin which stimulates multiple 5-HT receptor subtypes, and by acting as a positive modulator of sigma-1 receptors (16, 21–23). Fenfluramine has been approved by the FDA in June 2020 and is currently under evaluation by the European Medicines Agency (EMA). The drug proved significantly effective in reducing seizures in phase-3 trials on DS patients: the 0.8 mg/kg/day treated group did experience a mean 64% reduction in seizures as compared to 34% in the 0.2 mg/kg/day group. Notably, a >75% reduction in seizures occurred in 45% of patients under 0.8 mg/kg/day, in 20.5% of those on 0.2 mg/kg/day compared to 2.5% in the placebo group (23). Fenfluramine has then continued to provide a clinically meaningful reduction in convulsive seizure frequency over a median of 445 days of treatment. The median percent reduction in monthly convulsive seizures frequency was 83.3%. Overall, 62% of patients showed a 50% reduction in convulsive seizure frequency (16).

Together with the anti-seizure effect, FFA has also relatively few drug-drug interactions, primarily a moderate effect on stiripentol (STP), which requires the downward adjustment of FFA dosing to.5 mg/kg/day. No additional interaction with other drugs such as valproate, CLB, and CBD are known (15). The most common AEs reported under FFA treatment include decreases in appetite, weight loss, diarrhea, fatigue, lethargy, and pyrexia (16). The main AEs leading to FFA withdrawal as a weight-loss agent were the occurrence of valvular heart disease (VHD) and pulmonary arterial hypertension (PAH), for which 6-month-echocardiographic monitoring is required together with an ECG. However, at the anti-seizure dosages, no VHD or PAH was observed after a median duration treatment of 256 days. No ECG alterations indicative of atrioventricular conduction or cardiac depolarization alterations were seen, and no mitral or aortic valve regurgitation greater than "trace" was observed in any of the 232 patients with DS who participated in the open-label extension (OLE) study (21, 24, 25).

Cenobamate

Cenobamate (*Xcopri* or *YKP3089*) is a new ASM that has recently gained approval by the FDA for the treatment of focal-onset seizures in adults. The EMA is currently reviewing the drug for approval as an adjunctive treatment in focal-onset epilepsies. Cenobamate is a tetrazole-derived carbamate compound with a dual mechanism of action; the drug can both enhance the inactivated state of voltage-gated sodium channels, and act as a positive allosteric modulator of the GABA-A receptors, binding at a non-benzodiazepine site (26).

A multicenter, randomized study of patients with uncontrolled focal seizures (27) showed that the adjunctive cenobamate, with dosage groups of 100, 200, and 400 mg/day led to a consistent reduction in focal-seizures frequency after 18-weeks of treatment, with the greatest reduction observed in the 200 and 400 mg/day doses groups. A similar dose-effect relationship was seen when evaluating the responder rates ($\geq$50% in seizure reduction). *Post-hoc* analysis proved seizure frequencies decreased early during cenobamate titration; while, during the 12-week maintenance phase, significantly more patients under the active 200 or 400 mg/day harms achieved seizure freedom as compared to that receiving placebo. Cenobamate is overall well-tolerated, showing mild to moderate severity AEs on the CNS system, such as somnolence, dizziness, and disturbances in gait and coordination, with a linear

incidence-dose correlation and disappearance at maintenance. Four cases of hypersensitivity adverse reactions occurred during two RCTs, including one serious AEs of Drug Rash with Eosinophilia and Systemic Symptoms (DRESS) (26, 27). In this case, the rapid titration of 100 mg/week from 200 to 400 mg dose might have contributed to the higher rates of AEs in the 400 mg group; a lower starting dose and a slower titration rate have been shown to reduce the occurrence of hypersensitivity reactions, possibly through the development of immune tolerance (27). As cenobamate inhibits the P450 family cytochrome CYP2C19*18, dosing adjustment is needed when adding cenobamate to ASM regimens containing phenytoin or phenobarbital (28); moreover, a dose reduction of CLB should be considered, counteract the increase in plasma levels of desmethylclobazam, its active metabolite. Cenobamate has also been shown to decrease by 25% the plasma exposure to carbamazepine, through the induction of the CYP3A4. Cenobamate could shorten the QT-interval on the ECG in a dose-dependent manner. Hence, cenobamate is contraindicated in patients with familial short QT syndrome, and caution is required in co-administration with other drugs known to reduce the QT interval since a synergistic effect may occur (26, 27). In a short time, data will help to assess cenobamate active time-window on seizures control and real-life data will help to acknowledge whether freedom rates will be borne out in clinical practice. The mechanisms of action and the potential additive or synergistic interactions of cenobamate with concomitant ASMs also warrant further investigation (26).

NOVEL NON-PHARMACOLOGICAL TREATMENTS

Neurostimulation comprises different techniques, already implemented in the clinical practice, direct to deliver electrical or magnetic currents to the brain in a non-invasive or invasive way and hence modulating neuronal activity to achieve seizure suppression.

Vagal Nerve Stimulation

Vagal nerve stimulation (VNS) is approved both in Europe and in the United States as an adjunctive treatment in patients with refractory epilepsies, and it is routinely available in many epilepsy centers, with more than 100,000 patients treated worldwide (6). Vagal stimulation may then turn off seizures originating in regions susceptible to heightened excitability, such as the limbic system, thalamus, and thalamocortical projections. Moreover, an additional mechanism of action derives from the activation of the locus coeruleus and the raphe nuclei, and the regulation of the downstream release of norepinephrine and serotonin, both having antiepileptic effects (29).

Two large RCTs showed VNS efficacy in reducing seizures, achieving a 50% reduction in 31% of patients, and over 50% seizures reduction in 23.4% of the studied population. On the other hand, seizure freedom at long-term follow-up was observed in <10% of patients. Side effects are usually mild and include hoarseness, throat paraesthesia or pain, coughing, and dyspnea. This tends to improve over time or through the adjustment of setting parameters (6).

In conclusion, evidence suggests VNS is well-tolerated in both children and adults with drug-resistant partial epilepsies (30–32); moreover, the newest VNS models can detect ictal tachycardia and automatically deliver additional stimulation to abort seizures or reduce their severity (6).

Transcutaneous VNS

Developed as a non-invasive alternative to VNS, the transcutaneous VNS (tVNS) acts on the auricular branch of the vagus nerve (ABVN), targeting thick-myelinated afferent fibers in the cymba conchae, and hence activating the ipsilateral nucleus of the solitary tract (NTS) and locus coeruleus. This activation pathway overlaps with the classical central vagal projections, leading to a brain activation pattern similar to that produced by invasive VNS (33). The device consists of a programmable stimulation apparatus and an ear electrode (34). Stimulation setup is adjusted by applying decreasing and increasing intensity ramps and achieving a level just above the individual detection threshold, but clearly below that of pain. Patients usually apply tVNS for 1 h/three times per day (33) and adherence is usually high (up to 88%) (35). Trials converge in demonstrating up to 55% reduction in seizure frequency, with mild or moderate side effects mainly including local skin irritation, headache, fatigue, and nausea (6, 35).

Deep Brain Stimulation

Deep brain stimulation (DBS) is a minimally invasive neurosurgical technique, which through implanted electrodes can deliver electrical *stimuli* to deep brain structures. Patients with refractory focal epilepsies and not eligible for surgery are usually good candidates (29). Both stimulation of the ictal onset zone and the anterior thalamus have gained approval by the FDA as effective stimulation *sites*, providing a significant and sustained reduction in seizures together with the improvement of the QoL. Nowadays, both DBS and responsive neurostimulation (RNS) are available, being the latter a system able to monitor electrical changes in cortical activity and give small pulses or bursts of stimulation to the brain to interrupt a seizure (36). The interim results of a prospective, open-label, and long-term study did show that the median 60% or greater reduction in seizure frequency is retained over years of follow-up. Moreover, the majority of patients took advantage of treatment with the RNS® System, and 23% experienced at least one 6-month period of seizure freedom (37). The most relevant reported side effects were depressive mood and memory impairment, besides the local side effect of implantation. Nonetheless, it should be stated that RNS is a feasible option in most epilepsy centers in the US, but its use remains limited in other parts of the world. In these cases, DBS could be an option with targets and stimulation parameters selection are largely driven by the experience of the referred center (38, 39).

Trigeminal Nerve Stimulation

Trigeminal nerve stimulation (TNS) is a novel neuromodulation therapy, designed to deliver high frequencies stimulation in

a non-invasive way, hence modulating mood and relieving symptoms in drug-resistant epilepsies. The study of DeGeorgio et al. (40) found that the responder rate (at least 50% reduction in seizures) was 30.2% in the active group, while it was 21.1% in the control group. Moreover, the responder rate did further increase over the 18-week treatment period in the actively treated group. TNS was overall well-tolerated and, when occurring, treatment-related AEs were mild including anxiety (4%), headache (4%), and skin irritation (14%). However, long-term follow-up studies showed inconclusive results (6), meaning further studies and patient monitoring will be needed in the next years.

Transcranial Direct Current Stimulation

The transcranial direct current stimulation (tDCS) displays the use of two skull electrodes (anode and cathode) to induce widespread changes of cortical excitability through weak and constant electrical currents. Cortical excitability may increase following anodal stimulation, while it generally decreases after cathodal stimulation. Based on this principle, hyperpolarization using cathodal tDCS has been proposed to suppress epileptiform discharges. Major six clinical studies are promising with 4 (67%) showing an effective decrease in epileptic seizures and 5 (83%) exhibiting a reduction of epileptiform activity. However, some results may be misleading due both to the small and heterogeneous nature of the studied populations and to the different setting parameters applied. Hence, nowadays the major achievement is the demonstration that tDCS may be effective and safe in humans; however, further studies will be needed to define setting stimulation protocols and understand the long-term tDCS effectiveness (41).

Transcranial Magnetic Stimulation

The nerve cells of a brain to a maximum depth of 2 cm can be stimulated using transcranial magnetic stimulation (TMS). To this, low-frequency and repetitive magnetic stimulations have been shown to induce long-lasting reductions in cortical excitability and, hence, have been proposed as a treatment for drug-resistant epilepsies (4). Probably, it is the repeated nature of magnetic pulses which allows modulating the neuronal activity, wherein high frequencies (>5 Hz) would have an overall excitatory effect, while low-frequencies (0.5 Hz) would exert an inhibitory effect on neurons (29).

Despite the optimal stimulation parameters still needing to be clearly defined, they are likely of crucial importance because treatment intensity depends both on the number of pulses and the number of sessions applied over the treatment period. Superior results are achieved in patients with neocortical epilepsy, whit a calculated effect size of 0.71 and 58–80%. This makes sense taking into account the rapid decay of the strength of the magnetic field with distance hence no adequate secondary currents can be elicited in the deep cortex. However, evidence suggests the effects of repetitive TMS may not be restricted to the only site of stimulation but may spread from focal areas to wider areas of the brain.

In conclusion, results should be reproduced in larger cohorts with double-blinded randomized trials, but are promising if compared to the effects currently achieved with invasive neurostimulation techniques for the treatment of epilepsy (42).

NEUROINFLAMMATION AND IMMUNOMODULATION

Nowadays, the neuroinflammatory pathways are known to contribute to both the development and progression of epilepsy and could be targeted for disease-modifying therapies in epilepsies of wide-range etiologies. Studies on patients with surgically resected epileptic foci have demonstrated inflammatory pathways may be involved, hence the neuroinflammation is not merely a consequence of seizures or brain neuropathology but may induce seizures and brain anatomical damage itself (2).

Finally, any inflammatory response within the brain will be associated with the blood-brain barrier (BBB) dysfunction. Evidence indicates that BBB opening and the subsequent exposure of brain tissue to serum proteins induces upregulation of proinflammatory cytokines and complement system components: this suggests positive feedback between increased brain permeability, local immune/inflammatory response, and neuronal hypersynchronicity (43).

It should also be considered that overall neuroinflammation is a negative disease modifier in epilepsy, however, some inflammatory processes may be involved in tissue repair and brain plasticity after injury hence interference with these beneficial mechanisms should be avoided: anti-inflammatory intervention in the wrong patient and at the wrong time could be ineffective or even harmful. Yet, it is for this reason that evidence remains set at the preclinical level with few reports of use in the clinical practice. The discovery of non-invasive biomarkers of pathological neuroinflammation would enable physicians to identify patients who could benefit from the treatments, also providing a potential marker of therapeutic response.

IL-1R1-TLR4 Signaling

The Interleukin IL-1R1-TLR4 signaling pathway originates the neuroinflammatory cascade in epilepsy through increased levels of either the endogenous agonists or their receptors, or even a combination of both (2). These findings prompted the clinical use of anakinra, the recombinant, and modified form of the human IL-1Ra protein. Case report studies of Anakinra in patients with intractable seizures did result in a significant reduction of seizure activity and improvement of cognitive skills (44). Moreover, IL-1R1 and TLR4 signaling have been targeted by specific, non-viral, small interfering RNAs (siRNAs) to knock down the inflammasomes or caspase 1 in rats with kindling-induced SE (45).

Prostanoids

Prostanoids are a family of lipid mediators generated from the cell membrane arachidonic acid by cyclooxygenase enzymes 1 and 2 (COX1 and COX2). Prostanoids bind to specific G protein-coupled receptors (GPCR), hence regulating both innate and adaptive immunity (46).

Monoacyl Glycerol Lipase

The monoacyl glycerol lipase (MAGL) is a lipase constitutively expressed by neurons and a key enabler of 2-arachidonoylglycerol (2-AG) hydrolysis. 2-Arachidonoylglycerol is an endocannabinoid, which likewise prostaglandins are involved in seizures genesis. Hence, the upstream inhibition of the MAGL has the potential to be an effective target in epilepsy therapy (2). In 2018 Terrone et al. (47) did demonstrate CPD-4645 (a selective and irreversible MAGL inhibitor) was effective in terminating diazepam-resistant status epilepticus (SE) in mice. Moreover, clinically relevant outcomes such as reduced cognitive deterioration were ensured by CPD-4645 action: reducing post-SE brain inflammation to prevent neural cell damage. Lastly, the authors noted that SE was more promptly stopped in those mice concomitantly receiving the KD, hence suggesting brain inflammation is the common, final, target. Striking inflammation through different inflammatory pathways may enhance neuroprotection and seizure control.

COX2 Inhibitors and Prostaglandin Receptor Antagonists

Targeting the inducible enzyme COX2 to that of blocking the prostanoid cascade has been tested to interfere with acute seizures or SE. The importance of timing was demonstrated by early anti-inflammatory interventions showing worsening seizures as compared to late-onset interventions (2, 48). Prostaglandin $F_{2\alpha}$ (PGF), which is anti-ictogenic, is indeed predominant in the first hour after SE onset, then the ratio between PGF and the pathogenic prostaglandin E_2 (PGE) normalizes in association with an increase in COX2 synthesis (2). Hence, punctual COX2-related treatments have been considered to prevent epileptogenesis and reduce the frequency of seizures in epileptic patients. COX2 inhibition could either be selective (*coxibs* = selective COX2 inhibitors) or non-selective (*aspirin*). In two in-animal studies testing celecoxib and parecoxib over evoked SE, treatment with celecoxib or parecoxib did show to consistently reduce the number and severity of seizures, together with the improvement of spatial memory deficits (2).

Non-selective blockade of COX2 has been also tested in experimental models of epilepsy, and ASA administration over the chronic, latent, epileptic phase could consistently suppress recurrent spontaneous seizures and inhibit the seizure-induced neuronal loss, preventing aberrant neurogenesis in the hippocampus. Thus, ASA is being actively investigated and has the potential to prevent the epileptogenic processes, including SE occurrence, and may avoid pathological alterations in CNS areas (2, 49). Potential cardiotoxicity is the main limit, bordering COX2 inhibition in clinical practice.

Shifting attention downstream to prostaglandin receptors, highly potent PGE receptor (EP2R) antagonists administered from a 4 h-starting point after the onset of pilocarpine-induced SE, proved to mitigate deleterious consequences such as delayed mortality, functional deficits, alterations of the BBB permeability, and hippocampal neurodegeneration (50). The delayed timepoint of administration further brings evidence that EP2R blockade may allow obtaining neuroprotection later in SE stages, mainly reducing long-term sequelae (2).

Inflammatory Response Lipid Mediators

Specialized pro-resolving lipid mediators that activate GPCRs have a major role in controlling inflammatory responses in peripheral organs. G protein-coupled receptors activation leads both to reduced expression of pro-inflammatory molecules and increased synthesis of anti-inflammatory mediators which can modulate immune cell trafficking and restore the integrity of the BBB. Neuroinflammation was reduced after the intracerebroventricular injection of the omega-3 (n-3) docosapentaenoic acid-derived protectin D1 ($PD1_{n-3DPA}$) in mouse models of epilepsy. Interestingly, recognition of memory deficits after SE also gained improvements (2, 51). Since $PD1_{n-3DPA}$ derives from n-3 polyunsaturated fatty acids (PUFAs), in humans, it may be possible to non-invasively increase $PD1_{n-3DPA}$ levels through the dietary intake of n-3 PUFAs, which are found in flaxseed, walnuts, marine fish, and mammals (52). Another way may then be the developing stable analogs of pro-resolving lipids (51).

Oxidative Stress

Activation of the Toll-like receptors (TLRs) can lead to reactive oxygen species (ROS) production, hence promoting and sustaining inflammatory pathways. The detrimental effects of ROS are usually counteracted through the activation of the nuclear factor E2-related factor 2 (Nrf2). Activated Nrf2 translocates to the nucleus where it heterodimerizes with the small Maf proteins (sMaf) and binds to the antioxidant response element (ARE 5′-TGACXXXGC-3′) battery activating transcription of genes that are involved in antioxidant and cytoprotective tasks (53).

Transient administration of *N*-acetyl-cysteine (NAC), a glutathione precursor, did prove to activate Nrf2 in mouse models of SE, thus inhibiting high mobility group box 1 (HMGB1) cytoplasmic translocation in the hippocampal neural and glial cells and preventing the linkage between oxidative stress and neuroinflammation for which the redox-sensitive protein HMGB1 is central (2). Also, high doses (4–6 g/day) of NAC were used in Unverricht-Lundborg disease (ULD), progressive myoclonus epilepsy (PME), showing overall improvement of myoclonus, ataxia, and generalized tonic–clonic and absence seizures. Neuroprotection and improvements in spatial learning abilities were also observed with retained beneficial effects during treatment (54, 55).

Adeno-associated viral (AAV) vectors gene delivery may provide long-term, persistent, induction of Nrf2 expression in a variety of cell types in the brain, with minimal toxicity. The injection of AAV coding for human Nrf2 in the hippocampus of mice with spontaneously recurrent seizures resulted in a reduction in the number and duration of generalized seizures, which interestingly was performed in the already established epileptic phase, highlighting the direct potential of such interventions in the treatment of epilepsy (56).

INHIBITION OF P-GLYCOPROTEINS

One of the major neurobiological mechanisms proposed to cause drug resistance in epilepsies lays in the removal of

ASMs from the epileptogenic tissue through the expression of multidrug efflux pumps such as the P-glycoproteins (P-gps). P-glycoproteins are the final encoded product of the human multidrug resistance-1 (*MDR-1*) gene, and play a role in treatment response possibly inducing MDR (57, 58). The increased activity of P-gps reduces clinically effective concentrations of ASMs despite adequate serum concentrations, reversing the anti-seizure effects on epileptogenic areas in the parenchyma of the brain (3).

Following the general rule that the higher the lipophilicity of a drug, the faster the entrance into the brain (59), available ASMs are very lipophilic, but more than one-third of the patients do not respond to treatment. The possible reason may be ASMs serve as P-gps substrates; secondly, the P-gps levels are higher (3). Different clinical studies had shown poor prognoses associated with MDR1 gene products, which gave rise to extensive experimental research on the P-gps (3). The adjunctive use of a P-gps inhibitor might counteract drug resistance and efficiently decrease seizure frequency. In addition to verapamil, other first-generation P-gps inhibitors include nifedipine, quinidine, amiodarone, nicardipine, quinine, tamoxifen, and cyclosporin A. It is primarily due to the lack of selectivity and the pharmacokinetic interactions that trials using such agents failed to rule out P-gps inhibition efficacy in other fields such that of oncology (60, 61). First-generation MDR inhibitors required high concentrations to reverse MDR and thus were associated with unacceptable toxicity. In recent years, second and third-generation compounds have been developed which are more selective, highly potent, and non-toxic. Notwithstanding second-generation agents have better tolerability, they still have unpredictable pharmacokinetic interactions (i.e., valspodar is a substrate for cytochrome P450, altering plasma availability of co-administered drugs) and may inhibit other transport proteins. Third-generation inhibitors have more advantages such as high specificity for P-gp, lack of non-specific cytotoxicity, relatively long duration of action with reversibility, and good oral bioavailability. However, despite their selectivity and potency, also this last generation of MDR modulators is far from being perfect and further studies will be needed to outline their effectiveness and safely overcome drug resistance (3, 60). As pertains to clinical research, Iannetti et al. (62) first demonstrated the action of verapamil in a case of prolonged refractory SE and then, subsequently on small series of other types of drug-resistant epilepsies (63, 64).

A novel, yet preclinical, approach for reversing multidrug resistance in epilepsy may derive from the modulation of P-gp by herbal constituents. Nowadays, several herbal formulations and drugs which act by modulating P-gps are available and can be explored as alternative treatment strategies. For example, curcumin (the natural dietary constituent of turmeric) orally administered to pentylenetetrazole-kindled epileptic mice models is known to prevent seizures and related memory impairments (65). The mechanism of action may lie on that curcumin and can reverse multidrug resistance. Hence, curcumin synthetic analogs, which hold more favorable pharmacodynamic properties, have been developed (i.e., GO-Y035); or curcumin has been encapsulated in nanoparticles (NPs) enhancing its solubility and sustaining release inside the brain (66).

Again, piperine (an alkaloid present in black pepper) and capsaicin (the active component of chili peppers) are known to increase curcumin and other P-gps substrates bioavailability and can be therefore used as basic molecules for the development of non-toxic P-gps inhibitors (67, 68).

In conclusion, the identification of an optimal P-gps inhibitor that is potent, effective, and well-tolerated, is desirable to reverse MDR in epileptic patients and will be the challenge of the upcoming years.

GENE THERAPIES

Currently lying at the preclinical evidence, gene-based therapy modulates gene expression by introducing exogenous nucleic acids into target cells. The delivery of these large and negatively charged macromolecules is typically mediated by carriers (called vectors) (69). In treating epilepsy, the main hitch is the BBB, which prevents genetic vectors from entering the brain from the bloodstream. Consequently, an invasive approach may be needed (29). Moreover, several considerations need to be taken into account when translating gene therapy into clinical practice, namely the choice of the viral vector, promoter, and transgene (6).

Viral Vectors

Viral gene therapy may employ three classes of viral vectors, namely, adenovirus (AD), adeno-associated virus (AAV), and lentivirus. All these three viral vectors have successfully demonstrated to attain high levels of transgene delivery in *in-vivo* disease models and clinical trials. However, the risks of immunogenic responses and transgene mis-insertions, together with problems in large-scale production are still a deal to face (70).

Adeno-associated viruses belong to the Parvoviridae family and proved to retain favorable biology, leading their recombinant forms (rAAVs) to become the main platform for current *in-vivo* gene therapies (29). A limited clinical trial on patients with late-infantile neuronal ceroid lipofuscinosis (LINCL) did prove neurosurgical gene therapy to be practical and safe, supporting the potentialities of this kind of approach (71). However, in the view of removing invasiveness, interest was moved to engineered capsid which can confer the ability to cross the BBB and transduce astrocytes and neurons, allowing direct intravenous injection. This was achieved through a process of directed selection in a mouse strain, and further work would be needed to develop a similar variant for use in humans (6, 72).

Retroviruses such as lentivirus share with AAVs the ability to infect neurons and lead to a stable expression of transgenes. Lentiviral vectors (lentivectors) are RNA viruses and the transgenes can integrate into the host genome through the reverse transcriptase gene. However, possible insertional mutagenesis may be reduced by using integration-deficient lentivectors, which simultaneously ensure stable transduction (73). Lastly, lentivectors can package larger genes or regulatory elements as compared to AAVs (6).

Different viral vectors intrinsically tend to infect different neuronal and glial subtypes, but the high specificity of the target is far from their properties. Hence, several efforts have been

made that to identify specific neuron-type targeting promoters: the calcium/calmodulin-dependent protein kinase II (CamKII) promoter is suitable to manipulate excitatory neurons in the forebrain; on the other hand, targeting inhibitory interneurons may be difficult as promoters for specific GABAergic neurons are poorly defined (6). Finally, the optimal promoter should provide the expression of a level of transgene which is sufficient to moderately alter cell properties but avoids cytotoxicity (6, 74).

As for the transgene, gene therapies have been commonly built on the basis that the excitation–inhibition balance is altered in epilepsy. Hence, on a general principle, gene therapy may work through modulating the expression of neuropeptides, and regulation of the neuropeptide Y (NPY) did already show promise, acting both on pro-excitatory Y1 and pro-inhibiting Y2 receptors in the hippocampus (6, 75). Another way may be that of regulating potassium channels; overexpression of the potassium channel Kv1.1 proved effective in preventing epileptogenesis in a mouse model of focal epilepsy, the physiological basis may lie on the modulation of both neuronal excitability and neurotransmitter release (76, 77). Lastly, *chemogenetics* refers to the possibility to use gene transfer to express receptors that are insensitive to endogenous neurotransmitters but highly sensitive to exogenous drugs, in a receptor-to-drug therapeutic approach. This promising approach will also allow adjusting the activating drugs to find the optimum dosage with low interference with normal brain function but efficiently suppressing seizures (6). Further refinements of *chemogenetics* have jet got underway, which may use receptors detecting out-of-range extracellular elevations of the concentration of glutamate and, therefore, inhibiting neurons, preventing drug administration. Although attractive, this strategy will need further work to assess the risk of immunogenicity (6).

Non-viral Strategies

Some of the issues of viral vector-based gene therapy may be overcome by non-viral gene strategies, which provide advantages with regards to the safety profile, localized gene expression, and cost-effective manufacturing. Non-viral gene delivery systems are engineered complexes or NPs composed of the required nucleic acid (pDNA or RNAs) and other materials, such as cationic lipids, peptides, polysaccharides, and so on (70). These vectors have low production costs, can be topically administered, can carry large therapeutic genes, use expression vectors (such as plasmids) that are non-integrating, and do not elicit detectable immune response also after repeated administrations (29, 70). Cationic lipid-based vectors are currently the most widely used non-viral gene carriers. Limitations may include low efficacy due to the poor stability and rapid clearance, or the possible generation of inflammatory or anti-inflammatory responses. Hence, cationic polymers, such as poly(L-lysine) (PLL) or modified variants (PEGylated PLL), constitute alternative non-viral DNA vectors that are attractive for their immense chemical diversity and their potential for functionalization (69).

Antisense Oligonucleotides Therapies

Oligonucleotides are unmodified or chemically modified single-stranded DNA sequences (of up to 25 nucleotides) that hybridize to specific complementary mRNAs. Once bound to targeted mRNAs, oligonucleotides can either promote RNA degradation or prevent the translational machinery through an occupancy-only mechanism, referred to as *steric blockage*. Anyhow, the process leading to protein formation is inhibited. Synthesizing antisense oligonucleotides (ASOs) must deal with making a structure that must be suitable for a stable and selective oligonucleotide/mRNA complex. Moreover, oligonucleotides are rapidly degraded by endo- and exonucleases and the mononucleotides products may be cytotoxic (29, 78). Hence, the use of ASOs in clinical practice requires overcoming problems related to the design, bioavailability, and targeted delivery (78). To date, few *in-human* studies have been conducted that primarily addressed invariably progressive and fatal diseases such as PMEs (79, 80). The authors proved the feasibility of the ASOs-based approach by specifically customizing oligonucleotides over the genetic defect of patients. This opens the way to N-of-1 trials, which will hopefully be the road of the next few years not only in oncology but also in epileptic patients (81).

STEM CELL THERAPY

Recurrent seizures are associated with the loss of inhibitory GABAergic interneurons. Herby, the replacement of lost interneurons through grafting of GABAergic precursors might improve the inhibitory synaptic and reduce the occurrence of spontaneous seizures (6).

Currently, in a pioneering way, progenitors from the medial ganglionic eminence (MGE) derived either from fetal brains or, to avoid the need for immune suppression, from human induced pluripotent stem cells (hiPSCs) proved the most suitable for treating epilepsy, particularly with temporal lobe onset features. Medial ganglionic eminence cells show pervasive migration, differentiate into distinct subclasses of GABAergic interneurons, and efficiently get incorporated into the hippocampal circuitry improving inhibitory synaptic neurotransmission (82, 83). An important point is that MGE progenitors from fetal brains hoist ethical issues, and it is also a challenge to obtain the adequate amount of cells required for clinical application (82). Consequently, the MGE progenitors derived from hiPSCs appear the most suitable donor cell type, as they do not raise ethical problems and are also compatible with patient-specific cell therapy in non-genetic epileptic conditions. However, it will be important to understand whether the suppression of spontaneous recurrent seizures is transient or enduring after the GABAergic progenitor cells grafting (82); moreover, it will be important to assess the safety profile of these hiPSCs, hence they may either exhibit genomic instability or cause undesired differentiation raising concerns for *in human* application (6). In conclusion, the results are exciting, but some points need to be addressed in the next years, before starting a true *in human* application.

CONCLUSIONS

A variety of drugs are being investigated for the treatment of epilepsy, many of whom target previously neglected pathophysiological pathways but demonstrate a favorable efficacy

TABLE 1 | Advanced RCTs on new drugs for epilepsy treatment.

References	Type of RCT and treatment	Study population (n° of pts, type of epilepsy, mean age ± SD)	Previously tested vs. concomitant ASMs (n°)	Primary end point	Outcomes
Devinsky et al. (17)	Double-blind, placebo-controlled RCT 20 mg/kg/d CBD oral solution	214 DS pts M 9.8 ± 4.8 years	4.0 3.0	Change in CSF	- 38.9% reduction in CSF in the CBD group vs. 13.3% in reduction in the placebo group - ≥50% reduction in CSF in 43% pts in the CBD group vs. in 27% pts in the placebo group - 5% pts sz-free in the CBD group vs. 0% sz-free in the placebo group
Thiele et al. (18)	Double-blind, placebo-controlled, phase 3 RCT 20 mg/kg/d CBD oral solution	171 LGS pts M 15.4 ± 9.25 years	6.0 3.0	Change in monthly frequency of drop sz	- 43.9% reduction in monthly drop sz frequency in the CBD group vs. 21.8% reduction in the placebo group - ≥50% reduction in drop sz frequency in 44% pts in the CBD group vs. in 24% pts in the placebo group - Improved overall condition in 58% pts in the CBD group vs. in 34% pts in the placebo group
Devinsky et al. (19)	Multicenter, double-blind, placebo-controlled, phase 3 RCT 10 or 20 mg/kg/d CBD oral solution	225 LGS pts M 15.6 ± 9.9 years	6.0 3.0	Average change in drop sz frequency	- 41.9% reduction in drop sz frequency in the 20 mg CBD group vs. 37.2% reduction in the 10 mg group vs. 17.2% reduction in the placebo group - 50% reduction in drop sz frequency in 39% pts in the 20 mg CBD group vs. in 36% pts in the 10 mg group vs. in 14% pts in the placebo group - Improved PGIC in 57% pts in the 20 mg CBD group vs. in 66% pts in the 10 mg group vs. in 44% pts in the placebo group
Devinsky et al. (20)	OLE 20 up to 30 mg/kg/d CBD oral solution	264 DS pts M 9.8 ± 4.4 years	na 3.0	Long-term safety and tolerability of CBD	- 37.5% reduction in CSF retained for up to 48 w; - 4.8% pts were convulsive sz free and 2.9% pts were totally sz-free in the last 12 w of treatment - ≥50% reduction in CSF observed in more than 40% of pts - 93.2% of pts reported AEs: 36.7% mild; 39.0% moderate; 29.2% severe
Lagae et al. (23)	Double-blind, placebo-controlled RCT 0.2 or 0.7 mg/kg/d of fenfluramine HCl oral solution	119 DS pts M 9.0 ± 4.7 years	na M 2.4 ± 1.0	Change in monthly CSF	- 74.9% reduction in CSF in the 0.7 mg/kg/d group vs. 42.3% reduction in the 0.2 mg/kg/d group vs. 19.2% reduction in the placebo group - ≥50% reduction in CSF observed in 68% pts in the 0.7 mg/kg/d group vs. in 38% pts in the 0.2 mg/kg/d group vs. in 12% pts in the placebo group - 8% pts were sz-free in the 0.7 mg/kg/d group vs. 8% in the 0.2 mg/kg/d group vs. 0% in the placebo group - Improved CaGI in 55% pts in the 0.7 mg/kg/d vs. in 41% pts in the 0.2 mg/kg/d vs. in 10% pts in the placebo group
Lai et al. (25)	OLE 0.2 up to 0.7 mg/kg/d of fenfluramine HCl oral solution (up to 0.4 mg/kg/d if concomitant STP)	232 DS pts M 9.1 ± 4.7 years	na na	Number of pts with VHD or PAH during treatment (median 256 d)	- No pts developed VHD or PAH - 23% pts showed trace of mitral regurgitation (mostly transient)
Krauss et al. (27)	Multicentre, double-blind, placebo-controlled, dose-response RCT 100–200–400 mg/d cenobamate oral solution	437 pts with drug-R focal epilepsy M 39.8 ± 11.8 years	2.0–3.0 2.0–3.0	Change in monthly focal sz frequency	- 55.0% reduction in focal sz frequency in the 200 and 400 mg/d group vs. 35.5% reduction in the 100 mg/d group vs. 24.0% reduction in the placebo group - ≥50% reduction in sz frequency observed in 64% pts in the 400 mg/d group vs. in 56% pts in the 200 mg/d group vs. in 40% pts in the 100 mg/d group vs. in 25% pts in the placebo group

(Continued)

TABLE 1 | Continued

References	Type of RCT and treatment	Study population (n° of pts, type of epilepsy, mean age ± SD)	Previously tested vs. concomitant ASMs (n°)	Primary end point	Outcomes
Sperling et al. (28)	Multicenter, ongoing, phase 3, OLE 12.5 up to 400 mg/d cenobamate oral solution	1,339 pts with drug-R focal epilepsy M 39.7 ± 12.84 years	2.0–3.0 2.0–3.0	Long-term safety of cenobamate	- At least one AE was reported in 84.2% of pts: 77.8% were mild-moderate - At least one serious AE was reported in 8.1% of pts: seizures; pneumonia; fall; dizziness - No cases of DRESS were identified when starting at low dose and titrating every 2 w

AEs, adverse events; ASMs, antiseizure medications; BDI, beck depression inventory; CaGI, caregiver global impression; CBD, cannabidiol; CSF, convulsive seizure frequency; d, day; drug-R, drug-resistant; DS, Dravet syndrome; LGS, Lennox-Gastaut syndrome; LINCL, late infantile neuronal ceroid lipofuscinoses; M, mean; n°, number; na, not assessed; OLE, open label extension; PAH, pulmonary arterial hypertension; PGIC, patient global impression of change; Pts, patients; RCT, randomized clinical trial; Ref, reference; SD, standard deviation; SF, seizure frequency; SUDEP, sudden unexpected death in epilepsy; STP, stiripentol; sz, seizures; VHD, valvular heart disease; w, weeks; y, years.

TABLE 2 | Advanced RCTs on new non-pharmacological treatments for epilepsy.

References	Type of RCT and treatment	Study population (n° of pts, type of epilepsy, mean age ± SD)	Previously tested vs. concomitant ASMs (range, mean)	Primary end point	Outcomes
Orosz et al. (31)	Retrospective, open-label, multicenter study VNS Therapy, Cyberonics	347 pts with DRE of any type M 2.7 ± 3.0 y	1–27, M 6.9 1–6, M 3.0	Change in the "predominant sz type" frequency at 12 months of FU	- 5.5% pts became sz free (i.e., no sz of the "predominant sz type") - 32.1% pts achieved ≥50% sz reduction - 17.1% pts had a 25–49% sz reduction - The percentage of responders increasing over time: 32.5%, 37.6%, and 43.8% at 6, 12, and 24 months of FU
Boon et al. (32)	Prospective, observational, unblinded, multicenter study Model 106 VNS Therapy System	31 pts with focal-onset sz, iTC, and DRE M 39.6 ± 13.4 y	na na	≥80% sensitivity for iTC sz in at least one CBSDA, and investigate FP rate	- 37/66 (56%) sz were associated with a ≥20% heart rate increase - 11/66 (17%) sz were associated with iTC (55% or 35 bpm heart increase from baseline, minimum 100 bpm) - ≥80% sz detection sensitivity achieved in multiple CBSDA - FP rate ranged from 0.5 to 7.2/h
Bergey et al. (37)	Prospective, open-label, multicenter study RNS System, NeuroPace	230 pts with focal-onset sz, sGTC sz, and DRE (feasibility and pivotal studies already completed) M 34.0 ± 11.4 y	na 0–8, M 2.9	Long-term efficacy and safety of RNS	- 66% median reduction in sz at 6 y of FU with a RR of 56% - Improvements in QoL were maintained at 5 y of FU ($p < 0.05$) - Most common serious device-related AEs (5.4 y of FU) were implant site infection (9.0%) and neurostimulator explantation (4.7%)
DeGiorgio et al. (40)	Double-blind, parallel-group, phase 2, multicenter RCT External pulse generator for eTNS	50 pts with focal-onset sz, sGTC sz, and DRE M 33.7 y	na, M 3.35 na	Change in mean monthly SF, and RR (>50% sz reduction), time to the fourth sz	- 16.1% reduction in sz frequency for the treatment group vs. 10.5% reduction for the control group - 30.2% RR for the treatment group vs. 21.1% RR for the control group - Net increase 2.5 d (20%) to fourth sz in the treatment group vs. decrease 5 d (21.7%) in the control group ($p = 0.73$)

AEs, adverse events; ASMs, antiseizure medications; bpm, beats per minute; CBSDA, cardiac-based seizure detection algorithm; DRE, drug-resistant epilepsy; eTNS, external trigeminal nerve stimulation; FP, false positive; FU, follow-up; h, hours; iTC, ictal tachycardia; M, mean; n°, number; na, not assessed; Pts, patients; RCT, randomized clinical trial; Ref, reference; RNS, responsive neurostimulation; RR, retention rate; SF, seizure frequency; sGTC, secondarily generalized tonic-clonic; sz, seizures; VNS, vagal nerve stimulation; y, years.

profile, together with low to mild grade AEs (15). Traditional ASMs, given alone or in a fair combination, are invariably the initial therapeutic approach; afterward, if drug resistance occurs, more than one underlying pathophysiological mechanism may likely contribute (14). Currently, uncontrolled epilepsy is often disabling, with patients experiencing increased comorbidity, psychological, and social dysfunction, combined with an increased risk of premature death. In younger patients, cognitive and neurodevelopmental impairments are severe consequences of recurrent spontaneous seizures, impacting the QoL and future

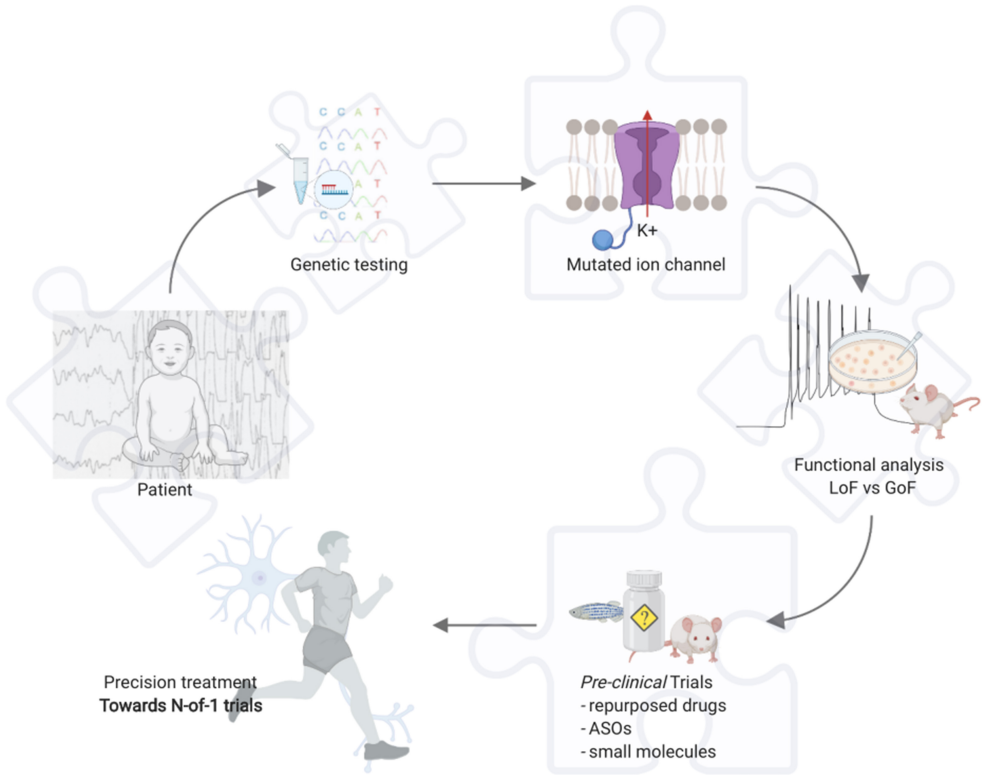

FIGURE 1 | Example for precision medicine in epileptic channelopathies. Toward N-of-1 trials. Created with BioRender.com. ASOs, antisense oligonucleotides; GoF, gain of function; LoF, loss of function.

independence (44). Accordingly, gaining a reduction of either the severity or frequency of seizures might have benefits (44) and hitherward new therapeutical strategies are in the pipeline.

Cannabidiol, FFA, and cenobamate have been shown to efficiently control seizures and are generally well-tolerated; particularly, an increase in the number of seizure-free days was observed with positive outcomes on the QoL of patients (16). Comparison of treatments such as VNS, DBS, and TNS are needed to decide which modality is the most effective; moreover, data collection on promising *non-invasive* neurostimulation modalities will allow getting a precise estimate of their therapeutic efficacy and long-term safety (30) (**Tables 1**, **2**).

Evidence on the role of neuroinflammation in epilepsy suggests that drugs that modulate specific inflammatory pathways could also be used to control seizures and improve neurological comorbidities, such as cognitive deficits and depression. Notably, many anti-inflammatory drugs are already available and could be repurposed in patients with epilepsy. Another mechanism likely involved in drug-resistant epilepsies is the undue expression of multidrug efflux transporters such as P-gps (52); however, the use of P-gps inhibitors in the clinical practice did prove disadvantageous for inseparable systemic toxicity (3). This arises the need to directly modulate not the transport but the expression of the P-gps (3). Finally, epilepsy represents a field suitable for the development of personalized approaches, requiring integration of clinical measures with both genomics and other *-omics* modalities (14).

Today epilepsy carries restrictions in the everyday life of the affected people, together with social burdens, and eventually high-level burdens for caregivers in EE. Hitherward, the continuous pursuit of the best treatment approach that nowadays, with the widening understanding of the pathophysiological basis of the epilepsies, is inevitably moving toward a "*precision*" approach. Gene hunting and new genes discovery proved essential in this way, but further support derives from functional *in-vitro* and *in-vivo* studies, i.e., in epileptic channelopathies it is crucial to understand whether the phenotype is caused by the loss- or gain-of-function mutations in the encoded protein through *patch-clamp* studies (**Figure 1**). Likewise, if a novel gene is identified it is fundamental to understand through which mechanism it may cause the disease, consequently identifying the best treatment to reverse the functional defect. However, given a PM-based approach, this may not yet be enough, and a holistic evaluation of the patient involves the clinician to deeply know an own expected vulnerability to drugs through *pharmacogenomics*; thus, avoiding potential AEs.

Targeting the biological mechanism responsible for epilepsy could lead either to repurpose as ASMs and adjust dosages of drugs yet used in other fields of medicine (i.e., FFA, COX2

inhibitors, or inhibitors of P-gps) or even to develop outstanding treatments such as gene therapy. Great advances have been achieved in gene-based therapies, ranging from the development of new delivery material to the improved potency and stability of delivered nucleic acids. However, this field is still actually limited by the little understanding of exogenous-endogenous DNAs interaction and the invasive nature of some neurosurgical approaches. Moreover, targeted approaches (i.e., gene therapy, but also innovative drugs) currently carry high economic costs, which are covered by pharmaceutical industries during clinical trials but are hardly affordable for patients. In the new few years, the standardization of drug development, together with a larger use, and faster approval by regulatory agencies will probably make these treatments cheaper for patients.

The inflammatory pathways are common over epilepsies of different etiology and may therefore be reliable targets for treatment. However, targeting such complex and cross-interacting pathways of the human system may prove difficult, potentially altering basic life signals and causing a *plethora* of AEs further impacting the QoL of patients. Hence, also from this site, the next few years will be important to expand our knowledge and act consciously or even early, having fully comprised the red flags (biomarkers) of altered pathways through *-omics* studies.

Overall, research has changed our approach to epileptic patients, but PM is not always straightforward, and the pathophysiology of diseases may be more complex than what we can *model*, as different concomitant genetic variants, epigenetics, or the environment may modulate phenotypes in unintelligible and irreproducible ways. Moreover, nowadays patients are still often belatedly diagnosed raising the need to better define the way clinicians address *phenotyping*, which if incomplete could lead primarily toward the application of NGS epilepsy panels and then to whole-exome or genome sequencing, but invariably delaying diagnosis. Hence, also newer and standardized means of *phenotyping* will be needed, and wide opportunities in this are opened by the human phenotype ontology (HPO), a standardized vocabulary to describe phenotypic abnormalities. The hope will remain that of early diagnosis, early and non-invasive treatment to heal symptoms, improving the QoL of patients, and, in encephalopathies, improving the learning curve of patients.

AUTHOR CONTRIBUTIONS

AR: conceptualization, writing-original draft, writing-review, and editing lead. AG: writing-original draft. GB: writing-review and editing support. EA, MV, GP, MI, SL, VS, and CM: writing-review and editing support. PS: conceptualization, funding acquisition, supervision, writing-review, and editing. All authors contributed to the article and approved the submitted version.

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De novo STXBP1 Mutations in Two Patients With Developmental Delay With or Without Epileptic Seizures

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Objectives: Mutations in the STXBP1 gene have been associated with epileptic encephalopathy. Previous studies from *in vitro* neuroblastoma 2A cells showed that haploinsufficiency of *STXBP1* is the mechanism for epileptic encephalopathy. In this *ex vivo* study, *STXPB1* DNA mutations and RNA expression were assessed from two patients to help understand the impact of *STXBP1* mutations on the disease etiology and mechanism.

Methods: Microarray analysis and DNA sequencing were performed on two children with development delay, one with and one without infantile spasms. Different pathogenic mutations of *STXBP1* were identified in the patients and RNA expression of *STXPB1* was then performed by RT-Q-PCR on RNA extracted from blood samples of each patient.

Results: Pathogenic deletion [of exons 13–20 and 3′ downstream of *STXBP1*] and nonsense mutation [c.1663G>T (p.Glu555X) in exon 18 of *STXBP1*] were detected from the two patients, respectively. RNA analysis showed that 1) the deletion mediated RNA decay, and that 2) no RNA decay was identified for the nonsense mutation at codon 555 which predicts a truncated STXBP1 protein.

Significance: Our RNA expression analyses from the patient blood samples are the first *ex vivo* studies to support that both haploinsufficiency and truncation of STXBP1 protein (either dominant negative or haploinsufficiency) are causative mechanisms for epileptic encephalopathies, intellectual disability and developmental delay. The RNA assay also suggests that escape from nonsense-mediated RNA decay is possible when the nonsense mutation resides <50 nucleotides upstream of the last coding exon-exon junction even in the presence of additional non-coding exons that are 3′ downstream of the last coding exon.

Keywords: *STXBP1*, haploinsufficiency, dominant negative, nonsense mutation, RNA expression

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INTRODUCTION

Missense, nonsense, frame shift mutations and deletions of all or part of the *STXBP1* gene have been reported in the literature in more than 280 patients affected with epileptic encephalopathy patients with early infantile onset-4 (OMIM # 612164). All patients with germline pathogenic variants and deletions of *STXBP1* have global developmental delay, intellectual disability and cognitive dysfunction. The majority of affected patients present with seizures (1). Mutations or deletions of *STXBP1* have been reported primarily in patients affected with Ohtahara syndrome (2, 3), West syndrome (4), and less frequently in patients affected with early myoclonic epileptic encephalopathy (5, 6), Dravet syndrome (7), Lennox-Gaustaut syndrome (8), Angelman/Pitt Hopkins-like syndrome phenotype (9, 10), and atypical Rett/Rett-like phenotypes (11). Ohtahara syndrome is characterized by neonatal onset severe seizures, tonic spasms, burst suppression pattern on EEG, intellectual disability and developmental delay (2). West syndrome is defined by infantile spasm onset between 3 and 12 months of age with atypical hypsarrhythmia (4). Recently, mutations or deletions of *STXBP1* have been reported in 14 patients with intellectual disability without epileptic seizures (12, 13). Somatic mutation of *STXBP1* was also identified in a patient with focal cortical dysplasia (14).

STXBP1 encodes syntaxin binding protein 1 which is highly expressed in brain and plays a role in neurotransmitter release as part of the synaptic fusion machinery. There are two isoforms of the *STXBP1* gene. One isoform (isoform a) contains 20 exons (NM_003165) encoding 603 amino acids (P61764-2) and another isoform (isoform b) has 19 exons (NM_001032221) encoding 594 amino acids (P61764-1), respectively. The difference between the two isoforms at the amino acid sequence level is from positions 576 to 594 or to the C-terminal end (3). Mutations in *STXBP1* can be detected by sequencing analysis for 83% of cases, by targeted deletion/duplication analysis for 5% of cases and by microarray analysis for 12% of cases (7). Haploinsufficiency of *STXBP1* has been proposed as the mechanism for the epileptic encephalopathies based on expression experiments of mutant STXBP1 proteins in cultured neuroblastoma 2A cells (3). Recently, a dominant negative mechanism has been suggested from missense mutant studies (15).

In this study, we report genomic and RNA findings on two unrelated children with different *STXBP1* alterations. The results are presented for each proband in the Genetic Anomalies and *STXBP1* Expression sections. Our findings from patient blood samples are the first *ex vivo* assay to support that both haploinsufficiency and truncation of STXBP1 protein (either dominant negative or haploinsufficiency) are causative mechanisms for epileptic encephalopathies, intellectual disability and developmental delay.

MATERIALS AND METHODS

Patients

Proband 1: A 2 year old male child was referred for microarray testing due to clinical findings of developmental delay, intellectual disability and hypotonia. An intragenic deletion of the *STXBP1* gene was detected by microarray analysis. Parental blood samples were obtained for follow-up studies to determine if the deletion was inherited or *de novo*.

Proband 2: A 6 year old child, with a history of infantile spasms, was tested with a 51-gene infantile epilepsy panel (GeneDx, Gaithersburg, Maryland, USA). A nonsense mutation, c.163G>T (p.Glu555X), was identified in exon 18 of *STXBP1*.

DNA and RNA Extractions

DNA was extracted from peripheral blood cells from the probands, their parents, and five normal control males using the MagNA pure compact instrument and MagNA Pure LC DNA isolation kit 1 (Roche Diagnostics, Laval, Quebec, Canada). RNA was extracted from blood samples from the probands, and two normal male controls of similar age (1 and 6 years) using the RNeasy mini kit (QIAamp® RNA Blood Mini, Qiagen, Hilden, Germany).

Microarray Analysis

Approximately 200 ng of genomic DNA was used for the ThermoFisher High Resolution CytoScan HD Array studies according to the manufacturer's protocol. Genomic copy number variants (CNVs) and absence of heterozygosity (AOH) were identified using ChAS software from Affymetrix/ThermoFisher Scientific (Waltham, MA, USA).

Relative Quantitative-PCR (Q-PCR) Analysis

To confirm the small deletion and determine the inheritance of the deletion, Q-PCR studies were performed on the genomic DNA extracted from proband 1, parents and five normal controls. Two pairs of primers designed from within the hemizygously deleted region and undeleted region were used, respectively for the Q-PCR studies. Primer pair 1 is located in a non-deleted region in *STXBP1* intron 1: 5′ GACATTTGCAAAACGGCATC 3′-F and 5′ TGTTGGTGATGAGAAAGGTCA 3′-R (**Figure 1A**). Primer pair 2 is located within the deleted region in intron 19: 5′TTGCTTGTAACGAGGAAGCT 3′-F and 5′ TGAAGAGTG AACCATTGCCA 3′-R (**Figure 1A**). A *FOXP2* gene amplicon was used as a two-copy reference control for the relative Q-PCR calculation. It was amplified using primer pairs: 5′-TGC TAGAGGAGTGGGACAAGTA 3′-F and 5′ CAAAAGCCACAG CAATCCTT 3′-R (courtesy of TCAG, Hospital for Sick Children, Toronto, Canada). The relative Q-PCR amplification using 9 ng genomic DNA in a total 15 ul reaction volume was performed on the Roche LightCycler®480 real time PCR instrument using the LightCycler® 480 high resolution melting master protocol. Briefly, the DNA was denatured at 95°C for 10 min, amplified at 95°C for 10 s, 60°C for 15 s, 72°C for 15 s for 45 cycles. The PCR products were melted at 95°C for 10 s, 65°C for 1 min, continuous at 95°C. The products were cooled to 37°C for 10 min. The LightCycler®480 software v1.5 using the ΔΔ Ct method determined the ratio of the amplified target sequence (*STXBP1*) to the amplified reference sequence (*FOXP2*) and normalized with the five pooled normal control samples (16–18). Each run contained triplicates for each sample and the mean number was obtained from the triplicates. Copy numbers were

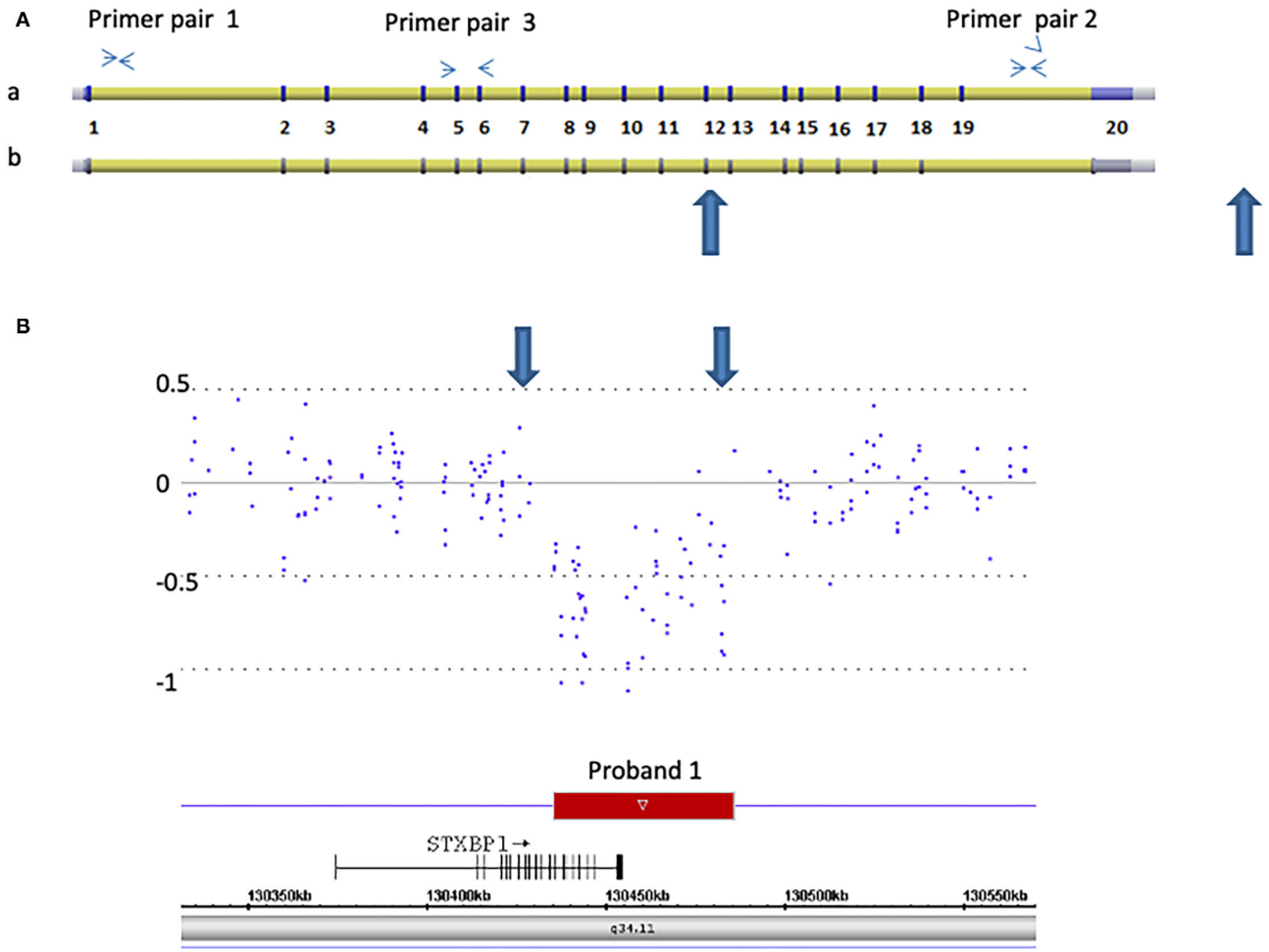

FIGURE 1 | Detection of a 50 kb deletion of *STXBP1*, exons 13 through 20 and 3′ downstream genomic region in proband 1. **(A)** Schematic representation of two isoforms of *STXBP1* (a: NM_003165. b: NM_001032221) (not scaled). The exons (numbered 1–20) and introns are shown. Primer pairs 1 and 2 are located at introns 1 and 19, respectively. They were used for Q-PCR analysis. Primer pair 3 is located at exons 5 and 6 and was used for RT-Q-PCR analysis. **(B)** Microarray scan of ∼250 kb region encompassing the *STXBP1* region. CytoScan HD array probes over the region are represented as dots. The numbers on the Y-axis represent Log2 values. The rectangle represents the region deleted. The *STXBP1* gene relative to the deletion (small arrow indicates the orientation from 5′ to 3′ of the gene), genomic location [hg19], and chromosome 9 band is indicated. Large blue arrows demarcate the region deleted in both **(A,B)**.

interpreted as loss (1n), normal (2n), and gain (3n) while the ΔΔ Ct ratios were 0.4–0.7, 0.8–1.2, and 1.3–1.7, respectively.

Reverse Transcription-Q-PCR (RT-Q-PCR) Analysis

To determine if the deletion in proband 1 and the nonsense mutation in proband 2 mediate RNA decay, RT-Q-PCR studies were performed on RNA isolated from each proband and similar age- and gender- matched controls. Complementary DNA (cDNA) was obtained from reverse transcription (RT) of 2.5 ug total RNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen/ThermoFisher Scientific, Waltham, MA, USA). One pair of primers, outside of the *STXBP1* genomic deletion region, was designed on exons 5 and 6 (primer 3): 5′ TCTCATCAGTGACTTTAAGGACC 3′-F, 5′ AGTTTTGATGACTTTGGCTGCT 3′-R (**Figure 1A**) and was used for the RT-Q-PCR analysis.

PCR amplification was performed with the Applied Biosystems GeneAmpR PCR System 9700 (Applied Biosystems/ThermoFisher Scientific, Waltham, MA, USA) for 38 Cycles at 95°C denaturation for 10 s, annealing at 59°C for 15 s and extension at 72°C for 15 s. The PCR amplification was completed during the linear phase at cycle 38. The PCR products were separated on the TapeStation 2200 instrument with a High Sensitivity D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany) as per the manufacturer's specifications for analysis of the RT-Q-PCR products. Data were obtained and images were captured using TapeStation Analysis Software (A.02.01) (Agilent Technologies, Waldbronn, Germany). The tests were repeated more than three times.

Consent forms were obtained from each family for this study. Research Ethics Boards (REB) approval was not required by our Institution based on the nature of the study (https://www.uwo.ca/research/_docs/ethics/hsreb_guidelines/Case_report_vs_research.pdf).

RESULTS

Clinical Profiles

Proband 1: This male child presented with feeding difficulty and generalized hypotonia noted in the first few weeks after birth, delayed motor milestones (sitting at 12 months, walking with assistance at 24 months) as well as delays in both fine motor and language domains. At 2 years of age, he displayed behavioral stereotypies (hand flapping) and autistic traits (preoccupation with spinning objects). He has no history of seizures. His growth parameters were: height of 85 cm (10–25th%ile), weight of 11.85 kg (10%ile) and occipital frontal head circumference of 47 cm (3%ile) with a brachycephalic skull shape. Mild bilateral 5th finger clinodactyly was noted on physical examination, but no other significant dysmorphic features. Imaging studies of the brain showed increased periventricular T2 signal suggestive of delayed myelination. At age 3 years he was investigated for staring spells with an electroencephalogram (EEG) that showed mild background slowing, without any evidence of abnormal epileptiform activity. There were no sleep related changes documented on the available record. No other prior EEG recordings were available for review.

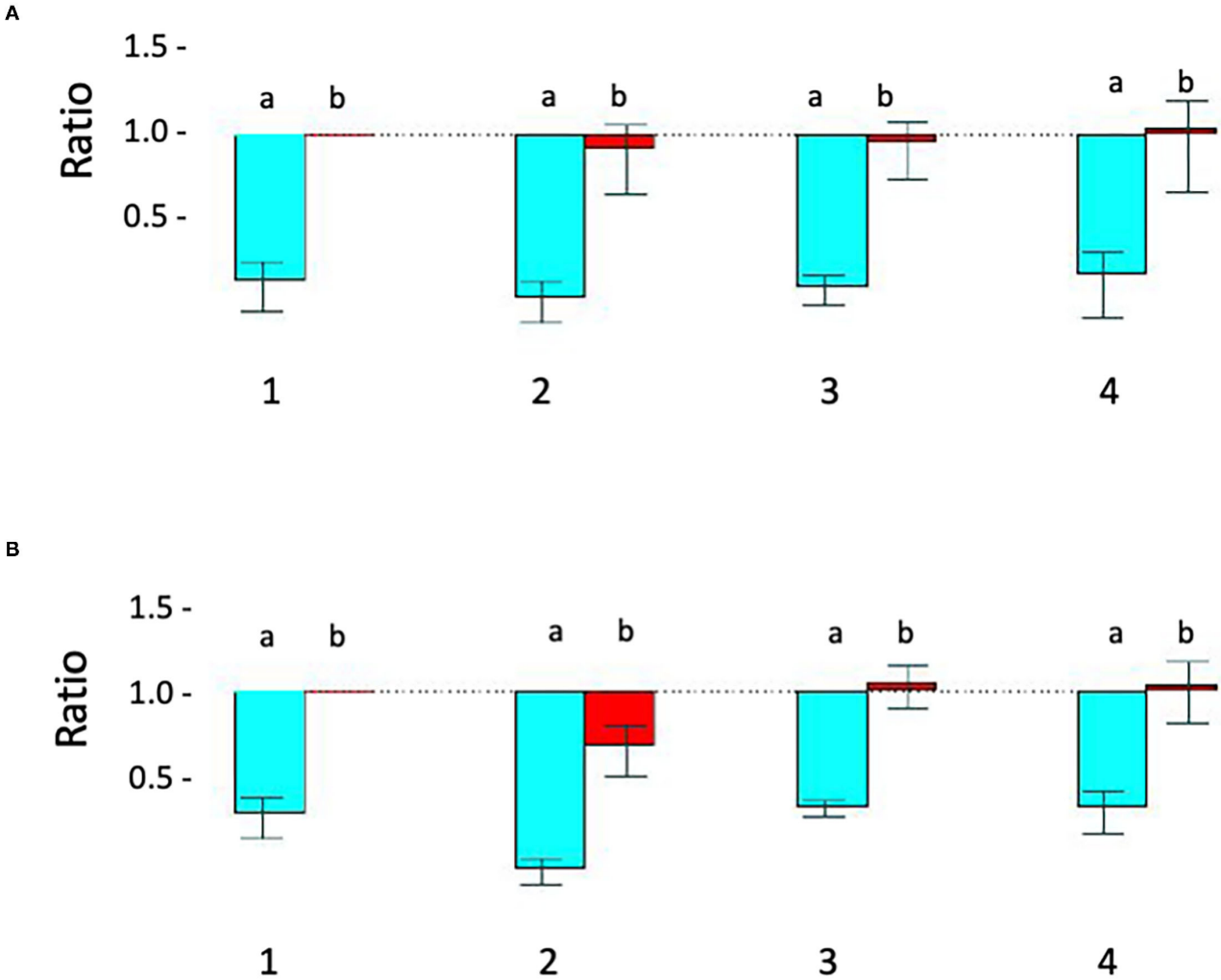

FIGURE 2 | Relative quantitative –PCR [Q-PCR] confirms *STXBP1* deletion in proband 1 and demonstrates that it is *de novo*. **(A)** Q-PCR product using primer pair 1 (intron 1) did not detect the deletion in the proband 1 (#2), his parents (#3-mother, #4-father) and a pooled normal male sample control (#1). **(B)** With primer 2 (intron 19), Q-PCR product confirmed the *STXBP1* deletion in proband 1 (#2b) detected by microarray. No deletion was detected in the proband's mother (#3) and father (#4) or the control (#1). a = target/reference ratio (i.e., *STXBP1/FOXP2*); b = normalized ratio (i.e., = proband's a/normal controls a, mother's a/controls a and father's a/controls a). Error bars indicate average standard error of the mean obtained from normalization of triplicates for each sample. The test was reproducible and was repeated more than three times.

TABLE 1 | Q-PCR analysis results for proband 1, his parents and the five pooled male controls using primer pair 1 at intron 1 of *STXBP1*.

Sample	Reference gene	Target Cp-mean	Reference Cp-mean	Target/reference	Normalization
Pooled male control	FOXP2	31.47	27.54	6.55E-02	1
Proband 1	FOXP2	31.64	27.46	5.54E-02	0.8458
Mother	FOXP2	31.73	27.22	6.43E-02	0.9817
Father	FOXP2	31.12	27.51	8.19E-02	1.2504

The analyses showed that proband 1 and his parents have no deletion at intron 1 of the STXBP1 gene.

TABLE 2 | Q-PCR analysis results of proband 1, his parents and the five pooled male controls by using primer pair 2 at intron 19 of *STXBP1*.

Sample	Reference gene	Target Cp- mean	Reference Cp-mean	Target/reference	Normalization
Pooled male control	FOXP2	31.52	28.39	0.1138	1
Proband 1	FOXP2	32	27.46	4.32E-02	0.3796
Mother	FOXP2	30.73	27.77	0.1285	1.1292
Father	FOXP2	30.48	27.51	0.1276	1.1213

The analysis confirmed the deletion detected by microarray analysis.

Proband 2: This 6 year old male presented to the neurogenetic clinic with a history of epileptic spasms with onset at 2 months of age, global developmental delay, poor visual attention (cortical visual impairment) and bilateral clubfeet with no major facial dysmorphism. His EEG findings at age 3 months confirmed the presence of discontinuous suppression burst pattern with multiple independent spike foci, features consistent with an epileptic encephalopathy. Subsequent follow up recordings done at regular intervals showed variable features with slow background rhythms, regional (occipital) expression of spike activity, with further enhancement and activation of spiking in sleep. By age 12 years the EEG recordings had begun to show features of generalized slow spike wave activity (2–2.5 Hz), paroxysmal fast (recruiting rhythms) in keeping with evolution of his epilepsy. Imaging studies showed non-specific features of generalized loss of gray matter volume. Extensive investigative work-up including biochemical studies for inborn errors of metabolism were negative. The patient was initially treated with Vigabatrin titrated to a maximum of 120 mg/kg/day, and further medication changes were dictated by appearance of refractoriness to therapy. Ophthalmological monitoring for retinal toxicity was continued. Valproic acid, and Levetiracetam were added sequentially and withdrawn due to a lack of benefit in terms of seizure control. Eventually, by age 4 years he was weaned off Vigabatrin. He did show a period of seizure remission for about 18 months around age 5 years, after which tonic spasms made a reappearance at age 7 years. At age 12 years, he continues to report nocturnal tonic seizures sometimes in clusters, and is currently maintained on Rufinamide 31 mg/kg/day in three divided doses daily and Clobazam 0.25 mg/kg at bedtime.

Genomic Anomalies

Proband 1: A 50 kb deletion including 56 oligonucleotide probes in chromosome region 9q34.11 was detected using CytoScan HD Array and ChAS analysis (**Figure 1B**). The genome coordinates of the deletion are 9q34.11(130 435 492–130 485 618)x1 [hg19]. This deletion resulted in loss of exons 13 through 20 and 3' downstream of the *STXBP1* gene (NM_003165, NM_001032221, OMIM# 602926). The Q-PCR studies confirmed the deletion in the proband and revealed the deletion was *de novo* (**Figures 2A,B** and **Tables 1**, **2**). No identical deletion has been previously reported in literature or databases.

Proband 2: Extracted DNA was sent to GeneDx, Inc (Gaithersburg, MD, USA) for testing with their infantile epilepsy 51-gene panel in 2013. A c.1663G>T (p.Glu555X) in exon 18 of *STXBP1* was detected. This variant is predicted to result in a premature stop codon at position 555 (nonsense mutation) in domain 2 (13). This variant has not been previously reported in the literature. Parental DNA sequencing studies revealed that this nonsense mutation was *de novo*.

STXBP1 Expression

Proband 1: RT-Q-PCR products from exons 5–6, outside of the deleted genomic region, showed that the expression level of the *STXBP1* was reduced to ∼half of the expression level of the similar age normal control 2. This indicated that the deletion of exons 13–20 mediated one allele of RNA decay (**Figure 3** and **Table 3**).

Proband 2: The expression level of *STXBP1* was not reduced (**Figure 3** and **Table 3**). It is predicted to result in a truncated STXBP1 protein product with stop codon at amino acid position 555 in domain 2.

DISCUSSION

STXBP1 protein plays an important role in regulating the release of neurotransmitters during synaptic docking and fusion of the vesicle at the synaptic membrane. Mutations of *STXBP1* have been associated with autosomal dominant patterns associated with diverse neurodevelopmental phenotypes

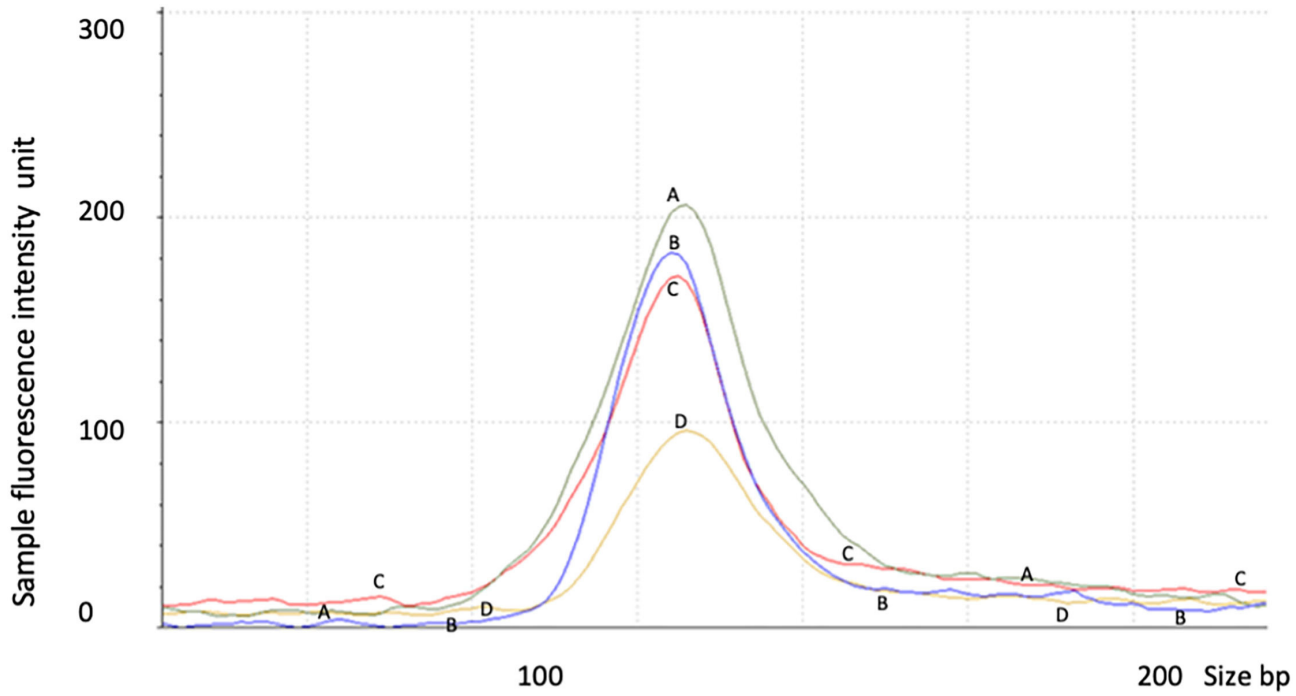

FIGURE 3 | RNA decay observed in proband 1 with *STXBP1* deletion not observed in proband 2 with nonsense mutation. Control 1 (A) = 6 year old male, control 2 (C) = 1 year old male, proband 1 (D) = 2 year old male with partial deletion of *STXBP1*, and proband 2 (B) = a 6 year old male with c.1663G>T nonsense mutation of *STXBP1*. The data were averaged from triplicates for each sample. The test was repeated for at least three times.

TABLE 3 | RT-Q-PCR analyses of RNA expression from probands 1 and 2 relative to age-matched controls using primer pair 3 located at exons 5 and 6 of *STXBP1*.

Sample	Age	Area	Proband1/sample (%)	Proband 1/control average (%)	Proband 2/sample (%)	Proband 2/control average (%)
Control 1	6	0.118	44.10		96.60	
Control 2	1	0.102	51.00		111.80	
Proband 1	2	0.052	100.00	47.60	219.20	
Proband 2	6	0.114	45.60		100.00	104.20

Proband 1 had ∼51% of STXBP1 expression compared to the similar age control 1 (Expression=[Area of proband 1] / [Area of control 2]), or 47.6% of expression compared to the average of both controls (Expression=[Area of proband 1] / [Area of control 1 + area of control 2]). Proband 2 had ∼97% of STXBP1 expression compared to same age control 1, or ∼104% expression compared to the average of the both controls. Thus, the analysis detected RNA decay in proband 1 but not in proband 2. The results were reproducible. The tests were repeated at least three times.

that include autism, intellectual disability, developmental delay and early infantile epileptic encephalopathy (7, 14). Approximately 136 point mutations (including splice, nonsense, missense), small indels (<100 kb, including insertions, deletions and duplications) and 26 cases with large copy number variants (CNVs) affecting *STXBP1* have been reported in the literature (14). Estimated incidence rate is 3.3–3.8 per 100,000 births (14). The mutations were distributed throughout all domains (domains 1 through 3) of the STXBP1 protein, and either reduced the amount of functional protein (haploinsufficiency) or caused an abnormal structural protein. An abnormal amount or structure of STXBP1 protein impairs the release of neurotransmitters that lead to uncontrolled excitation of neurons and seizures. However, the cause of developmental delay, intellectual disability and other phenotypes by altered STXBP1 protein is unknown. No established genotype and phenotype correlation has been established (15).

Our RT-Q-PCR assay demonstrated that the nonsense mutation detected at amino acid position 555 of STXBP1 in proband 2 did not lead to RNA decay. This mutation is located in exon 18, the second to last exon and ∼40 nucleotides upstream of the last exon (exon 20) of *STXBP1* in *isoform b* and is consistent with the mechanism of escape from nonsense-mediated RNA decay where it is <50 nucleotides upstream of the last exon-exon junction (19). For *isoform a*, the mutation is located at the third last exon and approximately 37 nucleotides upstream of the second last exon (exon 19). However, the

exon 20 in *isoform a* is not a coding exon, thus exon 19 is the last coding exon. It indicates that escape from nonsense-mediated RNA decay is possible where the nonsense mutation resides <50 nucleotides upstream of the last coding exon-exon junction even in the presence of additional non-coding exons at the 3′ downstream end of the coding exon. The escape of nonsense-mediated RNA decay predicts that truncated STXBP1 proteins could be produced. The predicted truncated proteins are short of 39 and 48 amino acids at the C-terminal end in domain 2 for *isoforms b and a*, respectively. This indicates that domain 2 is critical for the normal function of STXBP1. It has been proposed that missense mutations result in STXBP1 protein destabilization and are prone to misfolding, aggregation and degradation (20). Based on missense mutations that can cause symptoms as severe as frameshift and heterozygous deletion mutations, it was proposed that missense mutants likely act to deplete the normal SYXBP1 protein in a dominant negative way (14). Similarly, the truncated STXBP1 protein predicted in proband 2 may work in a dominant negative way that could lead to normal protein destabilization or inappropriate binding with syntaxin-1 protein or other SNARE (soluble N-ehtylmaleimide-sensitive factor attachment receptor) proteins required in synaptic vesicle docking, priming and fusion (20). Alternatively, the truncated STXBP1 protein in proband 2 may lose its function completely and result in haploinsufficiency as discussed in a patient with truncated STXBP1 with atypical Rett/Rett-like phenotypes (11). More studies of structural and functional assays on the truncated STXBP1 protein will be needed to clarify whether dominant negative or haploinsufficiency is the causative mechanism for epileptic encephalopathies.

In proband 1, who had a deletion of exons 13 through 20, our RT-Q-PCR assay suggested mediated RNA decay, expected to result in haploinsufficiency of STXBP1 protein. This finding demonstrates the haploinsufficiency mechanism for STXBP1 related disorders directly from RNA extracted from patient blood samples.

Proband 1 and proband 2 have common clinical features of developmental delay and intellectual disability. However, proband 2 presented with infantile spasms which were not noted in proband 1. Whether the difference is due to the different nature of mutations in *STXBP1* (deletion vs. nonsense mutation) remains unclear. Several studies revealed no correlation between the type of mutations or position within the gene with respect to epilepsy, cognitive abilities and other phenotypes (7, 20). Thus, the differences may have contribution from each individual's genetic background. Epistasis is a possible explanation for this, which the phenotypes depend on the presence of one or more modifier genes (21).

CONCLUSION

We believe our RT-Q-PCR assay represents the first *ex vivo* assay to demonstrate that both truncation and deletion of *STXBP1*, resulting in truncated STXBP1 protein and reduced protein product, cause STXBP1 related disorders. Domain 2 of STXBP1 protein is critical for the normal function. The findings support haploinsufficiency and truncation/structure anomaly (either acts as dominant negative or haploinsufficiency) as mechanisms for the disorders. Our RNA assay also indicates that escape from nonsense-mediated RNA decay is possible where the nonsense mutation resides <50 nucleotides upstream of the last coding exon-exon junction even in the presence of additional non-coding exons at the 3' downstream of the coding exon. STXBP1 RNA and protein is highly expressed in brain, pancreas, soft tissue and lowly expressed in white blood cells (https://www.genecards.org/cgi-bin/carddisp.pl?gene=STXBP1). Our RT-Q-PCR assay supported that *STXPB1* RNA is also expressed in peripheral blood, and this provides a much more accessible way for *ex vivo* study.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/clinvar/variation/932281/.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PY contributed to conception, design of the study, and wrote the first draft of the manuscript. ANP wrote clinical sections of the manuscript. ANP, CP, and SL contributed clinical and treatment data and edited the manuscript. SG contributed clinical data. RB conducted the experiments and analyzed the data. JHK discussed the study and edited the manuscript. All authors contributed to manuscript, read, and approved the submitted version.

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frontiers in Neurology

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Epileptic Phenotypes Associated With SNAREs and Related Synaptic Vesicle Exocytosis Machinery

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SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptor) are an heterogeneous family of proteins that, together with their key regulators, are implicated in synaptic vesicle exocytosis and synaptic transmission. SNAREs represent the core component of this protein complex. Although the specific mechanisms of the SNARE machinery is still not completely uncovered, studies in recent years have provided a clearer understanding of the interactions regulating the essential fusion machinery for neurotransmitter release. Mutations in genes encoding SNARE proteins or SNARE complex associated proteins have been associated with a variable spectrum of neurological conditions that have been recently defined as "SNAREopathies." These include neurodevelopmental disorder, autism spectrum disorder (ASD), movement disorders, seizures and epileptiform abnormalities. The SNARE phenotypic spectrum associated with seizures ranges from simple febrile seizures and infantile spasms, to severe early-onset epileptic encephalopathies. Our study aims to review and delineate the epileptic phenotypes associated with dysregulation of synaptic vesicle exocytosis and transmission, focusing on the main proteins of the SNARE core complex (STX1B, VAMP2, SNAP25), tethering complex (STXBP1), and related downstream regulators.

Keywords: SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor), epilepsy, seizures, mutations, vesicle fusion, epileptic encephalopathies

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INTRODUCTION

Epilepsy is defined as a large heterogeneous group of diseases in which individuals have an enduring predisposition to seizures (1), characterized by many different seizure types and epilepsy syndromes (2, 3).The term "epileptic encephalopathy" refers to a group of disorders in which unremitting epileptic activity contributes to progressive cerebral dysfunction (4). The epilepsies have a wide range of etiologies, which include genetic, metabolic, immune, and inflammatory factors; acquired or congenital brain abnormalities, infections, trauma or hypoxic-ischemic insults due to brain injuries (3). A genetic cause has been found in more than 50% of epilepsy phenotypes, particularly in developmental epileptic encephalopathies (5, 6). Pathogenic variants in genes encoding several voltage-gated K+, Na+, and Ca^{2+} channels subunits are the most common genetic cause of epileptic encephalopathies, accounting for a group of diseases defined as "channelopathies" (7). Another class of functionally related proteins have been discovered by James E. Rothman in 1994 and given the name SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) (8). Since then, several mutations in each subunit of the SNARE complex have been associated with an heterogenous group of neurological disorders, together referred to as SNAREopathies. SNAREs are a protein family involved in transport and release

mechanisms of synaptic vesicles inside the neuron. They mediate the fusion of membranes by localizing both at the vesicular and target membrane. The core SNARE machinery consists of VAMP2 (synaptobrevin-2), the only vesicular binding SNARE (v-SNARE) of the complex and a combination of target membrane SNARE proteins (t-SNAREs): syntaxin1-A (STX1A) and synaptosomal-associated protein 25 kD (SNAP25) (9). The assembly of the SNARE machinery is carefully arranged by the assembly complex, which is composed of MUNC18-1 and MUNC13-1 and plays an essential role in the fusion of synaptic vesicles (10). Pathogenic biallelic and monoallelic variants disrupting proteins of the SNARE complex and associated regulators are a known cause for neurodevelopmental disorders consisting of an overlapping phenotype of developmental delay (DD), intellectual disability (ID), movement disorders, and epilepsy. The SNARE phenotypic spectrum associated with seizures ranges from simple febrile seizures and infantile spasms, to severe early-onset epileptic encephalopathies. Here we review the epileptic phenotypes associated with dysregulation of synaptic vesicle exocytosis and transmission, focusing on the main proteins of the SNARE core complex (STX1B, VAMP2, SNAP25), assembly complex (STXBP1, UNC13A), and related downstream regulators.

PROTEINS OF THE MAIN SNARE COMPLEX

STX1B

Sintaxin-1B (*STX1B*) codes for a presynaptic plasma membrane protein that belongs to the syntaxins family and is predominantly expressed in neurons. *STX1B* is part of the SNARE complex and its main role is to mediate the calcium-dependent synaptic vesicle release (11, 12). The crucial role of *STX1B* is underlined by studies performed on animal models. In fact, *STX1B* KO mice presented with impaired brain development, disrupted motor coordination, and only survived the first 14 days. Hippocampal cell cultures viability was lower compared to controls (13). Interestingly, heterozygous *STX1B* presented with a less severe phenotype (14), while heterozygous zebrafish models showed jerks and paroxysmal movements. Epileptic episodes were observed in approximately 50% of the animals, with increased events associated with higher temperature which is in concordance with the occurrence of febrile seizures (15).

A total of 40 different heterozygous or *denovo* mutations in *STX1B* have been described so far. These included 4 missense, 2 indels, 6 nonsense, 7 frameshift, 7 splice variants and 4 large indels (**Table 1**). Most of these mutations were predicted to cause haploinsufficiency of *STX1B,* resulting in early termination of the protein. Sixty-two individuals have been described so far and the mainly reported phenotype was epilepsy (**Table 2**) (15–28). Wolking et al. (23) divides STX1B-associated epileptic phenotypes in four different groups: (1) benign epilepsy syndrome with febrile and afebrile seizures corresponding to "genetic epilepsies with febrile seizures plus," (2) "genetic generalized epilepsy" phenotype, (3) "developmental and epileptic encephalopathy" syndrome with refractory seizures and moderate to severe developmental deficits, and (4) focal epilepsy phenotype. Seizures have been described in almost all the individuals (61/62, 98%) and, where specified, they were the main symptom at onset in the majority of the individuals (43/48, 90%). Generalized seizures were the most common type of onset, presenting in 53 individuals (53/57, 93%), while focal-onset seizures occurred in 14 individuals (14/39, 36%). Seizure type ranged from tonic-clonic seizures (40/56, 71%) absence seizures (16/47, 34%), tonic or atonic seizures (24/56, 43%) to myoclonic seizures (16/30, 53%). Infantile spasms and status epilepticus were infrequent, both being reported in only two individuals. Out of the 47 electroencephalography recordings available, 42 showed epileptiform or non-epileptiform abnormalities (42/47, 89%).

Global developmental delay was documented in 15 individuals (15/47, 32%), and 23 individuals manifested various degrees of intellectual disability (23/59, 39%). Motor impairment, mainly ataxia, has been reported in 15 individuals (15/48, 31%). Behavioral or movement abnormalities were infrequent, accounting for 10% and 4% of the cases, respectively (5/45 and 2/51, respectively). When available, magnetic resonance imaging (MRI) was unremarkable for the majority of the cases (22/27, 81%).

VAMP2

VAMP2 encodes synaptobrevin-2, a major neuronal v-SNARE protein responsible for fusing synaptic vesicles at mammalian central nerve terminals (29, 30). *VAMP2* KO mice presented with abnormal body shape and died shortly after birth, brain abnormalities were not detected (31). Moreover, synaptic vesicle observed under the electron microscope from VAMP2 KO mice presented abnormal morphology and size (32).

De novo mutations in VAMP2 have hitherto been reported in 11 individuals with neurodevelopmental disorder (**Table 1**) (33–35). VAMP2 has been first described as a causative gene by Salpietro et al. (33), who reported 5 unrelated individuals with *de novo* heterozygous mutations. Simmons (34) and Sunaga (35) further expanded the cohort with 6 additional unrelated individuals carrying *de novo* heterozygous mutations in VAMP2. The mutations described so far include 7 missense, 2 indels, 1 nonsense and 1 frameshift. The entire cohort of variants is localized in the highly conserved SNARE motif.

All the affected individuals showed moderate to severe global developmental delay and intellectual disability (11/11; 100%). Behavioral abnormalities, including Autism Spectrum Disorder (ASD) or Rett-like features, were virtually present in all the reported cases (9/9; 100%). Movement abnormalities were present in 6 individuals (6/11, 55%) and included chorea ($n = 3$), dystonia ($n = 1$), myoclonic jerks ($n = 1$), tremor ($n = 1$) and hyperkinetic movements ($n = 1$). Even though seizures have only been reported in 6 individuals (6/11, 55%), electroencephalography (EEG) recordings, where available, showed abnormalities in 7 individuals (7/9; 78%). Generalized-onset seizures were the most prevalent type, occuring in 5 individuals (5/6, 83%). Among those, seizure type varied from tonic-clonic ($n = 2$), tonic/atonic ($n = 1$) and myoclonic ($n = 1$). Infantile spasms have been reported in 3 individuals (3/6, 50%), two of them in the West syndrome spectrum. Focal seizures

TABLE 1 | Mutations in SNARE proteins encoding genes.

			Type of mutation						
Gene	**Inheritance**	**Zygosity**	**Missense**	**Nonsense**	**Frameshift**	**Splicing**	**Indel**	**Large indels**	**Associated OMIM number**
VAMP2	AD	*Denovo*	7/11	1/11	1/11	–	2/11	–	185881
STX1B	AD	Heterozygous, Denovo	14/40	6/40	7/40	7/40	2/40	4/40	601485
SNAP25	AD	Denovo	15/19	2/19	–	2/19	–	–	600322
STXBP1	AD, AR	Heterozygous, Homozygous, Denovo	60/209	30/209	42/209	34/209	2/209	41/209	602926
MUNC13-1	AD, AR	homozygous, denovo	1/2	1/2	–	–	–	–	609894
GOSR2	AR	Homozygous, Compound Heterozygous	4/7	–	–	2/7	1/7	–	604027
SNAP29	AR	Homozygous, Compound Heterozygous	3/10	2/10	5/10	–	–	–	604027
STXBP5L	AR	Homozygous	1	–	–	–	–	–	609381
CPLX1	AR	Homozygous	1/3	2/3	–	–	–	–	605032

were less frequent, occurring in 3 individuals (3/6; 50%). Two individuals experienced status epilepticus (2/6, 33%). Magnetic resonance imaging was unremarkable for 6 individuals, otherwise MRI findings included corpus callosum thinning or hypoplasia ($n = 2$), periventricular FLAIR hyperintensities ($n = 1$) or mild brain atrophy ($n = 1$) (**Table 2**). Of the 11 individuals with *de novo* variants, seven different missense variants were identified, in addition to two single residue deletions and two stop gains. All variants were absent from GnomAD database blablabla to date, it seems that no clear correlation between the type of variant and the phenotype has been identified.

SNAP25

SNAP25 encodes the SNAP-25 protein, a t-snare widely expressed in the brain which is localized both at the presynaptic nerve terminal as well as to neuronal membranes (36). SNAP-25 is distinctive in the SNARE complex as it lacks a transmembrane domain and contains two SNARE motifs separated by a linker region (37). When trying to generate KO *SNAP25* mice, it was observed that heterozygous mice did not present any significant differences in comparison to their wilt-type littermates. However, no homozygous *SNAP25-/-* were generated from the heterozygous crosses. Analysis of the homozygous fetuses revealed smaller size, absence of movements and blotchy appearance likely caused by vascular abnormalities of the skin. Morphology of the brain appeared normal (38). There have been 19 different *de novo* mutations identified so far in *SNAP25* across 23 patients (39). Out of the total number of variants, 14 were missense, 2 nonsense and 2 splice-site (**Table 1**). The mutations are localized in the SNARE motifs and are predicted to disrupt the SNARE complex, but thorough functional studies are yet to be performed.

All individuals presented with global developmental delay and intellectual disability (23/23, 100%), ranging between profound (4/20; 20%), severe (5/20; 25%), moderate (6/20; 30%), and mild (5/20; 25%). Regression was reported in five individuals (5/17; 29%) with three of them showing signs of regression with the onset of seizures. All individuals showed variable degree of motor delay, with motor impairment being more evident in 12 individuals (12/15, 80%). Seizures have been reported in 17 individuals (17/23, 74%). The age of seizure onset ranged between the 7th day of life to 12 years. In 14 individuals, the onset was before 2 years of age. Most individuals showed a broad spectrum of epileptic spasms, generalized and focal seizures. Generalized-onset or focal to generalized-onset seizures were the most frequently occuring, being reported in 11 individuals (11/17, 65%), while focal seizures were described in 6 individuals (6/17, 35%). Seizure spectrum included tonic-clonic ($n = 7$), absence ($n = 6$), tonic or atonic ($n = 4$), myoclonic ($n = 4$) seizures and epileptic spasms ($n = 5$). Status epilepticus was reported in only one individual. EEG abnormal findings, generally multifocal epileptic discharges and generalized spike wave discharges, were documented in 15 out of the 16 available records (15/16, 94%). Less frequently reported features were abnormal movements (5/21, 24%) and behavioral abnormalities (3/18, 17%). MRI was performed on 21 individuals and was unremarkable in 15 individuals (15/21, 71%) (**Table 2**).

SNARE COMPLEX ASSEMBLY FACTORS

MUNC18-1 (STXBP1)

MUNC18-1, also known as Syntaxin-binding protein-1 (*STXBP1*), belongs to the Sec1/Munc18 (SM) family. MUNC18-1, together with MUNC13-1, plays a crucial role in the SNARE complex assembly. More specifically, MUNC18-1 forms an

TABLE 2 | Main phenotypes associated with mutations in the SNARE complex.

Gene	Disorder (#OMIM)	MOI	Cases	Age of onset	Seizures	EEG discharges	Global developmental delay	Intellectual disability	Motor impairment	Movement disorders	Autism/ behavioral abnormalities	MRI abnormalities
Main proteins												
STX1B	Generalized epilepsy with febrile seizures plus, type 9 (#616172)	AD	62	Early infancy	61/62 (98%)	42/47(89%)	15/47 (32%)	23/59 (39%)	15/48 (31%)	2/51 (4%)	5/45 (11%)	5/27 (19%)
VAMP2	Neurodevelopmental disorder with hypotonia and autistic features with or without hyperkinetic movements (#618760)	AD	11	Early childhood	6/11 (~55%)	7/9 (~78%)	11/11 (100%)	9/9 (100%)	8/10 (80%)	06/11 (~55%)	9/9 (100%)	4/10 (40%)
STXBP1	Developmental and epileptic encephalopathy 4 (#612164)	AD	>400	Early infancy	401/446 (~90%)	160/226 (~71%)	297/313 (~95%)	261/279 (~94%)	143/261 (55%)	130/261 (50%)	96/274 (35%)	111/257 (43%)
SNAP25	Myasthenic syndrome, congenital, 18; Developmental and epileptic encephalopathy (#616330)	AD	23	Early infancy to childhood onset	17/23 (73.9%)	15/16 (93.75%)	23/23 (100%)	23/23 (100%)	12/15 (80%)	5/21 (23.81%)	3/18 (16.67%)	6/21 (29%)
MUNC13-1	Not on OMIM	AR/AD	2	Early infancy	1/2 (50%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
Other associated proteins												
CPLX1	Developmental and epileptic encephalopathy 63 (617976)	AR	5	Early infancy	5/5 (100%)	3/3 (100%)	5/5 (100%)	5/5 (100%)	3/3 (100%)	–	–	3/5 (60%)
STXBP5L	Not on OMIM	AR	2	Early infancy	2/2 (100%)	–	2/2 (100%)	–	–	1/2 (50%)	–	2/2 (100%)
SNAP29	Cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma syndrome (609528)	AR	25	Late infancy/Childhood	9/25 (36%)	–	25/25 (100%)	25/25 (100%)	–	0/25 (0%)	–	21/22 (95%)
GOSR2	Epilepsy, progressive myoclonic 6 (#614018)	AR	34	Childhood	31/33(94%)	22/22 (100%)	6/26 (23%)	5/24 (20%)	32/32 (100%)	24/26 (93%)	–	5/16 (31%)

	Seizures	**EEG discharges**	**Seizures as first symptom at onset**	**Infantile spasms**	**Generalized onset**	**Tonic-clonic**	**Absence**	**Tonic and atonic**	**Myoclonic**	**Focal onset**	**Status epilepticus**
STX1B	61/62 (98%)	42/47(89%)	43/48 (90%)	2/54 (4%)	53/57 (93%)	40/56 (71%)	16/47 (34%)	24/56 (43%)	16/30 (53%)	14/39 (36%)	2/8 (25%)
VAMP2	6/11 (~55%)	7/9 (~78%)	0	3/6 (50%)	5/6 (83%)	2/5 (40%)	0	1/5 (20%)	1/5 (20%)	3/6 (50%)	2/6 (33%)
SNAP25	73.91%	93.75%	7/17 (41.18%)	5/17 (29.41%)	11/17(64.71%)	7/17 (41.18%)	6/17 (35.29%)	4/17 (23.53%)	4/17 (23.53%)	6/17 (35.29%)	1/17 (5.88%)
STXBP1	401/446 (~90%)	160/226 (~71%)	69/84 (82%)	162/260 (62%)	108/159 (68%)	77/258 (30%)	22/247 (9%)	84/246 (34%)	49/236 (21%)	140/274 (51%)	9/163 (6%)

inactive complex with syntaxin-1 to secure correct positioning of the latter. The formation of this complex is likely to represent the initial event for synaptic vesicle fusion (40).

KO mice have shown severe phenotype, dying immediately after birth and with no neurotransmission activity recorded. Moreover, degeneration was observed from cultured neurons from these mice (41, 42). Mutations in STXBP1 are the most commonly reported in literature, with a large difference in numbers compared to mutations in other SNARE proteins. So far, 163 distinct mutations and 41 large indels have been identified in *STXBP1*. The majority of this group is composed of 59 missenses, followed by 40 frameshift, 34 splice-site, 28 nonsense and 2 indels (**Table 1**). When missense variants are compared to all other types of mutations, there is no correlation to presence or absence of epilepsy. Cases reported to date were monoallelic, with the exception of two recently described siblings carrying a biallelic *STXBP1* L446F mutation (43).

More than 400 cases have been reported so far (19, 44–103). However, for many individuals, detailed clinical information was not available. Nevertheless, the key clinical findings in STXBP1-spectrum comprised global developmental delay and/or intellectual disability, seizures and variable presence of movement disorder, motor impairment or behavioral abnormalities. The most commonly described clinical feature is global developmental delay, presenting in 95% of the patients for whom information was available (297/313, 95%). The range of intellectual disability may vary: Stamberger et al. (56) reported that more than 80% of the individuals described until then presented with severe to profound intellectual disability.

Seizures were reported in 401 individuals (401/446, ∼90%). A wide spectrum of seizure types was described in most individuals. Where specified, epileptic spasms frequently occurred at some stage during the disease course (162/260, ∼63%). Other frequent seizure types were generalized-onset seizures (108/159, ∼68%) and focal seizures (140/274, ∼51%). Almost 90% of seizures occur in early infancy as the first symptom. When performed, EEG was reported abnormal in 71% of the cases (160/226, ∼71%).

Other less commonly reported features include motor impairment [143/261 (55%)], movement disorders [130/261 (50%)] and behavioral abnormalities [96/274 (35%)]. In 146 of the 257 individuals for whom brain magnetic resonance imaging (MRI) was available, no abnormalities were documented (146/257, ∼57%) (**Table 2**).

MUNC13-1

MUNC13-1 is a protein encoded by *UNC13A* and is highly expressed in the hippocampus, cerebellum, cortex, striatum, and olfactory bulb (104). By binding both to synaptobrevin-2 and syntaxin-1, it aids in the formation of the SNARE complex, thus making synaptobrevin-2 more accessible by the MUNC18-1/syntaxin-1 formation (105).

Studies on MUNC13-1 KO mice have shown absence of evoked and spontaneous excitatory and inhibitory neurotransmitter release and synapses reduction in docked vesicles (106, 107). Until now, only two cases have been reported with mutations in MUNC13-1 (108, 109). A homozygous nonsense mutation (p.Gln102Ter) in UNC13A was identified in a girl with microcephaly, cortical hyperexcitability, fatal myasthenia, global developmental delay and intellectual disability. Seizures were not reported, but EEG showed abnormalities. MRI documented thinning of corpus callosum. Subsequently, a *de novo* heterozygous missense mutation was identified in a boy with dyskinetic movement disorder, developmental delay, intellectual disability, autism and ADHD, who also experienced febrile seizures.

Other Associated Proteins

Epileptic phenotypes have also been associated with mutations in other SNARE-associated proteins, here we review GOSR2, SNAP29, STXBP5L, and CPLX1. The Golgi snap receptor complex member 2 (*GOSR2*), is part of a complex responsible for docking and fusion of newly synthesized proteins from the endoplasmic reticulum (110). To date, seven different biallelic variants have been reported in *GOSR2* (**Table 1**). All individuals share a similar phenotype with myoclonus epilepsy, ataxia, and usually relatively preserved cognition. SNAP29, acting in the autophagosome-lysosome fusion (111), has been found to harbor 10 distinct biallelic mutations (**Table 1**). Namely, 5 frameshifts, 2 nonsense and 3 missense (112). Mutations in SNAP29 have been linked to CEDNIK syndrome, whose clinical features include microcephaly, severe neurologic impairment, psychomotor retardation, failure to thrive, and facial dysmorphism, as well as palmoplantar keratoderma and late-onset ichthyosis (113). Global developmental delay and intellectual disability have been reported in all the affected individuals. Seizures are not very common and have been described in 9 of the 25 reported individuals (9/25, ∼36%).

The function of *STXBP5L* is still not clear, but it has been observed it plays a role in the inhibition of the formation of the SNARE complex. Only one homozygous missense variant has been reported so far in two siblings with seizures, global developmental delay and MRI abnormalities (114, 115). Complexin-1 (*CPLX1*) is a neuronal protein of the SNARE complex, which contributes to vesicle fusing. To date, 2 nonsense and 1 missense biallelic mutations have been reported in the gene. Only five cases have been described so far, all presenting with seizures and global developmental delay (116, 117).

DISCUSSION

In the previous paragraphs, the epileptic syndromes associated with mutations or variants in the SNARE complex were briefly reviewed. Since the identification of SNARE proteins, many studies have focused on the role of the single molecules within the whole complex. Mutations in each subunit of the complex and in the related upstream and downstream regulators have been identified in a heterogeneous group of disorders, mostly neurological disorders. We focused on the association between the SNARE complex and seizures. Only four proteins involved in the synaptic vesicle fusion have not been linked to epileptic phenotypes: α-synuclein, synaptobrevin-1, and synaptotagmin-1 and -2.

Deficits in the subunits of the core complex (synaptobrevin-2, syntaxin-1B and SNAP-25), Munc18-1 and complexin-1 are mainly associated with an overlapping spectrum of developmental delay, intellectual disability, epilepsy, and movement disorders. Overall, the most reported feature is epilepsy, presenting in almost 90% of the individuals with a SNARE dysfunction. Particularly, mutations in syntaxin-1B are most associated with epileptic phenotypes. The type of seizures may vary from generalized tonic-clonic, myoclonic or absence seizures to focal seizures. Infantile spasms or West syndrome were also reported in association with SNARE dysfunction. The epileptic phenotypes associated with the main vesicle fusion machinery have been characterized in **Table 2**. Given the significant overlap in seizure semiology, it is not possible to differentiate the genetic cause based on seizure type. Global developmental delay and intellectual disability are also frequent features, presenting in 85% of the individuals. However, dysfunctions in STX1B and GOSR2 are less commonly associated with developmental delay, as most of the affected individuals don't show intellectual impairment. Motor impairment and movement abnormalities, including ataxia, gait abnormalities, tremor, hyperkinetic movements, chorea and myoclonus, are variably present, affecting almost half of the individuals. Behavioral abnormalities, comprising Autism Spectrum Disorder, Rett-like phenotypes and stereotypies, were less commonly documented in the cohort. Brain MRI was performed and reported as normal in more than 50% of the overall cohort. We comprehensively reported the total cohort of mutations identified so far in the genes that form the core SNARE complex and some associated proteins. The zygosity of genes in the core SNARE complex was heterozygous or *de novo*, indicating the crucial role of these proteins. Studies on animal models have confirmed this, by showing that homozygous KO animal models are either incompatible with life or severely affected, not surviving their first days. Therefore, it is likely that the early onset of the epileptic phenotype might be the consequence of the disruption of the neurotransmitters release machinery. Except for *STXBP1*, where only one biallelic variant was reported out of the total 209, all other discussed genes presented biallelic and monoallelic variants. In conclusion, we comprehensively described a cohort of more than 600 individuals affected with dysfunction in proteins of the SNARE complex or synaptic vesicle machinery. We illustrated the phenotypic spectrum of the SNARE-associated disorders and focused on the epileptic phenotypes. This review underlines the key role of SNARE proteins in the pathogenicity of epilepsy and the prevalence of this phenotype. Limitations of this study are mainly attributable to its retrospective nature. One important limitation has been the lack of precise information on the main phenotype and on the neuroradiological features, particularly when the mutation was reported in big cohort studies. Clinical features were summarized through percentages, but we cannot exclude the risk of under or overestimation. We reviewed the function of the main SNARE proteins, taking in consideration the consequence of their disruption in animal models. However, to better understand the pathways involved in these disease mechanisms, further functional studies will be required.

AUTHOR CONTRIBUTIONS

EC, CR, VS, and HH: conceptualization, writing—review, and editing. HH: funding acquisition. EC and CR: writing—original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.806506/full#supplementary-material

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The Broad Clinical Spectrum of Epilepsies Associated With Protocadherin 19 Gene Mutation

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Protocadherin 19 (PCDH19) gene is one of the most common genes involved in epilepsy syndromes. According to literature data PCDH19 is among the 6 genes most involved in genetic epilepsies. PCDH19 is located on chromosome Xq22.1 and is involved in neuronal connections and signal transduction. The most frequent clinical expression of PCDH19 mutation is epilepsy and mental retardation limited to female (EFMR) characterized by epileptic and non-epileptic symptoms affecting mainly females. However, the phenotypic spectrum of these mutations is considerably variable from genetic epilepsy with febrile seizure plus to epileptic encephalopathies. The peculiar exclusive involvement of females seems to be caused by a cellular interference in heterozygosity, however, affected mosaic-males have been reported. Seizure types range from focal seizure to generalized tonic-clonic, tonic, atonic, absences, and myoclonic jerks. Treatment of PCDH19-related epilepsy is limited by drug resistance and by the absence of specific treatment indications. However, seizures become less severe with adolescence and some patients may even become seizure-free. Non-epileptic symptoms represent the main disabilities of adult patients with PCDH19 mutation. This review aims to analyze the highly variable phenotypic expression of PCDH19 gene mutation associated with epilepsy.

Keywords: PCDH19, epilepsy and mental retardation limited to female (EFMR), GEFS, Dravet syndrome, antiseizure medication (ASM)

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INTRODUCTION

The latest ILAE classification (1) emphasizes the importance of an etiological classification of epilepsy to improve prognosis and, whether possible, initiate a target therapy. In fact, besides allowing a stratification of risk based on the genotype-phenotype correlation, different patterns of gene mutations could present specific drug-response and lead to target therapy. The main genes associated with epilepsy can be classified based on five different functions: (I) ion transport; (II) cell growth and differentiation; (III) synaptic processes regulation; (IV) transport and metabolism within and between cells of small molecules; and (V) gene transcription and translation (2). One of the most commonly implicated genes in epilepsy is the protocadherin 19 (PCDH19) gene located on chromosome Xq22.1. According to Symonds et al. (2) PCDH19 is among the six genes most involved in genetic epilepsies (**Figure 1**).

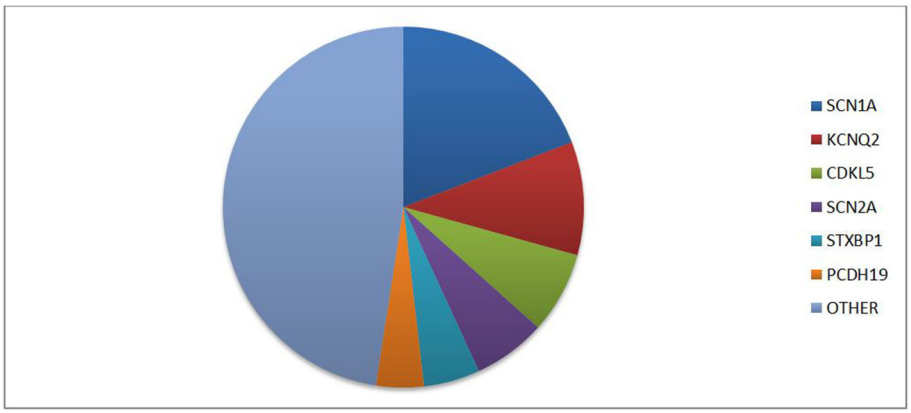

FIGURE 1 | Prevalence of different gene mutations in genetic epilepsies according to literature data [data from (2)].

PCDH19 gene is expressed in several organs, but primarily in the limbic areas of the nervous system (3). It has a six-exon structure that encodes a transmembrane adhesion molecule of the Cadherin family. The Cadherin superfamily comprises three subgroups of transmembrane cell adhesion molecules: cadherins, protocadherins, and desmosomal cadherins (4). Among these protocadherins represent the main subgroup with approximately 80 members involved in neuronal connections and signal transduction (5, 6). Almost 150 PCDH19 mutations have been described as either familiar clustering or *de novo* (7). Most mutations involve the extracellular protein domains encoded by exon 1 and are typically missense variations (7, 8). However, a few mutations of intracellular domains have also been reported, possibly affecting the intracellular signal pathway. Proper development of neural architecture and neuronal connectivity require efficacious cell-cell interactions and alterations of protocadherins could result in severe disruption in early brain morphogenesis. It is not well known how mutations of PCDH19 lead to the development of epilepsy. However, a role of this gene in the proliferation of neuronal progenitors and the regulation of cell motility during the early stages of neurulation has been proposed (9, 10). Recent *in vitro* studies, conducted with patient-derived induced pluripotent stem cells showed an accelerated differentiation in cells with PCDH19 mutation. It is also observed that increased neurogenesis occurs earlier in PCDH19-mutated culture with an increased neurite length and occurrence of premature neural rosettes. Accelerated neurogenesis is involved with a defect in the cell division plane at the stage of the neural progenitors. Moreover, it is possible that PCDH19 mutations can alter the correct positioning of the mitotic spindle causing a higher number of asymmetric divisions leading to accelerating neural differentiation. An altered equilibrium between symmetric vs. asymmetric cell division may contribute to the pathogenesis of the disease (11). The synaptogenesis role of PCDH19 was confirmed by Mincheva-Tasheva et al. who showed disruption of excitatory synaptic contacts between PCDH19-knock-out and wild-type neurons in "mosaic" neuronal cultures (12). In addition, PCDH19 mutation leads to a decrease in N-cadherin-dependent signaling resulting in an impaired mossy fiber synapse development (13). The interaction between the cytoplasmic domains of PCDH19 with the alpha subunits of the GABAa receptor could alter the excitatory-inhibitory balance underlying epilepsy (14). Besides regulating GABAa receptor surface expression, PCDH19 also regulates channel gating. Indeed, PCDH19-mutated cortical neurons have a spontaneous Ca^{2+} intracellular flux suggesting increase excitability of these cells (15). Another theory suggests PCDH19 involvement in blood-brain barrier (BBB) dysfunction. In fact, the gene is highly expressed in endothelial cells and the main epileptic foci involve the limbic region, close to the periventricular region where BBB is missing. Furthermore, this theory could explain the seizure remission with growth thanks to the maturation of BBB (16). The phenotypic spectrum of PCHD19 mutation is extremely variable involving neurological and psychiatric diseases. Although epilepsy and mental retardation limited to female (EFMR) is the most frequent clinical expression of PCDH19 mutation, other important clinical manifestations are genetic epilepsy with febrile seizure plus (GEFS+) and epileptic encephalopathies. This review aims to analyze the phenotypic expression of PCDH19 gene mutation associated with epilepsy.

LITERATURE SEARCH

We reviewed the papers (English language only) on PCDH19-related epilepsy through a Literature search on PubMed until August 2021. The terms "PCDH19" and "PCDH19" epilepsy were used in this systematic search. We included case reports and open-label studies. Moreover, we searched for additional articles through a review of the reference lists of published reviews.

EPILEPSY AND MENTAL RETARDATION LIMITED TO FEMALE

Epilepsy and mental retardation limited to female (EFMR) was first described by Juberg and Hellman (17) in 15 related females presenting epilepsy with cognitive impairment. This clinical manifestation was only later related to mutations of

the PCDH19 gene (8, 18). Brain MRI is generally normal and there are no peculiar electroencephalographic (EEG) features of PCDH19 mutation. **Figure 2** shows an EEG of a female patient with PCDH19 mutation and focal epilepsy. The phenotypic expression of EFMR is characterized by epileptic and non-epileptic symptoms. The hallmark feature especially in the early stages of the disease is cluster focal seizures with the tendency to prolonged episodes poorly responsive to antiepileptic therapy (19). The patient often displays early-onset seizures (6–36 months) generally sensitive to fever (20). Seizure types range from focal seizure to generalized tonic-clonic, tonic, atonic, absences, and myoclonic jerks. The severity of epilepsy is also extremely variable from drug-resistant and progressive forms to self-limiting ones. Seizures become less severe with adolescence while non-epileptic symptoms represent the main disabilities of adult patients with PCDH19 mutation (21). Non-epileptic features of EFMR include intellectual disability (ID) and behavior disturbances occurring in 75.4 and 55.4% of patients, respectively (21). In a study of 195 patients with PCDH19 mutations, only 28.2% had normal cognitive development, while patients with mild, moderate, and severe impairment were 27.2, 22.2, and 17.4%, respectively (7). A delay in the acquisition of language milestones represents a common feature of all patients and the absence of language before seizure onset represents a possible negative prognostic factor for cognitive development (22). Although most patients manifest cognitive delay after 2 years of age, 15% of cases present intellectual disabilities before the onset of epilepsy. Therefore, intellectual impairment is only partially related to epileptic encephalopathy and other genetic and/or environmental factors are among the causes of the phenotypic spectrum. The peculiar exclusive involvement of females seems to be caused by a cellular interference in heterozygosity. According to this theory, the coexistence of mutated cells with wild-type cells causes the neural network alteration at the base of the disease (17, 23). Confirming this hypothesis, while hemizygotic males are asymptomatic carriers, affected mosaic-males have been reported (24). Indeed, postzygotic somatic variants in males would configure a picture overlapping with that of females in heterozygosity. A mutation penetrance of ∼80% was estimated for both conditions (7, 25). The small number of affected males makes difficult a phenotypic characterization of these patients. The phenotypic spectrum of mosaic-males resembles that of affected females. In addition, psychiatric comorbidity has also been described in two males with germline mutation, albeit the possible correlation with the mutation is questionable (25). Several studies have looked for a genotype-phenotype correlation without any result. According to a recent study, missense variants seem to be more commonly related to normal cognitive development compared to the loss of function mutations (26). Whole gene deletion appears as well associated with a worsened prognosis. According to Shibata et al. truncating variants located from extracellular domain 5 (EC5) to the cytoplasmic domain present a later seizure onset with less severe intellectual disability compared to missense variants and truncating variants from EC1 to EC4 (27). Cognitive impairment does not appear to be directly associated with seizure severity (22). To date, age at seizure onset and seizure frequency are the only unfavorable prognostic factors associated with cognitive function (7, 26).

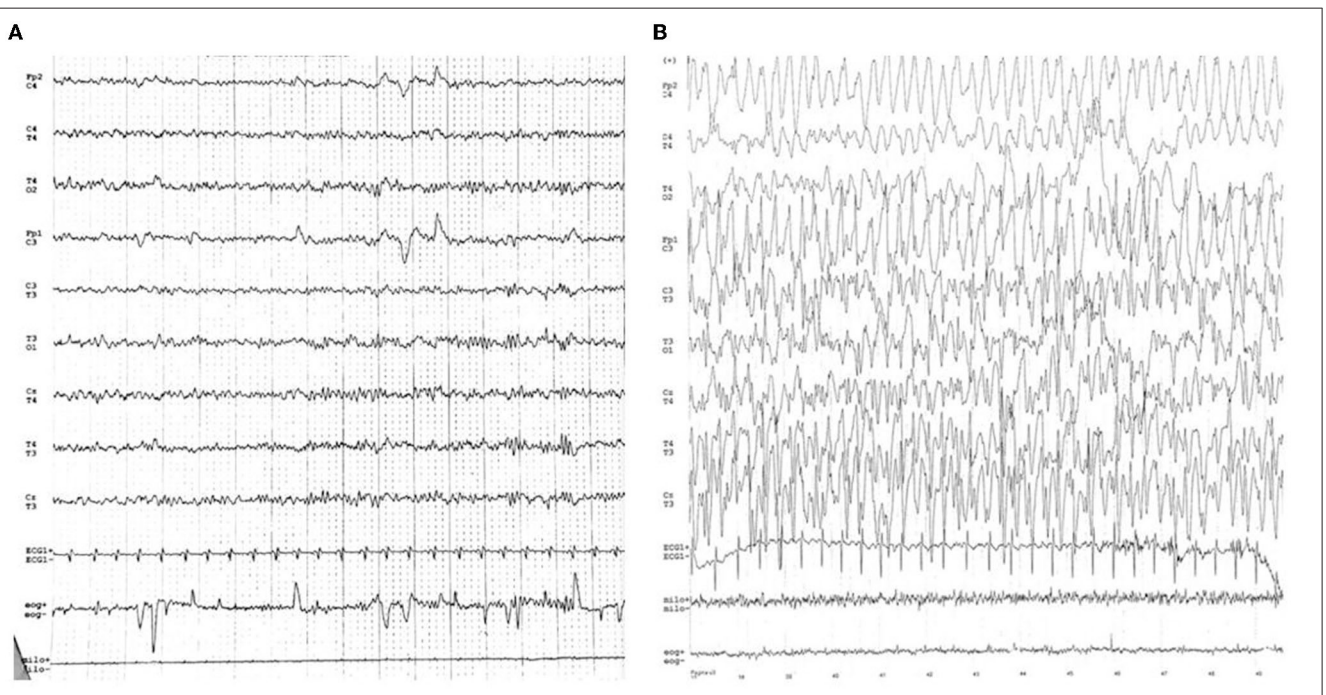

FIGURE 2 | (A) Normal inter-ictal EEG. **(B)** Ictal-EEG characterized by frontal theta activity of the left hemisphere followed by sharp waves on the same derivations. Slow waves spread on the frontal derivations of the right hemisphere, followed by diffuse slow waves (except on the right posterior deviations).

Indeed, the occurrence of new synapses' processing and changes in the frontal cortex predominantly in the first years of life may justify this correlation (7). In addition, the epileptic expression, also the neuropsychiatric profile is extremely heterogeneous, ranging from mild to severe forms with combination of autistic, attention-deficit/hyperactive, obsessive, or aggressive features (28). Approximately 25% of patients without intellectual disability presents psychiatric comorbidity (7). In addition, there is data that sleep alterations is a common feature in patients with EFMR. Both difficulty in maintaining sleep and in absorption have been reported (28). A more complete analysis of these disorders could be useful especially in light of the correlation between sleep disturbances and worse control of epileptic symptoms. The wide variability in the phenotypic expression of EFMR could be partially explained by X-inactivation in females. However, further studies are needed for a greater knowledge of the impaired biological processes in EFMR and to investigate possible genotype-phenotype correlations.

GENETIC EPILEPSY WITH FEBRILE SEIZURES PLUS

Febrile seizures (FS) are the most common clinical presentation of GEFS+, followed by febrile seizures plus (FS+). However, absence, myoclonic, atonic, or focal seizures combined with the most frequent phenotypes are also common. Confirming the various spectrum of this disorder, epileptic encephalopathy like Dravet syndrome (DS) and epilepsy with myoclonic-atonic seizures (MAE) are possible manifestations in GEFS+ families. The clinical presentation of PCDH19-related epilepsy often overlaps with that of GEFS+. In both, focal seizures with variable degrees of intellectual disabilities can be part of the phenotypic spectrum. To define a GEFS+ family are necessary two or more individuals with GEFS+ phenotypes including at least one with FS or FS+ (29–31). GEFS+ was initially described as a genetic disorder following an autosomal dominant pattern of inheritance with incomplete penetrance (29, 32, 33). However, some sporadic cases suggested that *de novo* mutation or polygenic pattern could also cause the disease (31, 33–36). With a better knowledge of the clinical presentation, it is now possible to recognize GEFS+ even outside a family context when *de novo* mutations in the GEFS+ gene are found (36). Several genes mutations are described underlying GEFS+ (37–48) including PCDH19 (49, 50). SCN1A is the most commonly involved gene, whose mutations are identified in 19% of affected families (30, 37). There is a known similarity between the phenotypic spectrum of PCDH19 and SCN1A, which includes DS and extends to GEFS+. Recognizing PCDH19 mutations in the context of a GEFS+ presentation can be difficult. However, seizure clusters prevalent in the females should point toward the search for PCDH19 mutations.

DRAVET-LIKE SYNDROME

Dravet syndrome (DS), originally named "severe myoclonic epilepsy of infancy," represents one of the most severe genetic epilepsy with childhood onset. According to the ILAE classification, the typical DS is defined by febrile and afebrile seizures that occur in the first year of life in an infant with normal development and the subsequent appearance of myoclonus, atypical absences, and focal seizures. Seizures become drug-resistant leading to poor prognosis with motor, cognitive, and psychiatric impairment of affected patients (1, 51). SCN1A is the most frequently involved gene occurring in 70–80% of DS (52, 53). DS-like phenotype is a similar condition due to the occurrence of other gene mutations involved in encephalopathy. In a recent study, mutations in the PCDH19 gene appear to be the underlying cause of DS-like phenotype in 16% of DS negative for SCN1A mutations (54). The variants in the SCN1A and PCDH19 genes show some similarities that link DS to DS-like, but they differ from each other for some peculiarities (55). Due to the unusual X-linked inheritance of PCDH19 gene mutations, DS-like is more common in females. The onset of symptoms is earlier in DS than in DS-like, with an average range of 3–6 and 8–54 months, respectively (56, 57). Fever represents the main triggering seizure factor for both DS and DS-like, however PCDH19 mutations show fewer provocation factors for seizure initiation (55). Clonic and hemiclonic seizures are mainly related to SCN1A mutations that also present a higher prevalence of generalized tonic-clonic seizures and atypical absences with more common status epilepticus. Whereas, seizure types associated with PCDH19 variants are often focal and hypomotor seizures with a higher prevalence of cluster seizures (57). Seizures with affective symptoms and fearful screaming have been described by many authors as a characteristic feature of DS-like (19). DS-like phenotype is less associated with photosensitivity compared to DS. Another relevant difference is the interval for the second seizure occurrence: 10 months for DS-like vs. 2–3 months for DS, probably due to the higher frequency of seizures in the first year of life and the earliest onset in DS patients (20, 57). Interictal EEG may have no abnormalities, however focal or generalized slow wave, sharp and polyspike discharges have been reported (22). DS-like phenotype carrying the PCDH19 pathogenic variants has greater variability in cognitive disability including some cases without intellectual impairment (20, 22, 57). In contrast, patients with DS present a greater degree of cognitive impairment, regardless of variant significance (55). Autism predominantly involves DS-like, occurring in 62.5% of patients with PCDH19 mutations vs. 37.5% of patients with SCN1A mutations (55).

TREATMENT OF PCDH19-RELATED EPILEPSY

Treatment of PCDH19-related epilepsy is limited by drug resistance and by the absence of specific treatment indications. These patients usually need polytherapy frequently with poor efficacy due to the natural fluctuating trend of seizures and to the various cluster triggers. The management of drug-refractory patients represents a great challenge for physicians, especially for syndromes with heterogeneous seizure semeiology and course (58). Currently, different drug associations have been tested and none has definitively proven to be superior (**Table 1**). However, familiar mutations show the same reactivity

TABLE 1 | Clinical, EEG and MRI characteristics and treatment efficacy in PCDH19-related epilepsy.

References	Sample size	Mean age at seizure onset	Seizure semiology	EEG patterns	Comorbidities	MRI findings	Treatments	Treatments efficacy
Scheffer et al. (18)	27	14 months	Tonic, tonic-clonic, partial, absence, atonic and myoclonic	Generalized spike wave and polyspike wave, focal discharges with more frequent frontotemporal involvement	ID (15/27) Autistic traits (6/13) Obsessive features (9/27) Aggressive behavior (7/27)	n.a.	VPA, LTG, PHT, PB,	n.a.
Depienne et al. (24)	13	9.5 months	Febrile and afebrile seizure, GTCS, absences, partial and hemiclonic	n.a.	ID (13/13) Behavioral disturbances (5/13) Autistic features (2/13)	n.a.	VPA, CLZ, CLB, TPM, STP, LTG	n.a.
Marini et al. (59)	13	8.5 months	Tonic-clonic, absences, myoclonic and focal	Centroparietooccipital activity (5/13) and frontotemporal activity (2/13)	ID (11/13) Autistic features (5/13)	Normal (13/13)	n.a.	n.a.
Depienne et al. (20)	25	2–54 months	GTCS, tonic, focal, hemiclonic, absence, myoclonic	Normal, focal and generalized seizures	ID (18/25), Behavioral disturbances (7/25)	Normal, frontal median dermoid cyst (1/25)	TPM, LEV, ZNS, CBZ, PB, VPA, LTG, PB, VGB, PHT, STP, CLN, CLB, NTZ	Seizures appeared highly resistant to ASM during the first years of life, the frequency and pharmacoresistance of seizures tended to decrease over time. The only drugs reporting a negative effect were CBZ, LTG and VGB
Marini et al. (19)	35	10 months	Clusters of focal febrile or afebrile seizures. Fearful screaming (24/35)	Prominent involvement of the frontotemporal regions (22/35)	ID (24/35) Autistic traits (11/35)	Normal (35/35)	GVG, OxCZ, LTG, LEV, VPA, PB, TPM, LCM, CZP, ESM, CLB, PHT, CLP, PGB, NZP, DZP	No specific drug or combination of drugs appeared to have been more effective than others. Oral, rectal, or intravenous benzodiazepines had been successful in arresting seizure clusters
Higurashi et al. (60)	18	8.6 months	Tonic, tonic-clonic and focal seizures often with subsequent generalization	Frontal and/or temporal activities (9/18), occipital involvement (4/18)	ID (15/18) Autistic traits (13/18)	Normal (13/18), Frontal heterotopia (1/18), Occipital atrophy (1/18), Hippocampal atrophy (1/18), White matter lesion (1/18)	PHT, BR, CLB, TPM, VPA, CZP, ZNS, PB, CBZ	MDZ showed efficacy in suppressing the ongoing seizure, but was insufficient to manage strong clusters. PHT, BR and CLB were beneficial for decreasing Seizures. CBZ had the poorest efficacy
Harssel et al. (61)	15	4–17 months	Tonic-clonic, tonic, hemiclonic, myoclonic, focal	Focal, multifocal or bilateral synchronous discharges, and background activity was either normal or showed slowing	ID (13/15) Behavioral disturbances (11/15) Autistic trait (6/15)	Normal (14/15), slight asymmetry frontal lobes (1/15)	n.a	n.a

(Continued)

TABLE 1 | Continued

References	Sample size	Mean age at seizure onset	Seizure semiology	EEG patterns	Comorbidities	MRI findings	Treatments	Treatments efficacy
Liu et al. (62)	21	5–18 months	GTCS, focal, myoclonic	Focal or multifocal seizures from the centroparieto-occipital regions or temporal region (5/21). Interictal focal or multifocal epileptic discharges in the centroparietooccipital or frontotemporal regions (14/21)	ID (17/21) Autistic trait (3/21)	Normal (21/21)	PB, LTG, LEV, VPA, TPM, CBZ, TPM, OXC, NZP	Seizures were refractory to antiepileptic drugs at onset in all patients. Seizure frequency and intractability tended to decrease over time
Lotte et al. (63)	58	11.2 months	GTCS (81%)	n.a.	Motor impairment (25/58) ID (48/58) Behavioral disorders (39/58)	Normal (38/58), focal cortical dysplasia (2/58)	BR, CBZ, CLB, CZP, ESM, GBP, CM,LEV, LTG, LZP, NZP, OXC, PB, PER, PGB, PHT, RFN,STM, STP, TPM, VGB, VPA, ZNS	CLB and BR decreased seizure frequency by more than 50% with a responder rate of 68 and 67%, respectively. A long-term response of 50 and 43% respectively was detected after 12 months. PHT resulted particularly ineffective.
Chemaly et al. (56)	13	4–14 months	GTCS, focal, atypical absence	temporo-occipital and frontal onset (8/13)	ID (12/13) Autistic traits (9/13)	Normal (13/13)	VPA,CLZ,VGB, LEV,CLB, PB, STP,TPM, CZP, CBZ, LTG, PHT, LVT, ETX	Clusters responded to benzodiazepines. STP decreased seizure frequency by more than 50%. VGB had a negative impact on behavior in two patients with seizure worsening and was stopped.
Smith et al. (26)	38	11.8 months	Focal, generalized seizures	n.a.	ID (30/38) Behavioral abnormalities (29/38) Autistic features (22/38) Abnormal sleeping patterns (20/25)	Normal	Most frequently used medications include BZP, OXC, VPA, LEV	Uncontrolled seizures with more than 3 medications (23/38), uncontrolled seizures with less than 3 medications (7/38), Controlled seizures with more than 3 medication (5/38), Controlled seizures with less than 3 medication (3/38)

(Continued)

TABLE 1 | Continued

References	Sample size	Mean age at seizure onset	Seizure semiology	EEG patterns	Comorbidities	MRI findings	Treatments	Treatments efficacy
Trivisano et al. (64)	61	10 months	Motor seizures were primarily tonic. Non-motor seizures were characterized by psychomotor arrest, loss of muscle tone, hypopnea, cyanosis and desaturation. Fearful expression was reported as one of the most common initial ictal manifestations.	Interictal epileptiform abnormalities (63.9%). Focal seizures arose from temporal (82.8%), frontal (6.2%), parieto-occipital (6.2%), and central (4.7%) regions. Diffuse onset (39.2%).	ID (36/61). Autistic features (36/61)	Normal	n.a.	During the first decade, epilepsy tended to be active and resistant to multiple antiepileptic drugs. Later on, a decrease in seizures regardless of treatment was observed.
Sadleir et al. (65)	Cohort A: 17 cohort B: 62	Cohort A: n.a. cohort B: 10.3 months	Focal, tonic, GTCS	n.a.	ID (13/17 cohort A) Autistic features (6/17 cohort A)	n.a.	CLB, CBZ, LTG, VPA, TPM, PRD, ACTH, PB, PHT, AZD, DZP, VGB, GP, NZP, TGB, OX, PRD	Levetiracetam resulted in at least 12 months' seizure freedom in 76% of cohort A and in 42% of cohort B

ACTH, adrenocorticotropic hormone; AZD, acetazolamide; BR, bromide; CBD, cannabidiol; CBZ, carbamazepine; CLB, clobazam; CZP, clonazepam; DZP, diazepam; ESM, ethosuximide; GBP, gabapentin; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; LZP, lorazepam; MDZ, midazolam; NZP, nitrazepam; OXC, oxcarbazepine; PB, phenobarbital; PHT, phenytoin; PER, perampanel; PGB, pregabalin; PHT, phenytoin; PRD, pyridoxine; RFN, rufinamide; STM, sulthiame; STP, stiripentol; TGB, tiagabine; TPM, topiramate; VGB, vigabatrin; VPA, valproate; ZNS, zonisamide.

to antiseizure medication (ASM), therefore the pharmacological choice can be oriented in cases of affected patients in the same family (50). Despite DS, the use of sodium channel blockers such as lamotrigine and carbamazepine in PCDH-19-related epilepsy has shown less seizure exacerbation (22). Bromide and clobazam revealed higher efficacy in reducing seizures after 3 months of treatment with a partial reduction in effectiveness during long-term follow-up (63). Valproate and levetiracetam resulted among the most efficacious antiepileptic drugs with a response rate of 61 and 57%, respectively after 12 months of use (63, 65). The effectiveness of phenytoin is unclear, Higurashi et al. showed a good response, not confirmed by Lotte et al. who reported a high grade of ineffectiveness and seizures worsening after phenytoin administration (60, 63). However, the small sample of patients treated with phenytoin in these studies raises doubts about the reliability of these results. Based on the similarities between DS and PCDH-19 related epilepsy, stiripentol was used in addition to valproate and clobazam in a female patient affected by PCDH19-related resistant epilepsy with a great efficacy (66). Stiripentol was later used, in six patients with PCDH-19 related epilepsy as an add-on to valproate and clobazam with a decrease of seizure frequency by more than 50% (56). It is not clear whether the efficacy obtained after stiripentol introduction was due to the intrinsic effect of the drug or to pharmacokinetic interactions causing an increase of clobazam and valproate blood levels (56, 66). PCDH19 gene mutation can be associated with reduced steroidogenesis. Confirming this hypothesis, Tan et al. identified dysregulated AKR1C1-3 which is involved in the production of allopregnanolone (67). Global decrease of neuroactive steroids such as allopregnanolone, pregnenolone sulfate, 17-OH progesterone, and cortisol could be related to seizures onset in PCDH19 mutation. Thus, restoring steroidogenesis can be a therapeutic goal that may improve the management of this disorder (68). Although corticosteroids can be used to control seizure clusters, lacks a long-term benefit with a high risk of recurrences after interruption (22). Oral corticosteroid prophylaxis during febrile episodes was used in a Japanese study with no recurrence of moderate/severe clusters (57). The ketogenic diet showed a positive response in 50% of patients. Vagus Nerve stimulation was used in only one case with a 75–90% seizure reduction at 3 months, persistent after 1 year (63). A single case report described an improvement in seizure control and development after leucovorin therapy in a patient with low cerebral folate levels (69). In conclusion, similarly to DS, GABAergic drugs are the most effective in the treatment of PCDH19-related epilepsy. In particular, first-line drugs that should be considered are bromide, clobazam, and valproate. Levetiracetam should be considered in patients with highly refractory clusters of seizures. Despite the predominance of focal seizures, carbamazepine does not appear as effective as expected, however sodium channel blockers showed relatively good effectiveness in some patients. Stiripentol may be effective especially in patients with DS-like, however, due to drug resistance, it is often necessary to use it in combination with clobazam and valproate. Aggravations of seizures were reported in connection with sodium channel blockers (58), with topiramate and valproate (60). The increasing number

of antiseizure medications in the last decades has led to the development of new successful therapies. Recently, fenfluramine and cannabidiol have proved to be well tolerated and effective in reducing seizures frequency in DS (70, 71). Based on the existence of a similar therapeutic response between DS and PCDH19-related epilepsy, these therapies could be considered in the treatment of the latter patients. Seizure clusters are a frequent clinical manifestation of PCDH19-related epilepsy, especially in the early stages of the disease. Midazolam infusion has shown marked efficacy. However, a high risk of seizure recurrence and worsening during dose reduction or early withdrawal was reported (60). Intravenous phenytoin and phenobarbital were also used during seizure clusters with good responses (22). The risk of seizure recurrence after ASM withdrawal in PCDH19-related epilepsy is significantly high. A recent study conducted on 42 patients with PCDH19-related epilepsy shows that 88.3% of ASM withdrawal leads to seizure recurrence. In 36.4% of cases, it was also necessary to increase the previous ASM dosage. Only in two cases, it was possible to totally withdraw ASM without seizure recurrence. Suspension of treatment was not only related to a high risk of seizure recurrence (72). Younger age and shorter previous seizure-free periods were risk factors for seizure recurrences, as reported from previous studies conducted on common epilepsies (73). Studies involving a wider population with PCDH19 mutation may improve the management and treatment of this disorder. Greater knowledge of gene variants and pathogenetic mechanisms underlying phenotypic expression could lead to a better understanding of the syndrome and a more effective pharmacological therapy.

CONCLUSION

The development of next-generation sequencing techniques has allowed us to bring the diagnosis to a deeper level recognizing specific gene variants and assessing any genotype-phenotype correlations. Besides SCN1A, PCDH19 is among the most relevant genes in epilepsy. The broad phenotypic spectrum of PCDH19-related epilepsy has been extensively studied in recent years and has led to improved and earlier recognition of symptoms. Further studies aimed at a better framing of the non-epileptic features and overall quality of life of these patients are needed. Especially cases of hemizygous males with psychiatric manifestations should be deeper investigated to better understand the expression of this gene and its peculiar inheritance pattern. Most mutations of PCDH19 occur in the extracellular domain, including whole and partial gene deletion and missense, nonsense, and frameshift mutation. The genotype-phenotype correlation has been investigated in several studies; however, it is not yet known how different gene variants can alter clinical manifestations. Protocadherin-19 is known to have an essential role through its extracellular domain in cell adhesion and neuronal architecture (74). However, the role of this molecule is not limited to cell-cell interaction and involves important mechanisms of signal transmission through its intracellular domain. Thus, to optimize precision therapies with the aim of targeting underlying pathogenesis, it is essential to have a greater knowledge of the biological processes in which PCDH19 is involved and how these are altered by the mutations studied so far. In conclusion, it is important to consider PCDH19 in the context of genetic epilepsies. Molecular testing for PCDH19 mutations is recommended especially for female patients who present seizure clusters with early onset, with familiarity or characteristics compatible with GEFS+ or DS, and in cases with cognitive and psychiatric comorbidities.

AUTHOR CONTRIBUTIONS

GD and VV put forward the conception of the review and wrote the manuscript. AV and EM participated in the proposal of the concept and revised the manuscript. AF, GT, GD, and PS proposed suggestions for revision. All authors approved the submitted version.

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BRIEF RESEARCH REPORT
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Cortical Visual Impairment in CDKL5 Deficiency Disorder

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Background: CDKL5 deficiency disorder (CDD) is a developmental encephalopathy caused by pathogenic variants in the gene cyclin-dependent kinase-like 5. Cerebral visual impairment (CVI) is frequent in patients with CDD. In addition to being recognized as a specific feature of the pathology, it has been suggested that visual impairment may correlate with neurodevelopmental outcome and epilepsy severity, but no systematic behavioral visual assessment has been performed. The aim of our study was to evaluate clinical and electrophysiological profile of CVI in patients with CDD, to correlate various aspects of visual function to neurodevelopmental and epileptic features.

Methods: The study included all patients with CDD from the National Pathology Registry. All patients underwent neurological examination, a disease-specific functional assessment, structured clinical evaluation of visual functions, including pattern reversal visual evoked potential (VEP), and a detailed monitoring of epileptic features, including video-EEG.

Results: All the 11 patients recorded in the CDKL5 national registry, 10 females and one male, age range of 1.5 to 24 years (mean 9, SD 7.7, median 6.5), were enrolled. Visual function is impaired in all patients; in particular, visual fields, visual acuity, contrast sensitivity, and stereopsis were consistently abnormal whereas other aspects, such as fixing and tracking, were relatively preserved. Pattern reversal VEP was abnormal in nearly 80% of our patients. No correlation was found among CVI severity, age, level of psychomotor development, EEG abnormalities, and pathology stages even if an overall less abnormal EEG pattern was more often associated with better visual results.

Conclusion: In conclusion, CVI can be considered as a major feature of CDD with a diffuse involvement in several behavioral and electrophysiological aspects. Larger cohorts will help to better clarify the possible prognostic role of EEG severity in predicting both visual and developmental abnormalities.

Keywords: cortical visual impairment, CDKL5 deficiency disorder, developmental and epileptic encephalopathies, VEP (visual evoked potential), EEG abnormalities

INTRODUCTION

CDKL5 deficiency disorder (CDD) is a developmental encephalopathy caused by pathogenic variants in the gene cyclin-dependent kinase-like 5 located on the short arm of the X chromosome (X22p13). The gene product, the CDKL5 protein, is highly expressed in the brain, predominantly in neuronal nuclei and dendrites. It has been found to have a role in cell proliferation, neuronal migration, axonal outgrowth, dendritic morphogenesis, and synapse development and function, also in the adult brain (1–5).

Clinical signs of CDD include early infantile onset refractory epilepsy, hypotonia, developmental intellectual and motor disabilities, cortical visual impairment, sleep disorders, associated with minimal dysmorphic features, and possible gastrointestinal and respiratory signs (6).

Median age of epilepsy onset is 6 weeks, with 90% of the patients having signs by 3 months.

Visual impairment is frequent in patients with CDD. It is mainly characterized by poor eye contact and the absence of visual tracking with normal ophthalmological assessment. Recent observational multicenter clinical studies (6, 7) reported fixation and tracking abnormalities, the presence of nystagmus, detection of roving eye movements (slow conjugated movements, mainly horizontal, of the eyes, similar to those observed in sleep), and abnormal optokinetic nystagmus. These findings were extrapolated by parental report of patient's functional abilities, neurologist's physical examination findings, and ophthalmological assessment. The impairment of at least one of these aspects was found in 76% of patients (6). The authors also found a direct correlation between visual deficit and neurodevelopmental outcome (6, 7). Because of this, visual impairment has been proposed as a marker of clinical severity or prognosis even if the pathophysiological mechanisms underlying this deficit in CDD are not clearly understood. A recent study on mouse model of CDKL5 disorder using pattern reversal intracortical visual evoked potential (VEP) reported a dramatic impairment of the cortical response in both juvenile and adult mice (8). The severity of reduction in VEP amplitude was related to the level of visual acuity and contrast sensitivity. The same group analyzed the morphology of the visual pathway from the retina to the primary visual cortex (V1) in CDKL5 null mice. They found reduced density and altered morphology of spines and excitatory synapsis of dorsal lateral geniculate nucleus and V1, but no anomalies in the anterior circuitry from the retina. The abnormal findings in the brain also suggest that there may be a common pathway with other clinical signs of central nervous system involvement and that other electrophysiological techniques, such as EEG, may be used to establish a correlation between brain electrical activity and cortical visual impairment.

It has been suggested (9) that visual impairment may correlate with the three epilepsy stages described in CDD: (I) early onset epilepsy, characterized by daily and polymorphous seizures, associated with a normal to destructured electrical background activity with or without focal anomalies; (II) epileptic encephalopathy; and (III) refractory multifocal and myoclonic epilepsy with destructured EEG and florid multifocal anomalies in wakefulness and sleep (10–13). In particular, a delay in maturation of visual abilities has been described in stage I whereas a regression of some aspects of visual function, such as visual attention, has been described at the beginning of stage II.

Because of the difficulties in obtaining reliable visual assessments in CDD children, no systematic behavioral visual assessment has been performed in correlation of epileptic features.

The aim of our study was to evaluate clinical and electrophysiological profile of CVI in patients with CDKL5-deficient encephalopathy to correlate various aspects of visual function and VEP and to establish whether both the clinical signs and the cortical responses are related to the severity of the clinical and EEG signs of epilepsy.

MATERIALS AND METHODS

The study includes patients who diagnosed with CDD identified through the "CDKL5 together toward the cure" association, as part of a project aimed to create a National Register of CDKL5 deficiency disorder. All patients in the registry were contacted. All families agreed to be a part of the study and signed an informed consent. The study was approved by the Ethics Committee of our institution.

Patients were assessed at the Child Neurology Unit and at the National Center of Services and Research for the Prevention of Blindness and Visual Rehabilitation of Visually Impaired, of the University Hospital "Fondazione Policlinico A. Gemelli IRCSS" in Rome.

All patients underwent neurological examination, a disease-specific functional assessment, structured clinical evaluation of visual functions, including pattern reversal VEP, and a detailed monitoring of epileptic features, including video-EEG.

Disease-Specific Functional Assessment

All patients were scored using the CDKL5 Development Score, a functional scale proposed by Demarest et al. (7) which provides a score from 0 to 7, obtained by adding the stages of psychomotor development reached by the patient (autonomous sitting posture, autonomous standing posture, autonomous walking, rake grip, gripper grip, lallation, and use of single words).

Assessment of Visual Function

This included an ophthalmological assessment, and a battery of tests assessing various aspects of visual function: fixation, saccades, acuity, visual fields, and attention at distance, was used, adding other aspects of visual function such as contrast sensitivity and stereopsis.

Ophthalmological assessment a single pediatric ophthalmologist examined all patients.

Anterior segment examination by handle slit lamp, indirect ophthalmoscopic of the fundus, and cycloplegic refraction by autorefractometry were performed. Myopia was defined as a cycloplegic refraction of −0.5 diopters (D) or less, hyperopia as +2.00D or more, and astigmatism was considered if more than 0.75D.

Slit lamp examination was performed searching for unrecorded alterations and to check lens transparency. Cycloplegic was performed 40 min after administration of tropicamide 1% mydriatic eye drops (1 drop for two times in 15 min) by means of Retinomax 3 Plus Handle Refractometer (Nikon). Fundus examination by indirect opthtalmoscopy with +28 and +20 diopters lens was performed. Fundus abnormalities (i.e., the presence of macular dystrophies or optic nerve alterations) were recorded.

Ocular motility was also observed, and the presence of nystagmus, strabismus, or abnormal ocular movements was recorded.

Fixation: The ability to fix was assessed by observing the ability of the infant to fix on a high-contrast target (black/white or colored) target. Fixation is stable if it lasts 3 s, is unstable if it is shorter, and is absent if it is not possible to elicitate.

Tracking or visual pursuit was assessed by observing the ability of the infant to follow a high-contrast target (black/white or colored) horizontally, vertically, and in a full circle. Tracking is considered complete if it covers the whole arc, incomplete if it goes for more than 50% of it, brief if it is less than 50% of it, and absent if it cannot be elicitate. For visual pursuit, in addition to quality, its presence in the three different arches was considered.

Saccadic movements were assessed using one target per hand. Child's attention was alternatively drawn on the targets, horizontally (right and left) and vertically (up and down). The item was repeated two times each side, noting if infant needed to move the head and did not move only the eyes.

Acuity was assessed binocularly by means of the Teller Acuity Card procedure (14–16). This method is based on an inborn preference for a pattern (black and white gratings of decreasing stripe widths depicted on cards) over a uniform field. The location of the left or right position of the test stimulus varies randomly. An observer judges the infant's reaction to the location of the test stimulus based on eye and head movements. The threshold of acuity is taken as the minimum stripe width to which the subject consistently responds. Acuity values were expressed in minutes of arc (or cycles per degree) and were compared with age-specific normative data reported in the literature (17, 18).

Attention at distance was tested by moving a colored toy (about 8–10 x 8–10 cm) backward in a small arc away from the child. The maximum distance at which the child still keeps attention on the toy is recorded (19).

Binocular visual fields were assessed using kinetic perimetry, according to the technique described in detail by van Hof-van Duin (20). The apparatus consists of two 4-cm wide black metal strips, mounted perpendicularly to each other and bent to form 2 arcs, each with a radius of 40 cm. The perimeter is placed in front of a black curtain, concealing the observer, who can watch the infant's eye and head movements through a peephole. The child is held sitting or lying in the center of the arc perimeter, with the chin supported. During central fixation of a 6° diameter white ball, an identical target is moved from the periphery toward the fixation point, along with one of the arcs of the perimeter, at a velocity of about 3°/s. Eye and head movements toward the peripheral ball are used to estimate the outline of the visual fields. Age-specific normative data for full-term and preterm infants are available (18, 21).

Contrast sensitivity was assessed using the Hiding Heidi test. It consists in four cards, one white and the other three with the image of a face on both sides with contrast reducing from 100 to 25%, 10, 5, 2.5, and 1.25%. The picture is presented by moving both the picture and the white card with the same speed, usually horizontally. The side the child looks is noted as response. The level of contrast sensitivity consists in the less-contrasted picture the child looks at.

Stereopsis was assessed using the Frisby stereotest. This test is used to assess stereovision at closer distances, requiring eye convergence (22, 23). The participant's task is to detect a circle containing a pattern of geometric objects (the target) visible within a mosaic of similar geometric shapes. The target and background are printed on opposite sides of a Perspex plate and so differ in their physical depth. The angular disparity depends on the thickness of the plate and the distance from the observer.

Pattern Reversal VEP

Patients were evaluated using the classic pattern reversal VEP protocol (24) with black and white checkerboard presented by a rectangular LED flat-screen monitor (4:3). The stimulation parameters were as follows: luminance 50 candles * m-2; contrast $> 80\%$. The chess shape was square, and regarding the chess size, three different measures were used in three different stimulation sessions: 1, 0.5, and 0.25 cm, with patient placed 57.3 cm from the screen to obtain angles of visual field of 60', 30', and 15', respectively. The phase change between black and white occurred without changes in screen luminance. The reversal rate pattern has been set at 2 reversals per second. Stimuli released for each test were 100 in total. In total, 4 recording electrodes (Oz, PO7, PO8, and Fz) were placed on the scalp, with reference electrode on Fz and ground electrode on the left ear lobe (A1). Copper disc electrodes were used. The electrode impedance was $< 3000\Omega$. The signal was filtered with a 1–250 Hz passband; 50 Hz notch filter active. For each stimulation, activity on the scalp was recorded between 50 ms before and 450 ms after stimulus release. The sampling rate was 4096 Hz. Three sessions were performed at a distance of 10 min from each other using different angles of visual field underlying the check. For each derivation on the scalp, the average of traces obtained after frequent and deviant stimulus was carried out. Potentials > 70 μV have been automatically excluded from the average. For data analysis, amplitudes and latencies of N75, P100, and N145 were evaluated and compared with normative data (25, 26).

Epilepsy

Epilepsy was classified according to Bahi-Buisson stages (9): (I) early onset epilepsy, characterized by daily and polymorphous seizures, associated with a normal to destructured electrical background activity with or without focal anomalies; (II) epileptic encephalopathy; and (III) refractory multifocal and myoclonic epilepsy with florid multifocal anomalies in wake and sleep. Details on onset of the seizures, progression, and pharmacological therapy were also collected.

Video-EEG

Patients underwent standard video-EEG during wakefulness and sleep. The recording was made through preassembled caps with 21 electrodes according to the International System 10–20. The EEG lasted about an hour and included, as activation tests, the intermittent photic stimulation (IPS). For IPS, a LED photic stimulator was used. The lamp was placed 30 cm in front of the nasion of the patient. White light flashes had an intensity of about 1 Joule (27). Due to poor patient cooperation, they were not expected to close their eyes during IPS. For the same reason, only increasing frequency protocol was performed (1–50 Hz). Each stimulation lasted 10 s with pauses of 10 s. In case of photoparoxysmal response, the protocol provided for the interruption of IPS. Once the parental consent was obtained, the repetition of IPS was provided at the end of the recording, starting from 50 Hz and decreasing to the frequency at which the photoparoxysmal response was observed.

Data were analyzed through the SystemView Micromed system, and a qualitative analysis of background activity in wakefulness and sleep was carried out. Any paroxysmal anomalies and characteristics of recorded electroclinical episodes were also examined. According to EEG features, the examination was classified as normal EEG; normal background activity and the presence of focal anomalies; abnormal background activity and the presence of focal anomalies; abnormal background activity and the presence of focal and generalized anomalies; and hypsarhythmia. Furthermore, according to seizures type and frequency and the results of EEG examination, the stage of pathology has been established for each patient, according to the classification of Bahi-Buisson et al. (9).

Other information included gender, family history of seizures or other diseases, genetic and imaging studies, and possible comorbidities.

For statistical analysis, continuous variables were expressed in means and standard deviations. Categorical variables were presented as frequencies and percentages. Due to the low sample number, only linear regression was performed to identify a possible correlation between the severity of the visual impairment and age, CDKL5 Development Score, and disease stage.

RESULTS

All the 11 patients in the CDKL5 national registry, 10 women and one men, age range of 1.5 to 24 years (mean 9, SD 7.7, median 6.5), were enrolled. All patients presented the minimum diagnostic criteria proposed by Olson et al. in 2019 (pathogenetic variants in the CDKL5 gene, severe global psychomotor retardation, and epilepsy onset in the first year of life).

All patients underwent a complete assessment.

Disease-Specific Functional Assessment

Only one patient was able to walk independently and used single words to communicate. Two patients had achieved the ability to sit and stand independently, but not to walk, mature grip, and use of single words. The other subjects had more impaired motor and verbal functions. Details of the CDKL5 Developmental Scale are shown in **Table 1**.

Assessment of Visual Function

All patients completed the assessment and showed clinical characteristics compatible with cerebral visual impairment (**Table 1** and **Figure 1**).

Ophthalmological assessment no abnormalities in anterior segment were found, and no media or lens opacity was observed. Optic nerve head slight pallor was observed in all but one patient. No other retinal abnormalities were recorded. Second-level ophthalmological examination (OCT, retinography) was not possible due to lack of cooperation. Hyperopia was found in six patients and myopia in two, associated with mild astigmatism. In the remaining three patients, results were not reliable due to lack of cooperation.

All patients were disturbed by light more than normal showing moderate photophobia.

Eye movements were conjugated in one of the 11 patients, and the remaining 10 patients presented strabismus, four esotropia, and six exotropia.

Fixation was present and stable in five of the 11 patients, unstable in four, brief in the remaining two patients.

Tracking or pursuit: Horizontal tracking was complete in seven out of 11 patients, incomplete in one, brief in two, and absent in the remaining patient.

Vertical tracking was complete in three patients, incomplete in four, brief in one patient, and absent in the remaining three.

Tracking in a circle was complete in two patients and absent in the remaining nine.

Saccadic movements were not elicitable in all patients, both horizontally and vertically.

Visual acuity was not testable in one out of 11 patients and reduced in the remaining 10 patients.

Attention at distance was impaired in all patients, with five of the 11 patients keeping attention for a distance between 1 and 2 m, four patients 50 $>$ 100 cm, and the remaining two patients $<$ 50 cm.

The *visual field* was not evaluable in eight patients and restricted in the remaining three patients.

Contrast sensitivity was not evaluable in seven patients and reduced in the remaining four.

Stereopsis was absent in all patients.

Visual attention was absent in 10 of the 11 patients and present only in the noncompetitive modality in the remaining patient.

Pattern Reversal VEP

In three out of 11 patients, it was possible to obtain a visual evoked potential of normal morphology and latency. In five patients, no response could be elicited, and in one patient, VEP was of low amplitude and altered morphology. The remaining two were too irritable to get reliable potentials due to movement artifact.

TABLE 1 | Detailed results for each subject.

ID	Age (y)	CDKL5 gene mutation	Visual functions															
			EYE MOV	Fix	Tracking			Attention at distance	Visual Acuity	Visual Fields	Constrast sensitivity	Visual Attention	Stereopsis	Saccades	Pattern VEPs	CDKL5 Dev Scale	CDD stage	EEG
				Hor	Ver	Circle												
1	1.5	c.1648C>T	Strabismus + roving movements upwards	Unstable	Complete	Complete	Complete	2m	<1/20	Bilateral reduction	25%	Absent	No reaction	No reaction	UT	2	II	Hypsarrhythmia
2	1.7	dup and del Xp22.13	strabismus	Stable	Complete	Uncomplete	Absent	50cm	<1/20	No reaction	No reaction	Absent	No reaction	No reaction	ABN	3	II	Abn background act + multifocal and generalized abn
3	3.5	c.433_433delC	Strabismus + nystagmus	Unstable	Brief	Brief	Absent	60cm	<1/20	No reaction	No reaction	Absent	No reaction	No reaction	Absent	4	II	Hypsarrhythmia
4	5.8	c.528G>A	strabismus	Stable	Complete	Uncomplete	Absent	1m	<1/20	Bilateral reduction	25%	Only NC	No reaction	No reaction	Absent	2	II	Hypsarrhythmia
5	6.3	c.7441 + G>C	strabismus	Stable	Complete	Uncomplete	Absent	50cm	<1/20	No reaction	No reaction	Absent	No reaction	No reaction	Absent	3	II	Abn background act + multifocal and generalized abn
*6	6.5	del exon 1	Strabismus + roving movements	Brief	Brief	Absent	Absent	10-15cm	UT	No reaction	No reaction	Absent	No reaction	No reaction	UT	0	II	Hypsarrhythmia
7	6.5	del exons 18-21	normal	Stable	Complete	Complete	Absent	1.5m	2/10	No reaction	25%	Absent	No reaction	No reaction	N	3	III	Abn background act + focal abn
8	12	c.533G>A	Strabismus	Brief	Absent	Absent	Absent	10cm	<1/20	No reaction	No reaction	Absent	No reaction	No reaction	Absent	3	III	Abn background act + focal abn
9	16.5	c.587C>T	Strabismus	Unstable	Uncomplete	Absent	Absent	1m	<1/20	No reaction	No reaction	Absent	No reaction	No reaction	N	6	III	Normal background act + focal abn
10	21	del exons 7-8	Strabismus + nystagmus	Unstable	Complete	Uncomplete	Absent	50cm	<1/20	Bilateral reduction	No reaction	Absent	No reaction	No reaction	Absent	6	III	Abn background act + focal abn
11	24	c.645 + G>A	strabismus	Stable	Complete	Complete	Complete	1.5m	3/10	No reaction	5%	Absent	No reaction	No reaction	N	7	III	Normal background act + focal abn

, male; UT, Untestable; ABN, abnormal; N, normal.

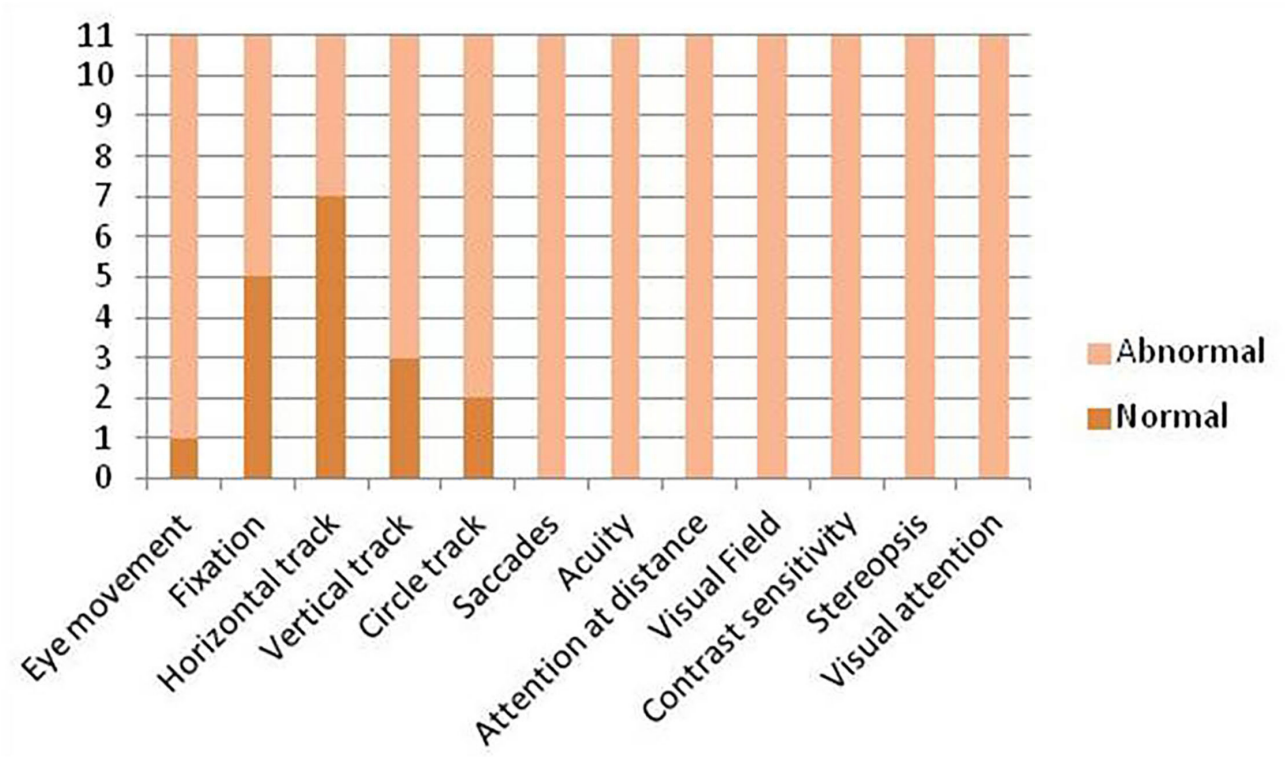

FIGURE 1 | Visual function overview.

Epilepsy

Six out of 11 patients were in the second stage of epilepsy, the other five in the third. As expected, patients in phase II present daily and polymorphic seizures (spasms, myoclonic, and tonic) and all but one (n ° 4) are on complex antiepileptic drug treatment (two or more drugs). Among the stage III patients, two have daily seizures, 3 every week. All are on complex antiepileptic treatment.

Video-EEG

No patient had normal EEG, two had recognizable background activity with focal anomalies, three altered background activity and focal anomalies, two altered background activity and multifocal and diffuse anomalies, and four presented with hypsarrhythmia.

None exhibited a photoparoxysmal response during IPS.

Statistical Analysis

For each patient, the number of impaired areas of visual abilities was used as an independent variable, and a linear regression was performed with age, CDKL5 Development Score, and CDD stage as dependent variables. No significant values of corrected R-squared were obtained for any of the three variables (severity of CVI and age: corrected R-squared = −0,11; severity of CVI and CDKL5 Development Score: corrected R-squared = −0.09; severity of CVI and CDD stage: corrected R-squared = −0,1).

DISCUSSION

Visual impairment has been reported to be frequent in patients with CDD (28), but the possibility to assess various aspects of visual function was limited by the poor collaboration of these subjects at the time they have to perform structured routinely used visual assessments. Most information on aspects of visual function comes from the ophthalmologic assessment or from caregivers' observation. In this study, using a battery of tests that have been specifically designed for young children with relatively poor collaboration, easy to be performed even in young children with multisensory or cognitive impairment (29–31), we were able to perform a detailed assessment in a cohort with a wide range of age and severity.

This allowed us to use, for the first time, a structured assessment of visual function in combination with a detailed ophthalmological assessment in patients with CDD and with the assessment of evoked potentials. Compared with previous studies, we combined the use of a structured visual assessment and electrophysiological examinations and this consented to define a wider description of visual abilities regardless of severity and pathology stage (6, 7).

Our data confirmed that visual function is impaired in all patients but the possibility to assess different aspects of visual function allowed providing more details on the extent and severity of the involvement.

More cortical aspects of functions, such as visual fields, visual acuity, contrast sensitivity, and stereopsis, which require the integrity of cortical–subcortical networks, were consistently abnormal.

Visual evoked potential was also abnormal in nearly 80% of our patients. These data are in agreement with previous studies assessing visual function in epileptic encephalopathy (30). Animal models of CDKL5 deficit suggest a possible role of an occipital cortical involvement connected to a specific impairment of visual acuity and contrast sensitivity (8, 32).

In contrast, other aspects, such as fixing and tracking, were relatively preserved. These aspects are typically considered to be mediated by subcortical structures but, even if relatively preserved, were still frequently affected in most patients. Although this was not systematically explored in our patients using imaging, the impairment of these aspects is likely to be in relation to the involvement in subcortical areas such as basal ganglia and lateral geniculate nucleus, as described by Mazziotti (8) and Lupori (31) in CDD animal models. It is of interest that while tracking horizontally and, partly, vertically was relatively spared, when increasing the complexity of action, requiring to track in a circle, most of the patients with CDD showed increasing difficulties.

Therefore, our data showed that visual impairment does not appear limited to more mature cortical functions, but may also involve more basic aspects that rely on subcortical structures.

The severity of visual deficit did not appear to be specifically related to age, as severe signs were found not only in infants assessed soon after diagnosis but also in the oldest ones and throughout the whole spectrum of age. Similarly, there was no obvious association between visual function and CDKL5 Development Scores. Interestingly, relatively sparing of these aspects was not always found in patients with less severe scores on the CDD developmental scores probably because these children, even if showing relatively normal tracking, still had severe involvement in all the other visual functions that are important for eye and hand coordination and other developmental aspects.

The relationship between visual function and EEG and disease stage was more complex. We were unable to observe a consistent association between severity of visual function and EEG abnormalities or pathology stages. This is possibly due to the relatively small sample size in our study and to the fact that we did not have any child in stage I. The results were too small, and the number of variables was too high to allow a meaningful analysis. Severe EEG patterns such as hypsarrhythmia were not always associated with the more severe diffuse visual impairment.

It should be noted however that, even if did not apply to all the individual cases, a more organized background activity and an overall less abnormal EEG pattern were more often associated with better visual results. This was found in all the three patients with normal pattern reversal VEP, with two of the three also having relatively more preserved aspects of visual function. These findings are partially in agreement with previous studies on animal models (8, 32) reporting that VEP correlated with visual function and more specifically with visual acuity and contrast sensitivity. Similarly, subjects with less abnormal EEG also had better CDKL5 Development Scores.

CONCLUSION

In conclusion, CVI can be considered as a major feature of CDD with a diffuse involvement in several behavioral and electrophysiological aspects. None of our patients had a normal profile of visual function and the impairment involved both cortical and subcortical aspects. Larger cohorts with a wider range of EEG abnormalities and disease stages will help to better clarify the possible prognostic role of EEG severity in predicting both visual and developmental abnormalities.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Università Cattolica del Sacro Cuore, Rome, Italy. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MQ, DR, DB, and EMM contributed to the conception of the subject of the manuscript and wrote and revised the manuscript. DR, MPet, SL, LO, and FA contributed to the visual assessment. MQ, DB, MG, CV, IC, MPer, and EM contributed to clinical and electrophysiology assessment. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Myoclonic Epilepsy: Case Report of a Mild Phenotype in a Pediatric Patient Expanding Clinical Spectrum of *KCNA2* Pathogenic Variants

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We report on the rare case of a male toddler presenting with myoclonic epilepsy characterized by daily episodes of upward movements of the eyebrows, and myoclonic jerks of both head and upper limbs. In addition, the child showed speech delay, tremors, and lack of motor coordination. Next Generation Sequencing analysis (NGS) performed in trio revealed in the proband the c.889C>T *de novo* missense variant in the *KCNA2* gene in heterozygous state. This is the first case of myoclonic epilepsy in a toddler due to a c.889C>T *KCNA2* missense variant. The patient was treated with valproic acid and ethosuximide with a good clinical response. At 6 years old, follow-up revealed that the proband was seizure-free with tremors and clumsiness in movements. According to the literature, this case supports the correlation between myoclonic epilepsy and *KCNA2* alterations. This evidence suggests that performing genomic testing including the *KCNA2* gene in preschool patients affected by myoclonic epilepsy, especially when associated with delayed neurodevelopment. Our goal is to expand the phenotypical spectrum of this rare condition and adding clinical features following a genotype-first approach.

Keywords: epilepsy, *KCNA2*, epileptic encephalopathies, genetic variants, genotype-first approach, epilepsy—abnormalities, classification, drug therapy

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INTRODUCTION

Among the genetically determined forms of epilepsy, many genes remain unknown. Cases of epilepsy caused by a *KCNA2* mutation are known in literature (as shown in **Table 1**) different from the one reported in this manuscript. In 2017, Sachdev et al. (8) described a case of genetically determined epilepsy carrying the same mutation of our patient, but with different phenotype, described in **Table 2**. We report for the first time a pediatric patient affected by myoclonic epilepsy due to the heterozygous c.889C>T missense variant in the *KCNA2* gene. According to the literature, this case supports the correlation between myoclonic epilepsy and *KCNA2* alterations. This evidence suggests that performing genomic testing including the *KCNA2* gene in preschool patients affected by myoclonic epilepsy, especially when associated with delayed neurodevelopment. Our goal is to expand the phenotypical spectrum of this rare condition and adding clinical features following a genotype-first approach.

TABLE 1 | Comparison between the characteristics of patients reported in the literature with mutation in the same triplet encoding an amino acid as the patient analyzed in this study.

	Pena and Coimbra (1)	**Syrbe et al. (2)**	**Corbett et al. (3)**	**Masnada et al. (4)**	**Masnada et al. (4)**	**Masnada et al. (4)**	**Masnada et al. (4)**	**Masnada et al. (4)**	**Canafoglia et al. (5)**	**Nashabat et al. (6)**	**Costain et al. (7)**
Case number	Case 1	Case 1	Case 1	Case 1	Case 2	Case 3	Case 4	Case 5	Case 1	Case 1	Case 1
Variant	c.890G>A, p.Arg297Gln *De novo*	c.890G>A, p.Arg297Gln *De novo*	c.890G>A, p.Arg297Gln *De novo*	c.890G>A, p.Arg297Gln *De novo*	c.890G>A, p.Arg297Gln (ovodonation)	c.890G>A, p.Arg297Gln *De novo*	c.890G>A, p.Arg297Gln *De novo*	c.890G>A, p.Arg297Gln (NR)	c.890G>A, p.Arg297Gln *De novo*	c.890 G > A, p.Arg297Gln (consanguinity)	c.890G>A, p.Arg297Gln *De novo*
Functional analisys	NR	Gain of function	Gain of function	NR	NR	NR	NR	NR	NR	NR	NR
Age of onset	15 m	5 m	12 m	10 m	15 m	since birth	6 m	12 m	36 m	20 m	8 m
Seizure type at onset	FS	Febrile SE	T, MC, AS	FS	FS	Infantile spasms	GTCS	GTCS	T	GTCS	T, GTCS, AS, MC
Other seizure types	MC, AS, GTCS	GTCS, AS	NR	GTCS, MC	MC, AS, GTCS	AS w/o MC, GTCS	AAS w/o MC	No	Prominent cortical myoclonus	No	NR
Clinical features	hypotonia and mild ataxia	Moderate-severe ataxia	psychomotor delay	Aggressiveness, stubbornness, psychomotor delay	Moderate ID, language delay, Stubbornness, difficulty of concentration, psychomotor delay	Moderate-severe ID and language delay, psychomotor delay (since birth)	Psychom. dev. delay (8–9 mo), ASD	Learning difficulties	Psychomotor delay, jerky movements of the upper limbs, clumsiness	N	N
Development at onset	Normal	Normal	N	N	Delayed	Delayed	N	Delayed	NR	N	N
EEG findings	BGS, irregular GSW; sleep activation	GSW and poly-Sp-W	GSW and polySp-W; bilateral posterior SW, BGS;	GSW, theta-beta activity + Sp in the midline	Slow background activity, Irregular GSW; sleep activation	BGS, GSW, posterior SW	BGS, right Occipital Sh-W, disorganized BG; irregular 2H GSW	BGS, GSW, Multifocal Epileptiform discharges	GSW; BGS, Sp on the posterior derivations, bilateral Sp during light sleep.	NR	Sp, SpW
Magnetic Resonance Imaging	NR	Normal	mild cerebellar atrophy	Severe cerebellar atrophy, small hippocampi	Severe cerebellar atrophy	N	Hyperintense subcortical white matter lesions	Cerebellar atrophy	Mild cerebellar atrophy, cisterna magna	Brain atrophy+cerebellar hypoplasia	Cerebellar atrophy
Neurological examination	ataxia and obvious delay of development	Moderate-severe ataxia, hyper-reflexia	Ataxia, cerebellar signs on examination, normal eye movement	Tremor, impaired coordination of fine motor skills, ataxia, dysarthria, myoclonia, pyramidal signs	N	Ataxia, finger tremor, impaired coordination	Tremor, ataxia, head titubation, axial hypotonia, pyramidal signs, impaired motor coordination	Impaired incoordination, mild dysdiadochokinesia, mild-moderate ataxia, dysarthria	Ataxia, irregularly repetitive myoclonic jerks during active hand movements	Ataxia	NR
Development at follow up	tremor of the extremities, loss of sphincter control and hyperkinetic behavior	Moderate ID	Slowing at 12 months	NR	NR	NR	NR	NR	Worsening of the movement disorder	Refractory to medications, still having seizure	NR

NR, Not reported; GSW, generalized spike waves; Sp-W, spike-waves; Sp, spikes; Sh-W, sharp waves; BGS, background slowing; N, normal; ID, intellectual disability; ASD, autism spectrum disorder; S, febrile seizure; MC, Myoclonic convulsion; AS, absence seizures; T, tonic; AAS, atypical absence seizures; GTCS, generalized tonic seizures; SE, status epilepticus; EEG, electroencephalogram.

TABLE 2 | Comparison between the characteristics of the patient studied with the only previous case reported in the literature with the same mutation.

	Sachdev et al. (8)	Our patient
Variant	c.889 C>T, p.Arg297Trp *de novo*	c.889 C>T, p.Arg297Trp *de novo*
Functional analisys	Not reported	Not performed
Age of onset	4 years	4 years
Seizure type at onset	Generalized tonic seizures	Absence seizures
Other seizure types	Status epilepticus	Myoclonic convulsion
Development at onset	Normal	Speech delay
EEG findings	Slow posterior dominant rhythm activity, delta activity with Sp, Spike and Slow Waves in the bioccipital regions, Parasagittal ShW and Sp during activity, GSW, bifrontal ShW; GBS	Slow posterior dominant rhythm activity, theta activity with Sp on the parieto-occipital regions, GSW
MRI	Normal	Ectopy of the cerebellar tonsils (6 mm), hyperintensity of the deep white matter in the suvra/paratrigonal, in subcortical area and in the temporal area bilaterally
Neurological examination	Non-fluent language	Tremor of the hands, clumsiness in movement and in fine motricity
Development at follow up	Normal	Normal

GSW, generalized spike waves; Sp, spikes; Sh-W, sharp waves; GBS, global background slowing; EEG, electroencephalogram.

CASE DESCRIPTION

The proband was born at 38 weeks of gestational age through Cesarean section, performed due to maternal-fetal disproportion. Psychomotor development was characterized by autonomous walking at 16 months (referred balance disorder) and mild language delay improved at the age of 30 months after schooling. The patient has been fed with homogenized food up to 24 months of age and showed selectivity in choosing new ones. The parents reported frequent episodes of vomiting (once a week) without nausea, mostly after physical activity. At the age of 2, the boy experienced head trauma after loss of consciousness. Subsequently, the child showed generalized hypertonus, cyanosis, and deviation of the buccal rhyme that lasted about 2 min with self-resolution. The CT scan highlighted a skull fracture without encephalic lesions and a Chiari type 1 malformation, confirmed during the MRI that showed caudal ectopia of cerebellar tonsils (6 mm from Foramen Magnum), hyperintensity of the deep white matter in the suvra/paratrigonal, in subcortical area, and in the temporal area bilaterally, compatible with terminal areas of myelination. Based on the evidence of cerebellar tonsils ectopia, the toddler underwent clinic evaluation for hypermobility, but did not meet Beighton Criteria for Ehlers Danlos Syndrome diagnosis.

At the age of 4 years (**Figure 1A**), the child started experiencing daily episodes of upward movements of the eyebrows associated with staring spells. The first neurological examination showed tremor of both hands at the end of the index-nose test. The toddler asks for support while climbing stairs and when walking. Electroencephalogram (EEG) recordings while awake showed a trace characterized by poorly organized brain electrical activity and differentiated by age, slow anomalies with interictal paroxysmal abnormalities on bilateral parieto-occipital regions, and abundant epileptiform anomalies with diffuse expression, the most prolonged associated with clinical correlates (**Figures 1B,C**). The physical examination does not underline any dysmorphism except for the presence of flaring of the lateral side of the eyebrows. After 3 months, frequency and duration of the seizures had increased (up to 20 episodes/day in clusters), subsequently associated with massive ictal myoclonus of the upper limbs or head of very short duration (80 episodes/day in clusters). A 48-h EEG recording showed multiple episodes of myoclonus and pathologic graphoelements.

Next Generation Sequencing analysis (NGS) performed in trio on a panel of 77 genes related to epilepsy revealed the missense variant c.889C>T; p.Arg297Trp: NM_004974 in the third exon of the KCNA2 gene (potassium channel, voltage-gated, shaker-related subfamily, member 2, chr1:110,593,579-110,631,535;GRCh38, MIM *176262) in a heterozygous state. Sanger sequencing did not detect this variant in parental DNA, suggesting a *de novo* origin of the alteration.

So, the toddler started antiepileptic therapy with sodium valproate at a dosage of 15 mg/kg/day which required, given the poor control of events, a raise to 21 mg/kg/day. This therapy did not result in any change of the clinical picture and EEG pattern. Therefore, after about 2 months of treatment, ethosuximide was added at a dosage of 25 mg/day with a reduction in the seizures and EEG abnormalities. Approximately a year later, due to the poor control of the therapy, valproic acid was raised to 30 mg/Kg/day and ethosuximide to 30 mg/Kg/day with complete resolution of the pathological picture.

At the age of 5 years and 11 months, the toddler has been tested with WPPSI-III, CPM, CBCL, and ABAS II. Based on the evaluation carried out, a normal cognitive functioning emerges (IQ 91), with abilities adequate to the expected level in the verbal performance and general language scale.

There is a significant decline in the domain that evaluates the processing speed. Non-verbal intelligence is adequate for the chronological age. Assessment of the adaptive profile, as reported by the mother of child, showed subnormal scores in

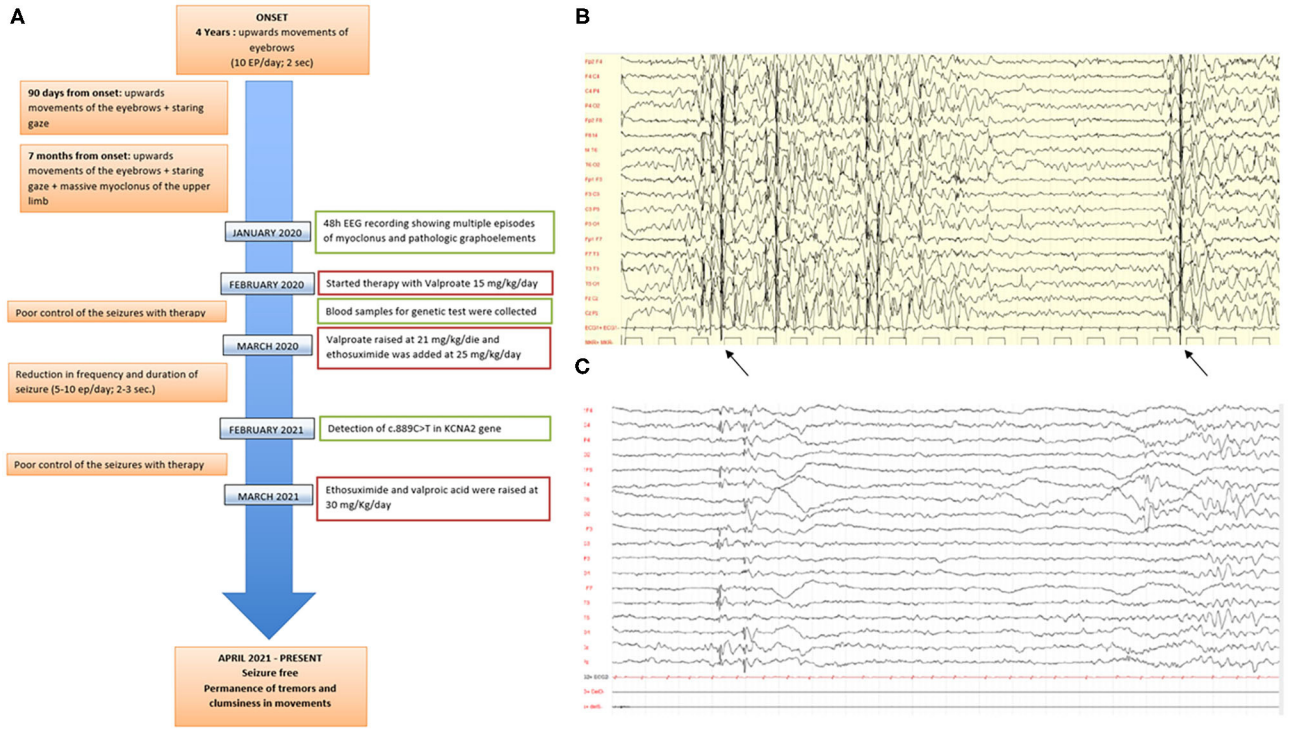

FIGURE 1 | (A) Timeline of clinical features, instrumental characteristics, and therapy approaches in the proband since the toddler started showing epilepsy. **(B,C)** Electroencephalogram (EEG) performed when the child was 4 years old. The trace is characterized by poorly organized brain electrical activity, slow anomalies with interictal paroxysmal abnormalities on bilateral parieto-occipital sites, and abundant epileptiform anomalies with diffuse expression.

the conceptual, social, and practical domains. Critical issues were observed in the categories related to preschool skills, communication, self-control, play, and use of the environment. Currently, the proband presents no epileptic events and improvement in the EEG picture, which still maintains theta-delta slow wave bursts in the occipital regions. Neurological examination appears to be normal, except for tremor of the hands and mild fine motor skills deficit. Some behavioral problems persist. In the EEG assessments carried out from 2017 to 2021, it was evident that the permanence of the slow theta-delta variable rhythm anomalies in the posterior regions, with isolated spike-and-wave complexes on the parieto-occipital sites bilaterally, sometimes fronto- or temporo-central that tend to present in diffuse paroxysms with diffuse expression of varying duration. The associated clinical correlation consists in a reduction in contact, ideomotor slowdown with spontaneous resolution, and asynchronous four-limb myoclonus. Overall, the EEG picture appeared disorganized for the age of child. After 7 months being seizure free, in the latest evaluation, the child no longer presents generalized epileptiform abnormalities.

The psychological tests carried out showed performance at the normal range in the ability to analyze and synthesize visual stimuli based on correct visual perception and visuomotor coordination. In addition, they highlighted results in the average range in logical reasoning and in the ability to abstract reason and categorize visual stimuli. The child showed good expressive and receptive language skills. The scale that assesses skills related to comprehension of verbal terms and instructions associated with long-term memory reached levels above normal. There was a decline in the ability to perform cognitive tasks smoothly and automatically especially under the pressure to maintain focused attention and concentration. Scores showed significantly below normal abilities related to the visual discrimination associated with visual short-term memory and visual-motor coordination and cognitive flexibility; even the ability to work quickly with unusual material achieved below normal performance. The toddler presented a cognitive profile that is adequate to the expected level for gender and age, although there is a significant drop in the domain that assesses the speed of processing. The adaptive framework presents criticalities that currently do not seem to affect the life of the child.

DISCUSSION

This is the first case of a toddler affected by myoclonic epilepsy due to a *de novo* missense pathogenic variant (c.889C>T) in the *KCNA2* gene in the heterozygous state.

The *KCNA2* gene encodes for 2 NCBI Refseq transcripts (NM_004974.4 and NM_001204269.2). Monoallelic *KCNA2* pathogenic variants, encoding the voltage-gated K+ channel Kv1.2, have been reported as a cause of developmental delay and

seizures (Developmental and epileptic encephalopathy 32, MIM #616366) (1–3, 9–13).

Mice models, carrying a *KCNA2* mutation, show motor incoordination, myoclonic jerks, tremor, and small body size (14), while null animals present increased seizure susceptibility (15). Previous functional studies showed that *KCNA2* mutations cause either a dominant-negative loss-of-function, or a drastic gain-of function (2), determining distinct phenotypes in patients (4, 16). Alterations, causing loss-of-function, are characterized by a better prognosis than gain-of-function and mixed forms. In dominant-negative loss-of-function, focal predominant seizures with greater sleep activation are reported in literature. In gain-of-function, critical events seem to be prevalently severe generalized seizures, most frequently are problems in neurodevelopment, such as ataxia. At a structural level, the cerebellar or whole brain atrophy has been frequently reported. Severe early onset epilepsies appear to be more frequent in mixed forms. Neonatal onset epilepsies with developmental impairments or generalized ictal events are rare (4, 16, 17). Recently the phenotypic spectrum has been expanded to include forms of progressive myoclonus epilepsy and myoclonic-atonic epilepsy (18) and functional studies of pathogenic variants had been performed (19).

The p.Arg297Trp variant, detected in the present case, is ranked as "Likely Pathogenic" according to the American College of Medical Genetics guidelines and "Pathogenic" according to ClinVar. Allele frequency is not available. The case we describe here represents the first in pediatric age and the second overall case of myoclonic epilepsy caused by this heterozygous pathogenic variant in *KCNA2*.

The toddler presented at the onset generalized epileptiform abnormalities that were often associated with upward movements of the eyebrows, fixation gaze, and ictal myoclonus of head and upper limbs. The toddler still presents difficulties in autonomous walking and while climbing stairs. The parents reported weekly vomiting episodes not preceded by nausea mostly associated with physical exercise, that could represent a clinical manifestation of seizure. After the therapeutical adjustments, given the considerable reduction in ictal events and the inability to perform a functional analysis of the gene, it has not been possible to attempt therapeutic strategies as recently suggested by the literature, such as 4-aminopyridine (20).

During sleep EEG showed fast/slow wave and polypoint/slow wave of medium- or large-voltage, on the fronto-central regions bilaterally, electrical status epilepticus during sleep (ESES) was not present as described by previous authors (21).

A literature review was conducted to identify the characteristics of reported patients who presented a mutation at the same triplet encoding an amino acid (as shown in **Table 1**). A case with the same mutation was described (8): the patient, differently from our toddler, was diagnosed with unprovoked generalized tonic-clonic seizures. Age of onset was 4 years old in a normal psychomotor neurodevelopment. After carbamazepine treatment, the boy showed seizure remittance. The toddler was worsened from 19 years old, when started presenting episodes of refractory status epilepticus strongly drug resistant. Similarly to our patient, EEG at the age of 4 was characterized by continuous polymorphic delta and theta slowing of the posterior dominant rhythm, intermittent bursts of rhythmic 2–2.5 Hz delta activity intermixed with spikes, spike-and-slow wave discharges over the bi-occipital regions, and isolated parasagittal sharp wave and spike activity during sleep.

Sachdev et al. (8) compared the similar clinical picture of their patient carrying the c.889 C>T mutation, to the reported cases detected with the c.890 G>A mutation in the same amino acidic site, differently from our patient that presents a mild clinical history. In **Table 2**, we compare the characteristics of the patient analyzed in this study with the only previous case reported in the literature with the same mutation (8).

Several cases detected with the c.890G>A p.(Arg297Gln) variant in the KCNA2 gene had been reported, expanding the clinical spectrum of developmental and epileptic encephalopathy and highlighting the variable expressivity. Progressive myoclonic epilepsy was described in one of the patients described by Canafoglia (5). This case showed ataxia and psychomotor delay, whereas epilepsy was delayed and limited to relatively rare tonic seizures, associated with prominent cortical myoclonus, occurred at 3 years. Syrbe et al. (2) reported the case of a toddler carrying a c.890G>A p.(Arg297Gln) *de novo* mutation in the *KCNA2* gene, whose functional analysis showed a gain-of-function alteration. The patient, differently from the reported case, experienced generalized tonic-clonic seizures, absences, status epilepticus often during febrile events. Moreover, the patient presented moderate-severe ataxia and moderate intellectual disability. The same nucleotidic variant was described in a patient with myoclonic and absence seizures. The age of onset, the clinical, and the neuropsychological deterioration was different from our case, resulting in a progressive worsening and drug resistance.

Masnada et al. (4) enrolled five patients carrying this mutation in the KCNA2 gene. Differently from the present case, all of them showed generalized tonic-clonic seizures, but they all presented tremors, ataxia, and a moderate intellectual disability. Two of them experienced myoclonic seizures associated with absences. Only one was seizure-free from 18 months of age. Corbett et al. (3) reported a patient that started having tonic seizures at 12 months, progressing in erratic myoclonic tremors. The proband showed different ictal phenotypes, while EEG and the neuropsychological evaluation showed similar results. Nashabat et al. (6) described the case of a patient that, differently from the present case, experienced earlier onset of generalized tonic-clonic seizures in a good global development, but with poor response to therapy. Costain et al. (7) reported a patient showing various ictal events, such as generalized tonic and tonic-clonic seizures, absences, and myoclonic seizures. This toddler presented an earlier age of onset, a good global development, and ataxia. Ngo et al. (22) described a case with apparently isolated ataxia as primary symptom, but no more clinical information was provided.

Interestingly, an unusual case of mosaicism of two novel missense variants in *KCNA2* had been reported, expanding the phenotypic spectrum associated with this mutations of gene, but not presenting with myoclonic epilepsy (23).

CONCLUSIONS

The present case aims to expand the clinical spectrum associated with heterozygous *KCNA2* pathogenic variants. This evidence suggests a genotype-first approach, performing genomic tests

that include the *KCNA2* gene, in patients with myoclonic epilepsy diagnosis. According to the literature, we highlight the variability in expression that can be clinically observed in patients with heterogeneous ictal events (15), even in those who show mutations in the same triplet encoding an amino acid. Indeed, the patient we describe presented a good prognosis characterized by a good response to therapy, and an improved neurodevelopment, in contrast with the previously reported cases, presenting with a more severe clinical picture (1, 2, 11).

We suggest that an accurate clinical examination improve the detection of genotype-phenotype correlations and expand the possibility of predicting the functional impact of *KCNA2* variants (18). Even if it is a rare condition, a *KCNA2* gene mutation test should be performed in patients over 3 years of age.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LP, GM, MM, IA, FA, AP, CG, and AS: conceptualization, data curation, resources, investigation, writing—original draft, methodology, visualization, supervision, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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A Review of Targeted Therapies for Monogenic Epilepsy Syndromes

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Genetic sequencing technologies have led to an increase in the identification and characterization of monogenic epilepsy syndromes. This increase has, in turn, generated strong interest in developing "precision therapies" based on the unique molecular genetics of a given monogenic epilepsy syndrome. These therapies include diets, vitamins, cell-signaling regulators, ion channel modulators, repurposed medications, molecular chaperones, and gene therapies. In this review, we evaluate these therapies from the perspective of their clinical validity and discuss the future of these therapies for individual syndromes.

Keywords: genetic epilepsy, gene therapy, anti-sense oligonucleotide, ketogenic diet, channelopathy, molecular chaperone

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INTRODUCTION

As of October 2021, the Online Mendelian Inheritance in Man database lists 1,285 genes or loci involved in epilepsy. Several of these discoveries have led to the development of gene-specific therapies—sometimes called "precision medicine"—ushering in a new era of gene-based therapies for epilepsy. By precision therapy or precision medicine, we mean any therapy that is either designed on the basis of the patient's underlying genetic diagnosis or which has been found through clinical trials to have a significant effect in a particular genetic epilepsy (even if the mechanism of the therapy is unknown). This broad definition of precision therapy is in contrast with an *ad hoc* treatment of seizures without any attention being paid to the genetic diagnosis.

In this review, we summarize the current state of precision therapies for monogenic epilepsy syndromes. These therapies are quite diverse, and include molecular chaperones, use or avoidance of ion channel blockade for channelopathies, repurposing of medications, diets, gene therapies including anti-sense oligonucleotides (ASO), RNA interference (RNAi), and inhibitors of over-active cellular signaling (1). While a genetic understanding of how individual patients metabolize and respond to anti-seizure medications are fast advancing (i.e., pharmacogenomics) and could be viewed as a personalized or precision therapy, these advances are outside the scope of this review (2). Throughout this review, we discuss monogenic epilepsy syndromes that are characterized by developmental and epileptic encephalopathies (DEE), according to the definition proposed by the ILAE (3).

We characterize the precision therapies by their documented efficacy as follows:

- *established*, based on randomized clinical trials having demonstrated their usefulness,
- *potential*, based on small or non-randomized clinical trials or case reports
- *hypothetical*, based on animal studies or *in vitro* data, but little-to-no clinical evidence available yet.

This review will summarize these approaches based on type (e.g., molecular chaperone, diet, gene therapy) rather than on documented success (i.e., established, potential, hypothetical), as this

creates a more logical scheme for categorization. Therapies were identified through recent reviews and additional literature review for monogenic epilepsy syndromes (1, 4–6). We conclude with a discussion on the future of precision medicine in genetic epilepsies.

THERAPIES FOR GENETIC EPILEPSIES

Diet and Vitamins

Specialized diets like the ketogenic diet and the modified Atkins diet have become a well-established therapeutic modality for severe seizure syndromes. Thanks to advances in our understanding of certain genetic epilepsies with underlying enzymatic or metabolic disturbances, specific diets and vitamins are increasingly becoming part of the precision medicine armamentarium (see **Table 1**).

One of the best-known examples of genetic disease that responds to a specialized diet is *GLUT1* deficiency syndrome (*GLUT1-DS*), a syndrome that often provokes global developmental delay, movement disorders, and epilepsy, associated with low glucose in the cerebrospinal fluid (CSF) or hypoglycorrhachia. The hypoglycorrhachia and associated symptoms are caused by variants in *SLC2A1* which encodes the type 1 glucose transporter located in the blood-brain barrier (BBB) (7). Because the lack of CSF glucose explains the symptomatology, providing ketones to the CNS *via* the ketogenic diet provides an alternate energy source for neurons. The ketogenic diet leads to improvements not only in the seizure burden but also in the motor and cognitive symptoms in *GLUT1-DS* (8, 9).

The use of ketogenic diet for other genetic epilepsies has expanded beyond the well-known application for GLUT1-DS. Two brothers with newly-identified disease-causing variants in phosphatidyl inositol glycan A (*PIGA*) resulting in X-linked recessive multiple congenital anomalies—hypotonia—seizures syndrome (MCAHS2) who had poorly controlled seizures on multiple anti-seizure medications (ASMs) were able to achieve seizure freedom with the ketogenic diet (10). Discontinuation of the diet led to seizure recurrence. This case report was the first to note a prompt seizure response with initiation of ketogenic diet in *PIGA*-associated early-onset epileptic encephalopathy (10).

Supplementation of pyridoxine (vitamin B6) and pyridoxine derivatives is another very impactful dietary measure for specific genetic epilepsies. Pyridoxine-dependent epilepsy (PDE) is a neurometabolic disorder that presents in the neonatal period with intractable seizures that respond sometimes in dramatic fashion to pyridoxine but not to typical ASMs (7, 11). While this form of epilepsy was known as early as the 1950's, its genetic origins were only identified in 2006 when biallelic variants in *ALDH7A1*, the gene encoding *antiquitin*, were found to be responsible for this neonatal epilepsy syndrome (7). Interestingly, *antiquitin* is a dehydrogenase involved in lysine catabolism that is primarily unrelated to B6 metabolism, but its deficiency results in the accumulation of metabolites inactivate pyridoxal 5'-phosphate (PLP), which is the active form of pyridoxine that is necessary for healthy neurotransmitter function (7). Pyridoxine administration overcomes the inactivation of PLP and then normalizes the metabolic abnormality that causes neonatal seizures.

In 2005, pyridoxal phosphate-dependent epilepsy was found to be caused by bi-allelic variants in the *PNPO* gene encoding the PLP oxidase (7). *PNPO* is involved in the last step of PLP synthesis (the active form of vitamin B6). Phenotypically, patients with *PNPO* deficiency present with epileptic encephalopathy, microcephaly, and developmental delay and respond well to PLP supplementation (7). Classically, patients with *PNPO* deficiency present in infancy and their epilepsy does not respond to pyridoxine, but the phenotypic spectrum of *PNPO* deficiency is expanding as there are documented cases of *PNPO* deficiency with delayed onset or response to pyridoxine (rather than PLP), suggesting that clinicians need to keep an eye open for atypical presentations of this epilepsy (12).

Several patients with vitamin-B6 responsive epilepsy but with genetic testing negative for disease-causing variants in *ALDH7A1* and *PNPO* were found to have bi-allelic variants in the *PLPBP* gene that encodes the proline synthetase co-transcribed homolog (PROSC) which was later renamed as PLP homeostasis protein (PLPHP) (7). This protein binds to PLP and is thought to be important for mitochondrial metabolism. As genetic sequencing technologies become more common in clinical practice, the list of genetic causes of vitamin B6-responsive epilepsy is likely to continue to increase.

Cerebral folate deficiency, caused by mutations in several genes implicated in the folate cycle (notably *FOLR-1* which encodes the cerebral folate receptor), manifests as developmental regression with ataxia, choreoathetoid movements, and myoclonic epilepsy starting around age 3 (13). CSF analysis shows low levels of 5-methyltetrahydrofolate (5-MTHF, <5 nmol/L) but normal serum levels of that metabolite (13). The condition responds very well to high-dose folinic acid, making it important to make the correct diagnosis. Other folate derivatives, like 5-methyltetrahydrofolate, have also been used with some success (14).

Lastly, a relatively new genetic epilepsy syndrome caused by an inborn error of metabolism for which dietary supplementation acts as a precision medicine has been reported recently. Researchers were able to identify four children across three families who presented to medical care for epileptic encephalopathy, global developmental delay, and anemia with anisopoikilocytosis (15). They were able to identify biallelic variants in *CAD*, a gene encoding a multifunctional enzyme involved in *de novo* pyrimidine biosynthesis, as the likely cause of this neurometabolic disorder (15). Two of the children had a worsening neurodegenerative course resulting in death at ages 4 and 5 respectively, but the remaining two children were treated with oral uridine supplementation (which allows recycling of pyrimidines) and showed significant developmental progress (15). The authors suggest that there may be an indication for adding *CAD* to the list of genetic conditions on the newborn

TABLE 1 | Diet and vitamins for monogenic epilepsy.

Gene	Epilepsy syndrome	Suggested precision medicine	Therapeutic rationale	Status as precision medicine
ALDH7A1	Vitamin B6-deficient epilepsy	Pyridoxine, lysine-restricted diet	Impairment of lysine breakdown	Established (11)
CAD	DEE	Uridine	Disruption of pyrimidine metabolism	Established (15)
Folate cycle genes: FOLR-1, MTHFR, DHFR, PCFT	Cerebral folate transporter deficiency (ataxia and refractory myoclonic epilepsy)	Folinic acid, 5-methyltetrahydrofolate	Supplementation of active metabolite missing in folate cycle	Established (13, 14)
*PIGA**	X-linked recessive multiple congenital anomalies – hypotonia – seizures syndrome (MCAHS2), epileptic encephalopathy	Ketogenic diet	Unclear	Potential (10)
PNPO	Vitamin B6 – deficient epilepsy	Pyridoxal-5-phosphate	Supplementation of deficiency	Established (12)
PLPBP	Vitamin B6 – deficient epilepsy	Pyridoxine, pyridoxal-5-phosphate	Supplementation of deficiency	Established (92)
SLC2A1 (GLUT1)	*GLUT1* deficiency syndrome	Ketogenic diet	Alternate energy source	Established (8, 9)

**Indicates the current absence of a molecular or genetic rationale for this particular therapy.*

TABLE 2 | Inhibitors of cellular signaling.

Gene	Epilepsy syndrome	Suggested precision medicine	Therapeutic rationale	Status as precision medicine
GATOR1 complex (DEPDC5, NPRL2, NPRL3)	Familial focal epilepsy with variable foci	mTOR inhibitors (everolimus)	Inactivation of mTOR pathway	Potential (1, 93)
GNAQ	Sturge-Weber-related epilepsy	mTOR inhibitors (sirolimus)	Inactivation of mTOR pathway	Potential (27)
PIK3CA	Intractable epilepsy	PI3K inhibitors	Suppression of PI3K signaling	Potential (29)
TSC1, TSC2	Tuberous sclerosis, focal cortical dysplasia	mTOR inhibitors (sirolimus, everolimus, 1,3,5-triazine derivatives)	Inactivation of mTOR pathway	Established (19)

screening as early detection of the disease could significantly impact the course of the illness.

Inhibiting Overactive Cellular Signaling

Several genetic epilepsy syndromes are the result of mutations in genes that regulate cellular proliferation. Medications that inhibit cellular overgrowth are rational drug candidates for these conditions and were found to be quite effective as adjunctive treatments (see **Table 2**). *TSC1/2* mutations, result in hyperactivity of mTOR complex 1 (mTORc1) with mTOR being a key regulator of cell growth and survival. The hyperactivity produces abnormal neuronal differentiation and migration. Up to 90% of patients with tuberous sclerosis from TSC1/2 mutations will develop epilepsy, with two-thirds of cases being medication-resistant (16). Everolimus, an mTOR inhibitor originally approved for the treatment of renal angiomyolipomas and TSC-associated subependymal giant cell astrocytomas, was subsequently approved as adjunctive therapy for TSC-associated focal seizures in children greater than age 2 (16). The randomized, double-blind, placebo-controlled phase 3 EXIST-3 study supported the use of everolimus as an adjunct if seizures could not be controlled with two ASMs (17). There is ongoing work to determine whether the indication for this drug should be extended to children younger than age 2, as some studies suggest a benefit for the infant age group (18).

Much of the research effort for mTORopathies has focused on everolimus but other mTOR inhibitors, specifically rapamycin or sirolimus, have also been studied (19). A mouse model of TSC with *Tsc1* conditional inactivation primarily in glia exhibits progressive epilepsy and premature death. Early administration of rapamycin in *Tsc1*-inactivated mice prevented the development of epilepsy and premature death compared to the untreated mice, while late administration suppressed seizures in mice that had already started having seizures and also prolonged survival (20). The efficacy noted in this animal model has also been documented in two pediatric case reports. The first reported on a 10 year-old girl who experienced a dramatic decrease in seizure frequency after 10 months of rapamycin therapy (21). The second case report documented significant improvement in seizure frequency in eight children with TSC during the 1st year but with worsening of seizure frequency in three of those patients (22). After discontinuation of rapamycin, three of five children experienced recurrence of seizures, suggesting an overall benefit at least for the 1st year (22).

In addition to case reports, two studies thus far have evaluated the role of sirolimus in TSC-related epilepsy. An open-label study of sirolimus in children with TSC-related epilepsy (23) found that sirolimus treatment led to a statistically

TABLE 3 | Precision therapies for channelopathies.

Gene	Epilepsy syndrome	Suggested precision medicine	Therapeutic rationale	Status as precision medicine
KCNA2	DEE	4-aminopyridine	Reducing current amplitudes	Potential (43)
KCNQ2	DEE	Sodium channel blockers, retigabine, gabapentin	Selective potassium channel Kv7 opener (retigabine), potassium channel Kv7 activator (gabapentin)	- Sodium channel blockers – established (44) - Retigabine – potential (47) - Gabapentin – potential (48)
KCNT1	Epilepsy of infancy with migrating focal seizures, nocturnal frontal lobe epilepsy	- Quinidine - ASO	- Potassium channel blockade in GOF variants - Gene silencing	- Quinidine – potential (7, 49) - ASO – potential (86)
PRRT2	Benign familial infantile epilepsy, paroxysmal kinesigenic dyskinesia	Sodium channel blocker	Failure of neurotransmission	Potential (64, 65)
SCN1A	Dravet syndrome	- Avoid sodium channel blockers - Stiripentol - Fenfluramine - Cannabidiol - ASO	Loss of function of NaV1.1 sodium channels	- Avoidance of sodium channel blockers – established (1, 6) - Stiripentol – established (35) - Fenfluramine – potential (36) - Cannabidiol – established (16, 37) - ASO – hypothetical (84)
SCN2A	Ohtahara syndrome, early encephalopathy	Sodium channel blockers	Gain of function of NaV1.2 channel	Potential (31)
SCN8A	DEE	- Sodium channel blockers - ASO		- Sodium channel blockers – potential (42) - ASO – hypothetical (85)

insignificant 41% decrease in seizure frequency compared to the standard-of-care. This result is in contrast with a larger pediatric study of sirolimus in TSC-related epilepsy that found a statistically-significant 78% decrease in seizure frequency, 47% of whom went on to be seizure-free (24). Subgroup analysis of patients with drug-resistant seizures also confirmed a statistically significant improvement in seizure control (24). Sirolimus may therefore become an important alternate for patients unable to tolerate everolimus. There are no randomized trials comparing sirolimus to everolimus but a retrospective multicenter study of patients with TSC-related epilepsy suggests that there were more adverse events and dosing issues with sirolimus use compared to everolimus (25). There are also newer 1,3,5-triazine derivatives under development that have shown promise as mTORc1/2 inhibitors as they have excellent tolerability profiles and demonstrate marked suppression of spontaneous recurrent seizures in two mouse models of epilepsy, including a mouse model of TSC-related epilepsy (26).

Other disorders of cellular proliferation—including Sturge-Weber syndrome caused by mutations in *GNAQ, NPRL3*-related cortical malformations, and *PIK3CA*-related overgrowth syndromes—also present with epilepsy that seems responsive to "rapalogs" like sirolimus and everolimus. A retrospective observation study of six patients with refractory epilepsy from Sturge-Weber syndrome reported complete seizure control in all patients with minimal side-effects (27). A case report of a neonate with NPRL3-related epilepsy reported 3.5 months of seizure control that allowed the patient to grow seizure-free until epilepsy surgery (28). Mutations in the catalytic subunit of phosphoinositide 3-kinase (*PIK3CA*) are associated with a phenotypic spectrum of bilateral dysplastic megalencephaly and focal cortical dysplasia causing pediatric epilepsy. In a mouse model of *PIK3CA*-related epilepsy expressing the most common human mutations and which recapitulates the human phenotype, a new inhibitor of PI3K signaling that is being trialed for solid tumors (BKM120, a 2,6-dimorpholino pyrimidine derivative) significantly increased the seizure threshold (29). While these studies are promising, there is an ongoing need for studies of mTOR inhibitors for other mTOR-opathies involving mutations in related genes such as the GATOR complex (*DEPDC5*, *NPRL2*).

Ion Channel Modulators

Many genetic epilepsies stem from mutations in genes encoding voltage-gated ion channels and are generally referred to as "channelopathies." This section will discuss the use of ion-channel modulators for these genetic epilepsies, specifically sodium and potassium channelopathies (see **Table 3**). It should be noted that precision therapies other than ion-channel modulation have also been developed for these channelopathies and these are covered in their respective sections.

Sodium Channelopathies

In order of population prevalence, *SCN1A*, *SCN2A*, and *SCN8A* are the four voltage-gated sodium channel genes that are most commonly associated with epilepsy (30–32). We will first consider therapies for *SCN1A* before addressing precision therapies for *SCN2A/8A*-related epilepsy because similar considerations regarding sodium channel blockade apply to these three conditions (7).

SCN1A mutations are associated with various forms of seizures and epilepsies on a wide spectrum of severity, including "isolated" febrile seizures as well as Dravet syndrome (DS), a

developmental and epileptic encephalopathy of poor prognosis. Eighty percent of DS cases are associated with loss-of-function (LOF) mutations in *SCN1A* that encodes the voltage-gated sodium channel Nav1.1, particularly in inhibitory interneurons (33). Precision therapy for DS involves avoidance of sodium channel blockers (e.g., carbamazepine, lamotrigine), as they can worsen symptoms in some patients, as well as the use of agents that enhance GABAergic neurotransmission (e.g., clobazam) (33, 34). Typically, seizures remain refractory to multiple drug combinations, and no single therapeutic approach has shown long-term efficacy in these patients, up to now. Though not yet developed for clinical use, a peptide derived from spider venom (Hm1a) seems promising as it selectively activates the Nav1.1 channel with consequent improvement in seizure and mortality outcomes in a mouse model of DS (33). Other precision therapies for DS [i.e., stiripentol, fenfluramine, and cannabidiol (35–37)] will be discussed in the next section on repurposed medications.

Disease-causing variants in *SCN2/8A* present in a very heterogenous manner that ranges broadly from developmental delay without epilepsy to severe early-onset DEE. Adding to this complexity, individuals with identical variants can present very differently (31, 32, 38). Despite this diversity of clinical presentations, a pattern seems to be emerging which suggests that patients with gain of function (GOF) variants exhibit the more severe early-onset epileptic encephalopathies (EE) while patients with LOF variants present later in life with autism or developmental delay (7, 39–41). This general rule about GOF vs. LOF variants will certainly need further studies to establish its validity, but for the time being it provides clinicians with a rationale for the current management of these sodium channelopathies [e.g., the use of sodium channel blockers like high-dose phenytoin for patients with early-onset *SCN8A* epilepsy (42)]. When it comes to the sodium channelopathies as a whole, their clinical response to sodium channel blockers remains an important area of research.

Potassium Channelopathies

4-aminopyridine is a potassium channel blocker that can antagonize GOF defects in the *KCNA2* gene that cause a DEE. In a study of n-of-1 trials in nine different centers, nine of 11 patients showed improvement in seizure burden, gait, ataxia, alertness, and cognition after starting 4-aminopyridine (43). Because of these findings, it seems a promising tailored treatment for KCNA2-encephalopathy caused by GOF variants.

Heterozygous variants (usually frameshifts or deletions) in *KCNQ2*, which encodes the alpha subunit of the potassium channel Kv7.2, underlie the majority of the autosomal dominant cases of benign familial neonatal epilepsy (BFNE). Variants in this gene associated with DEE are more commonly missense variants with a dominant negative effect (44). Most of these variants seem to induce loss of function of the voltage-gated potassium channel. Sodium channel blockers, which are not a form of precision medicine for this condition, have shown some efficacy, specifically for BFNE (45). Molecular insights into *KCNQ2* disease-causing variants are stimulating research into targeted therapies. Ezogabine (EZO), for example, increases the opening of *KCNQ2* channels, providing a rationale for its use in *KCNQ2* variants that decrease potassium channel activity (44). In 11 patients treated with EZO, seizure reduction and improvements in development were noted in three out of four patients treated before 6 months, and two out of seven patients treated after that age (44). Much like EZO, retigabine and gabapentin have been shown *in-vitro* to be selective Kv7 openers and seem poised to be used for *KCNQ2*-related epilepsy (46, 47). For example, initiation of gabapentin led to rapid and sustained improvement in seizures in a patient with *KCNQ2* DEE (48). Retigabine is an ASM that has been pulled from the market due to significant side-effects but had shown significant promise for refractory epilepsy – there is cause for optimism as new retigabine analogs with fewer side-effects are under development (47).

Pathogenic variants in *KCNT1*, also encoding a potassium channel, are responsible for a broad phenotypic spectrum that include autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), early-onset epileptic encephalopathy (EOEE), and epilepsy of infancy with migrating focal seizures (EIMFS) in neonates and infants, which are often refractory to conventional ASMs (49). Initial studies suggested that *KCNT1*-related epilepsy caused by a GOF variant responds to quinidine, a sodium and potassium channel blocker mostly used as an anti-arrhythmic (50). However, subsequent clinical experience found that it was mostly ineffective in early-onset EE (51). A subsequent study of patients with *KCNT1*-related epilepsy noted a $>$ 50% seizure reduction in 20% of patients, and with only a few achieving transient seizure freedom (49). While quinidine has had mixed results for this monogenic epilepsy syndrome, its robust efficacy in patients with specific variants in *KCNT1* does give hope that it may become standard therapy for those specific variant (49). Till then, *KCNT1* serves as an important reminder that, despite strong biomolecular evidence suggesting a certain therapy (e.g., ion channel modulation), the road to a successful therapy can sometimes be more complex than initially expected.

Repurposing Established Medications

As mentioned in the previous section, quinidine is an anti-arrhythmic agent that is being put to novel therapeutic use in epilepsy, an example of a repurposed drug. In this section, we discuss additional repurposed medications as examples of targeted therapies for monogenic epilepsy (see **Table 4**).

Dravet Syndrome

We have previously discussed the avoidance of sodium channel blockade in DS. Fenfluramine (36), cannabidiol (37), and stiripentol (35) have all been found to provide clinical benefit for this severe epilepsy syndrome in randomized clinical trials (52). These clinical findings are buttressed by animal studies, including a study of fenfluramine and norfenfluramine in a zebrafish model of DS (53) and a study of cannabidiol and stiripentol in hyperthermia-induced seizures in a mouse model of DS (54). We include these medications as "precision therapies" given their efficacy, even though the precise mechanism of action in DS patients is not fully understood. Fenfluramine was initially marketed as an appetite suppressant, typically in combination with the monoamine oxidase inhibitor phentermine. It was pulled from the market in the 1980's due to concerns of valvular

TABLE 4 | Repurposed medications for monogenic epilepsies.

Gene	Epilepsy syndrome	Suggested precision medicine	Therapeutic rationale	Status as precision medicine
*ARX**	Epileptic-dyskinetic encephalopathy	Valproic acid, estradiol	Unclear	Hypothetical (94, 95)
CACNA1A	Absence epilepsy with ataxia, DEE	- Aminopyridine (LOF) - Flunarizine (GOF)	Compensation in synaptic transmission (aminopyridine) calcium channel blockade (flunarizine)	Aminopyridine, flunarizine – potential (96)
CHRNA4/B2/A2	Sleep-related hypermotor epilepsy	Nicotine	Desensitization of nicotinic acetylcholine receptors	Established (66)
*FRRS1L**	DEE	Sulthiame	Unclear	Potential (67)
GABRB3	Lennox-Gastaut syndrome	Vinpocetine	Sodium channel modulation	Potential (68)
GABRG2	EE	Stiripentol	Increase GABA-A receptor activity	Hypothetical (97)
GRIN1/2A/2B/2D	Epilepsy with centrotemporal spikes, Landau-Kleffner syndrome, DEE	- NMDA receptor antagonists (memantine, dextromethorphan) - Ketamine (GOF) - Serine (LOF)	Modulation at the NMDA receptor	Potential (62, 63, 98)
*PCDH19**	DEE	- Ganaxolone - Stiripentol	- Compensation for altered steroidogenesis - Unclear	- Ganaxolone – potential (57, 99) - Stiripentol – potential (61)
*SLC13A5**	DEE	Stiripentol	- Unclear	Hypothetical (100)

**Indicates the current absence of a molecular or genetic rationale for this particular therapy.*

heart disease and pulmonary hypertension (16). However, subsequent studies have demonstrated its safety in DS patients (36, 55, 56). Regarding cannabidiol, there is a need for independent studies as the landmark study of this drug in DS was financed by GW pharmaceuticals (37).

Protocadherin 19 Female Epilepsy

Protocadherin 19 female epilepsy (PCDH19-FE) is an increasingly reported form of epilepsy whose pathophysiology is poorly understood. Disease-causing variants in *PCDH19* lead to early-onset DEE with clustering epilepsy (CE) as well as altered steroidogenesis and nuclear-hormone-related gene expression changes. The known involvement of hormonal pathways in the pathogenesis of PCDH19-related epilepsy has prompted researchers to look for steroid-based treatments for this disorder. Results for corticosteroid therapy are mixed (57–59). Because patients with PCDH19-FE have been found to have lower levels of allopregnanolone, it has been theorized that replacement of that hormone with a synthetic analog, ganaxolone, which acts as a human neurosteroid, could be therapeutic (60). Stiripentol has also been found to be beneficial as an adjunctive ASM in PCDH19-FE, as documented in a case report of a young girl who became seizure-free for an unprecedented 2 years and 10 months after starting stiripentol as an add-on to valproate and clobazam (61).

GRIN-Related Epilepsy

Mutations in *GRIN1/2A/2B/2D* are associated with childhood-onset epilepsy and developmental delay. Depending on the resulting change in the protein structure of the N-methyl-D-aspartate receptor (NMDAR), certain mutations cause increased charge transfer while other variants reduce current. Case reports have suggested a role for NMDAR blockade with dextromethorphan (typically used in cough syrup) or memantine (used for Alzheimer's dementia) for variants that cause gain-of-function (62), while a single case report suggests L-serine supplementation for loss-of-function variants (63). It should be noted that functional changes of receptor function (e.g., NMDA and GABA) or ion channel function, as determined with *in vitro* techniques or *in-silico* approaches, may end up being more relevant for the development of therapies than the current GOF/LOF dichotomy.

PRRT2-Related Epilepsy

PRRT2-related epilepsy provides a good example of repurposing drugs that had already been proved effective for a movement disorder caused by the same gene. *PRRT2* disease-causing variants are among the most common genetic causes of epilepsy. *PRRT2* encodes a pre-synaptic transmembrane protein that enables synaptic vesicle fusion (7). Mutations in *PRRT2* are associated with a broad clinical spectrum with three major phenotypes that include self-limited familial infantile seizures, paroxysmal kinesigenic dyskinesia (PKD), and infantile convulsions with choreoathetosis (64). Precision medicine therapy for *PRRT2*-related seizures with carbamazepine came from the common genetics shared between PKD and the infantile seizures. PKD had been treated effectively for years with carbamazepine and once it was understood that *PRRT2* variants explained both PKD and familial infantile seizures, carbamazepine and oxcarbazepine were used effectively for *PRRT2*-related infantile seizures (7, 65).

Miscellaneous Syndromes

In the last chapter of this section, we discuss a few monogenic epilepsy syndromes for which there is limited evidence (e.g., case series or reports) for certain repurposed medications.

TABLE 5 | Molecular chaperone treatments.

Gene	Epilepsy syndrome	Suggested precision medicine	Therapeutic rationale	Status as precision medicine
LGI1	Familial temporal lobe epilepsy	Phenylbutyrate, only for secretion-defective mutations	Molecular chaperone	Hypothetical (71)
STXBP1	Ohtahara syndrome, West syndrome	Phenylbutyrate in specific missense mutations and possibly LOF mutations	Molecular chaperone	Hypothetical (70)

Autosomal dominant sleep-related hypermotor epilepsy caused by mutations in CHRNA4 (cholinergic receptor, nicotinic, and alpha polypeptide 4) responds strikingly well to transdermal nicotine patches in a pediatric case series (66). Hypermotor seizures disappeared entirely, but sporadic arousals persisted. Ferric chelate reductase 1 like (*FRRS1L*) encephalopathy is a rare cause of DEE with only a few cases reported worldwide and with mostly drug-refractory seizures. A case report of a Malaysian child with *FRRS1L* encephalopathy documented excellent seizure response to sulthiame, a central carbonic anhydrase inhibitor used widely in Europe and Asia but not commonly used in North America (67). A woman with Lennox-Gastaut syndrome (LGS) from a disease-causing variant in *GABRB3* experienced a sustained, dose-dependent decrease in epileptiform activity on EEG after starting vinpocetine, a synthetic derivative of vincamine which is an alkaloid derived from the periwinkle plant. Its antiepileptic properties are likely from a combination of sodium channel modulation and GABA-A activity potentiation—future studies will be needed to determine its efficacy and pharmacokinetics in epilepsy (68).

Molecular Chaperones

Neurologists are very familiar with disorders of protein folding and aggregation, such as Huntington's disease and Alzheimer's disease, but may not be aware that certain genetic causes of epilepsy are also conformational disorders. For these genetic epilepsies, chemical chaperones—small molecules that correct these folding and aggregating abnormalities—represent an important avenue for precision medicine. These compounds work in a myriad of ways, including stabilization of misfolded proteins, reducing aggregation, preventing deleterious interactions with other proteins, and modifying the activity of endogenous chaperones to promote more efficient folding and translocation of proteins to the appropriate intracellular or extracellular destinations (69) (see **Table 5**). Recent examples of chemical chaperones for genetic epilepsies include studies of *Munc18-1* related epilepsy and *LGI1*-related epilepsy, which we will discuss next.

Heterozygous *de novo* mutations in *STXBP1*, which encodes *Munc18-1*, result in a syndrome consisting of epilepsy, intellectual disability and movement disorders that carries a poor prognosis (70). A recent study in yeast, worm, and mouse neurons has shown that several disease-linked missense mutations in this gene lead to the disease phenotype through a dominant-negative effect whereby the mutant protein becomes destabilized and forms aggregates that then deplete the functional levels of wildtype *Munc18-1* protein by co-aggregation (70). Use of chemical chaperones (4-phenylbutyrate, sorbitol, and trehalose) reversed the deficits caused by the mutations, both *in-vitro* and *in-vivo* in these animal models (70). Because this approach stabilizes the remaining wild-type Munc18-1 protein, the authors of the study predict that this strategy will also work for not only missense mutations but also non-sense and truncation mutations, suggesting a possible path forward for treating the multiple genetic causes of *Munc18-1*-related epilepsy (70).

Mutations in *LGI1*, which encodes a neuronally-secreted protein, cause autosomal dominant lateral temporal lobe epilepsy (ADLTE). Yokoi et al. classified 22 reported missense mutations in *LGI1* as either leading to defects in protein secretion (secretion-defective) or allowing proper secretion (secretion-competent), and then generated two mouse models of ADLTE encoding mutant proteins that were representative of the two groups (71). The secretion-defective mouse model expressed *LGIE383A* protein, which was found to be prematurely degraded by the endoplasmic-reticulum (ER) quality-control machinery. The secretion-competent mouse model expressed *LGI1S473L* protein, which dimerized abnormally and was defective in binding to ADAM22, one of its known receptors. These two mutations resulted in loss of function (LOF) through compromised intracellular trafficking or ligand activity of LGI1 to ADAM22. Use of 4 phenylbutyrate, a chemical chaperone, restored LGI1E383A's ability to fold and bind to ADAM22 and improved the seizure susceptibility of the LGI1E383A model mice but not the LGI1S473L mice. In addition to identifying *LGI1*-related epilepsy as a conformational disease, this study suggests a bright future for chemical chaperones as a precision therapy for certain monogenic epilepsies.

Gene Therapies

Gene therapy aims to alleviate disease by introducing genetic material into target cells to restore proper physiologic function (72). For monogenic epilepsy syndromes, gene therapies target neurons in the central nervous system (CNS), which requires either intrathecal delivery to bypass the BBB or systemic administration of the therapy that crosses the BBB to then reach the entire brain. Most gene therapies are packaged in adeno-associated viruses (AAV) that have tropism to the brain, typically AAV9 because of its BBB-permeable capsid. However, while being a prime delivery vector for gene therapies, viral capsid size leads to a limitation in terms of DNA cargo (<4.7 kB).

TABLE 6 | Gene therapies for monogenic epilepsies.

Gene	Epilepsy syndrome	Suggested precision medicine	Therapeutic rationale	Status as precision medicine
CDKL5	DEE	- AAV-mediated gene therapy - Fenfluramine - GSK3B-HDAC dual inhibitor - GABA receptor antagonist	- Genetic repair - Serotonin release (fenfluramine) - Histone deacetylase activity (GSK3B) - Decrease of excessive GABAergic transmission	Hypothetical (75, 101–104)
CSTB	Unverricht-Lundborg disease (progressive myoclonic epilepsy)	ASO	Restore normal gene splicing pattern	Hypothetical (88)
DNM1	DEE	AAV-mediated microRNA	RNA interference	Hypothetical (89)
GYS1	Lafora disease	ASO	Downregulation of glycogen synthase	Hypothetical (87)
KCNA1	Temporal lobe epilepsy	CRISPRa	CRISPRa-mediated upregulation of Kv1.1 channels	Hypothetical (105)

In addition to multiple delivery methods, there are also multiple approaches to gene therapy that we could subdivide loosely into gene editing, gene supplementation, and gene expression modification. We will discuss each of these approaches and the associated monogenic epilepsies that have been tackled using each approach (see **Table 6**). The details of each gene therapy are beyond the scope of this review, and the reader is directed to excellent reviews on each therapy (73).

Gene Editing

Gene editing methodologies, the most prominent of which is the CRISPR/Cas9 gene editing system, allow for direct correction of a genetic defect. Briefly, CRISPR/Cas9 is a gene editing approach in which DNA sequences called CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) allow the targeting and destruction of specific DNA targets by Cas (CRISPR-associated) proteins (74). CRISPR/Cas9 technology may be particularly important for genetic epilepsies since dominant heterozygous missense mutations or small insertion-deletions make up a large percentage of the known pathogenic variations. These variants make appealing targets for correction with CRISPR/Cas9 (74), but therapies have yet to emerge for clinical use.

Gene Supplementation

Gene supplementation usually involves the insertion of a supplemental transgene into a cell to make up for a LOF genetic defect, but does not correct the underlying defect *per se*. This approach has been trialed for cyclin-dependent kinase-like 5 (*CDKL5*) deficiency disorder, which is an X-linked dominant disorder caused by *de novo* mutations in *CDKL5* leading to a DEE. A gene supplementation study involving AAV delivery of the human *CDKL5* gene in *CDKL5* knock-out mice demonstrated improved behavior and restoration of synaptic function in neurons (75). While the study was limited (e.g., incomplete recapitulation of the human phenotype including seizures, weak effect of treatment on some parameters), it serves as a proof-of-concept for gene supplementation strategies for *CDKL5*-related epilepsy (72, 75).

Similar work has been done in animal models of tuberous sclerosis complex. The proteins hamartin and tuberin function within a complex to inhibit mammalian target of rapamycin (mTOR)-mediated cell growth and proliferation. Mutations in *TSC1* or *TSC2* (hamartin and tuberin, respectively) lead to tuberous sclerosis complex, a tumor suppressor syndrome characterized by overgrowth in multiple organs including the brain, resulting in intellectual disability, autism, and epilepsy. Several gene supplementation strategies have shown promise in mouse models of TSC. A mouse model of TSC with neuron-specific hamartin loss showed marked improvement in survival, weight, gain, and motor behavior after intracerebrovascular (ICV) injection of an AAV expressing tagged form of hamartin (76). Similar results were obtained with an IV. Follow up work by the same team showed that similar improvements in both phenotype and histology could be achieved through intravenous (IV) injection as opposed to ICV injection (77). Similar gene therapy efforts in a mouse model of TSC2, using AAV-mediated delivery of a "condensed" form of human tuberin, also showed significant increase in mean survival and reduction in histological evidence of brain pathology (78). These studies, taken as a whole, demonstrate the significant potential of AAV-mediated gene supplementation for TSC1 and TSC2.

Gene supplementation strategies have also been tested in various ion channelopathies that lead to epilepsy. As mentioned in a previous section, DS is mostly caused by LOF mutations in *SCN1A*, a gene that encodes the α subunit of the voltage-gated sodium channel Nav1.1 (72). As a result, most gene therapy efforts for DS have centered on increasing Nav1.1 expression. These efforts have been stymied by at least two factors. The first is that the *SCN1A* gene (6 kB) exceeds the packaging limit of AAV vectors. The second is that the proclivity to seizure in this syndrome seems to be caused by an inhibition-excitation imbalance from loss of *SCN1A* expression primarily in interneurons—restoration of healthy gene function would therefore have to specifically be targeted toward this specific cell population (79).

While the α subunit exceeds the packaging limit, the β1 multifunctional sodium channel auxiliary subunit does not exceed the limit. Moreover, the β1 subunit has been shown to increase ion flow through the α subunit and promotes the trafficking of α subunits from an intracellular pool to the cell

surface. Overexpression of *NaVβ1* should in theory improve the function of residual voltage-gated channels and thereby improve the DS phenotype. A murine study evaluated the effects of a bilateral ICV injection of an AAV vector coding for a truncated mouse promoter based on a GABAergic neuron expressing gene called *Gad-1* and mouse *NaVβ1*, with appropriate control mice (80). Treatment led to a partial rescue of the disease phenotype that was sexually divergent (80). The moderate efficacy of the treatment may have been due to the moderate specificity of the chosen promoter for GABAergic neurons—however, this study did provide a proof-of-principle for future treatments (72).

Gene Expression Modification

Gene expression modification seeks to increase or decrease the expression of a gene product, also known as activation and inhibition, respectively. There are various techniques that fall under this category including anti-sense oligonucleotides (ASO) (81), RNA interference (RNAi) (82), and dCas9-based CRISPRa/i (83).

Anti-sense Oligonucleotides

ASOs are short, single-stranded oligodeoxynucleotides, usually 8–50 nucleotides in length, which bind to a target mRNA *via* complementary base pairing. The base pairing then leads to endonuclease-mediated transcript knockdown and decreased expression of the associated deleterious protein (81). First-generation ASOs were limited by rapid turnover (i.e., RNA degradation) and insufficient intracellular concentration to impact target genes. Modifications to their chemical backbones led to 2nd and 3rd generation ASOs that, in addition to targeting mRNA, could also bind non-coding RNAs and toxic RNAs, leading to a much wider range of clinical uses. Their main limitation remains the need for intrathecal administration as they do not cross the BBB (81).

ASOs are being used in animal models of seizure disorders related to ion channelopathies. DS was discussed in a previous section and is predominantly caused by mutations in *SCN1A*. Expression of *SCN1A* is mediated by an anti-sense non-coding RNA (SCN1ANAT). Upregulation of the healthy *SCN1A* allele using oligonucleotide-based compounds (AntagoNATs) to target SCN1ANAT in a knock-in mouse model of DS and in a non-human primate led to significant improvements in seizure phenotype in the animals (84). AntagoNATs may therefore become a helpful treatment paradigm for DS, though will likely limited by the weekly intra-cerebral administration that is required (72).

GOF mutations in the *SCN8A* gene encoding Nav1.6 results in DEE (85). Reduction of the levels of *Scn8a* transcript to 25–50% of typical expression levels using an ASO resulted in delayed seizure onset and increased survival in mouse models of both *SCN8A* encephalopathy and DS (85). This success in animal models will pave the way for further studies of ASOs to decrease the pathological increase in transcript expression for GOF mutations in *SCN8A* and *SCN2A*. LOF mutations in *SCN8A* and *SCN2A* resulting in pathological decrease in transcript expression would benefit from a CRISPRa strategy, to be discussed in a subsequent section (72).

A similar ASO strategy has been developed for GOF variants in *KCNT1* that lead to epilepsy of infancy with migrating focal seizures (EIMFS) (86). A single ICV injection of a *Kcnt1* gapmer (i.e., short DNA strands flanked by strands of RNA mimics) ASO in a mouse model of KCNT1-epilepsy led to significant reduction in seizure frequency and increased overall survival compared to mice treated with a control (i.e., non-hybridizing) sequence (86). These results suggest a promising road ahead for ASO-based therapies in *KCNT1*-associated epilepsy.

Lafora disease is a fatal, genetic progressive myoclonic epilepsy from mutations in *EPM2A* and *NHLRC1* resulting in abnormal branching patterns in a subgroup of glycogen molecules. These abnormal glycogen molecules then precipitate and accumulate as Lafora bodies that then generate a neuroinflammatory response and neurodegeneration. Glycogen synthase, encoded by the *GYS1* gene, is the enzyme responsible for glycogen branch elongation. While mutations in *GYS1* are not responsible for Lafora disease, down-regulation of glycogen synthase with an ASO could lead to decreased elongation of glycogen polymers, thereby decreasing the formation of Lafora bodies (87). ICV injection of an ASO targeting the mRNA of brain-expressed *GYS1* in a murine model of Lafora disease led to inhibition of further accumulation of Lafora bodies in older mice that had already formed bodies and prevented Lafora body formation in young mice that had not yet formed any (87). The inhibition of Lafora body formation correlated strongly with improvements in neuroinflammatory markers, suggesting all-in-all that this approach could prevent and stop the progression of this currently fatal form of epilepsy that strikes otherwise-healthy teenagers (87).

Mutations in the cystatin B gene (CSTB), which encodes an inhibitor of several lysosomal cathepsins, result in Unverricht-Lundborg disease (ULD), a form of progressive myoclonic epilepsy with limited pharmacological treatments (88). ASO-based therapies uniquely designed for particular CSTB mutations seem to be on the horizon. An ASO therapy for a newly-identified splicing mutation causing ULD restored the normal splicing pattern in a dose-specific manner in cultured cells from the affected patient, providing proof-of-principle for mutation-specific anti-sense therapy in ULD and similar genetic epilepsies (88). It should be noted, however, that the most common mutation causing ULD is an unstable expansion of a dodecamer repeat in the promoter region, leading to down-regulation of *CSTB* mRNA levels (88), and the remaining mutations are missense, non-sense, frameshift, and splice-site.

Before moving to a discussion of other gene therapy strategies, it should be noted that, in addition to the challenge of intrathecal administrations of the gene product mentioned previously, another limitation of ASOs is the need for multiple administrations per year—in contrast with the one-time administration of gene supplementation strategies.

RNA Interference

RNA interference (RNAi) has also emerged as an important player in the modification of gene expression for therapeutic

purposes. In most cases, RNAi is used to silence dominant toxic genes in an allele-specific way, meaning that the technology ideally silences the toxic allele while allowing healthy expression of the normal allele (82). Briefly, RNAi refers to a process whereby double-stranded RNA (dsRNA) is processed into small-interfering RNAs (siRNAs) by the ribonuclease-III (RNase-III) enzyme called Dicer. Measuring about 21–23 nucleotides in length, siRNAs are dsRNA intermediates that then pair up with a protein complex called the RNA-induced silencing complex or RISC. As the siRNA duplex is unwound, one strand called the "guide strand" is incorporated into RISC while the other strand, called the "passenger strand," is selectively eliminated. The elimination (i.e., cleavage of the targeted transcript) is done by the "slicer" component of RISC called Argonaute-2 (Ago2). AAV-mediated delivery and liposome-mediated delivery remain the most common forms of siRNA delivery but several other exciting approaches are being actively developed (82).

To date, there are no human studies of RNA interference for the treatment of epilepsy. However, animal studies show some promise. *De novo* mutations in dynamin-1 (*DNM1*) cause a severe DEE with both infantile spasms (IS) and LGS, that is highly resistant to current ASMs (89). Prior to the association of pathogenic human variants in *DNM1* and severe epilepsy, a spontaneous missense mutation in the mouse ortholog, called "fitful," had already been associated with epilepsy, and fitful homozygotes exhibit a DEE-like phenotype with developmental delay and severe seizures (89). A *DNM1*-targeted microRNA delivered by self-complementary AAV9 into fitful homozygous mice led to a decrease in the seizure phenotype and improved cellular features on brain histology. As hopeful as this initial study is, more work is needed since causative mutations in *DNM1* in humans are usually *heterozygous* dominant negative, in contrast with the *homozygous* mouse model in this study. But as was discussed previously, RNAi is a good approach to envisage when dealing with dominant negative alleles that need to be silenced while maintaining normal expression of the healthy allele.

CRISPRi/a

Another approach to gene activation and inhibition relies on nuclease-deficient (or catalytically dead or deficient) dCas9 protein, in conjunction with various effector domains such as transcriptional activators to activate (called CRISPRa) or transcriptional inhibitors to inhibit (called CRISPRi) gene expression (83). Briefly, this process involves conversion of Cas9 protein from a DNA "scissor" into a gene activator by disrupting its nuclease activity. This is done by introducing mutations into the evolutionarily-conserved nuclease domains (RuvC and HNH) of Cas9 to make dCas9, essentially converting the protein into an RNA-guided DNA-binding protein. From there, dCas9 can be fused with an effector (i.e., a transcriptional activator such as VP64 or VPR, or a transcriptional repressor such as the Kruppel-associated box domain of Kox1). The resulting dCas9-effector fusion, when paired with a target-specific single guide RNA (sgRNA) then operates as an artificial transcription factor to activate or inhibit gene expression.

CRISPRa/i approaches to monogenic epilepsy have only been attempted in animal models but appear promising. A CRISPRa approach in a mouse model of DS led to significant decrease in febrile seizures compared to the wildtype control mice, but did not completely suppress all seizure activity (90). A similar result was obtained by a different team using a CRISPRa methodology delivered *via* AAV (91). Both studies suggest that upregulation of *Scn1a* in inhibitory neurons leads to phenotypic improvement, even after the juvenile murine stage, opening a path for future therapies along these lines (90, 91). On a more cautious note, and has been noted in other reviews, these Cas9-based treatments do require long-term expression of exogenous Cas9 proteins in neurons, potentially leading to off-target immunogenic effects (72).

CONCLUSION

As genetic causes of epilepsy continue to be discovered, it will be important for clinicians and researchers to leverage genetic and molecular insights to produce precision therapies for these patients. This review highlighted the many ways that monogenic epilepsies can be treated in a targeted manner based on pathophysiological insights. Areas of ongoing promising research include phenotype-treatment studies meant to delineate the specific roles of genetic variants and advances in gene therapies including RNAi, ASO, and CRISPRa/i. Another important area of investigation is discovering the degree to which seizure burden contributes to developmental delays in these syndromes that commonly present as DEE—i.e., does the reduction of seizure frequency lead to an improvement in the developmental aspects of the condition (7, 31, 40)? As personalized therapies improve, the answer to this question will hopefully emerge.

AUTHOR CONTRIBUTIONS

VZ wrote the article. CK and BM reviewed and corrected the article. All authors contributed to the article and approved the submitted version.

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Etiologic Classification of 541 Infantile Spasms Cases: A Cohort Study

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Objective: To explore the etiology of infantile spasms (IS) in a large Chinese cohort based on the United States National Infantile Spasms Consortium (NISC) classification.

Methods: In the present study, we recruited IS patients diagnosed at a single center (Xiangya Hospital, Central South University) between Jan 2010 and Aug 2019. Thereafter, we collected their clinical and genetic information retrospectively. Their underlying etiologies were classified according to the NISC classification and then compared in different scenarios to understand their distribution.

Results: A total of 541 patients with IS from 18 provinces were included in this study. The underlying etiology was identified in 53.2% of the cases: structural-acquired, 25.3%; genetic, 12.9%; genetic-structural, 7.2%; structural-congenital, 5.0%; metabolic, 2.4%; infections, 0.4% and immune, 0%. Whole-exome sequencing (WES) provided the highest diagnostic yield (26.9%). In structural-acquired IS, the proportion of hypoglycemic brain injuries was significant, second only to hypoxic-ischemic encephalopathy. There was no patient discovered to have Down syndrome. *STXBP1*, *CDKL5*, *TSC2*, *KCNQ2*, *IRF2BPL*, and *TSC1* were the most frequently implicated genes. Genetic causes were found to be the most common cause of IS in the early onset group, while structural-acquired etiologies were common in males and preterm babies. Patients with pre-spasm seizures were associated with a higher proportion of identified causes than those without. Non-acquired structural etiologies were more common in patients without hypsarrhythmia than in those with hypsarrhythmia.

Significance: The most prevalent cause of IS was structural acquired followed by genetic causes. When brain MRI fails to detect the etiology, we propose WES as the next step. Structural-acquired IS and cases with genetic disorders are characteristic of the Chinese cohort, however, the etiology differs with the patient's age of onset, gestation age at birth, sex, and the presence/absence of both pre-spasm seizures, and hypsarrhythmia.

Keywords: infantile spasms (IS), etiologies, spectrum, variants, whole-exome sequencing (WES)

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HIGHLIGHTS

- More than half of the IS cases in China have an underlying etiology.
- The most common cause is structural acquired followed by genetic.
- Both males and preterm babies independently have higher proportions of structural-acquired etiologies than their counterparts.
- Early-onset IS patients have a larger proportion of genetic reasons than other onset ages, with *STXBP1* being the most prevalent causative gene.

INTRODUCTION

Infantile spasms (IS) is a type of developmental and epileptic encephalopathy (DEE), with an incidence of 0.43 per 1,000 live births, occurs mostly between the ages of 3 and 12 months, and with a peak occurring around 4–7 months (1–3). Studies have shown that nearly 60% of the cases have an underlying etiology (4–6), however, data from the Chinese population is lacking. Moreover, only a few studies have explored the distribution and etiology of IS in different scenarios (5, 7).

In 2015, the National Infantile Spasms Consortium (NISC) in North America divided the etiologies into eight groups, including genetic, genetic-structural, structural-congenital, structural-acquired, metabolic, immune, infectious, and unknown etiologies (8). Considering the rationality and operability of this consortium, we adopted it as the standard etiological classification and used it to investigate the underlying etiology of 541 cases of IS in the central south of China. Moreover, this study also explored the distribution of the patients based on different scenarios, such as the age of onset, gestation age at birth, sex, and the presence/absence of both pre-spasm seizures, and hypsarrhythmia to guide clinical management.

MATERIALS AND METHODS

Study Participants

All individuals with IS seen at Xiangya Hospital, Central South University between Jan 2010 and Aug 2019 were retrospectively enrolled in this study. We included cases with: (1) epileptic spasms manifested within 2 years of age and (2) typical findings in electroencephalography (EEG), such as hypsarrhythmia or modified hypsarrhythmia. Cases without hypsarrhythmia, but with a background characterized by multifocal spikes and electroclinical spasms were also included.

Diagnostic Protocol

A stepwise approach was employed to identify the underlying etiology for each case based on the NISC classification (**Figure 1**). It's worth noting that individuals with acquired intracranial lesions resulting in epilepsy were classified into the structural-acquired group, including those caused by an intracranial infection in this study. Besides, identification of the probable etiology was dependent on the neurologists' judgment, which varied among the patients and period.

Patient follow-up: a repeat brain imaging was performed for patients who had focal clinical seizures or focal EGG abnormalities**.** For patients suspected to have genetic disorders, raw data of genetic tests were re-analyzed periodically. Recently published literature reviews and scientific studies on bioinformatics analysis, RNA-seq, *in vivo,* and *in vitro* experiments helped with the interpretation of sequencing data.

Data Collection

The following data were retrieved during the patient's visit to the hospital: demographics, seizure-related information, relevant medical history, and medical record abstraction were performed. Individuals with insufficient medical records or lacked baseline assessments were excluded from the study. All data were independently reviewed by two neurologists. Furthermore, at least one neuro-radiologist reviewed the brain images, and all genetic results were interpreted by two geneticists following the American College of Medical Genetics (ACMG) guidelines (9, 10). A third geneticist was consulted to reach a consensus in case of disagreement. Patients with pathogenic/likely pathogenic copy number variations (CNVs) were included in the group with identified causes, while the others were categorized as unknown IS. Patients with pathogenic/likely pathogenic/uncertain significance variants were referred to neurologists for a literature review of genotype-phenotype correlation. Specific etiologies were recorded and classified into appropriate NISC categories based on the preceding work.

A systematic search of all the identified genes in the Online Mendelian Inheritance in Man (OMIM) and PubMed databases was performed. A thorough literature review was conducted to present the general perspective of all the causal genes, including the gene types, functions, and treatment conditions.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Science (IBM, SPSS Statistics Version 25). Categorical data were summarized in the form of frequencies and proportions and analyzed with the Chi-square test ($p \leq 0.05$ indicated statistically significant differences between groups) or Fisher's exact test with a Bonferroni adjustment ($p \leq 0.0167$, $k = 3$) where applicable.

Ethical Standards

This study was reviewed and approved by the Institutional Ethics Committee of Xiangya Hospital, Central South University, and it was performed following the World Medical Association Declaration of Helsinki adopted in 1964. Written informed consent was obtained from the parents/guardians of the subjects.

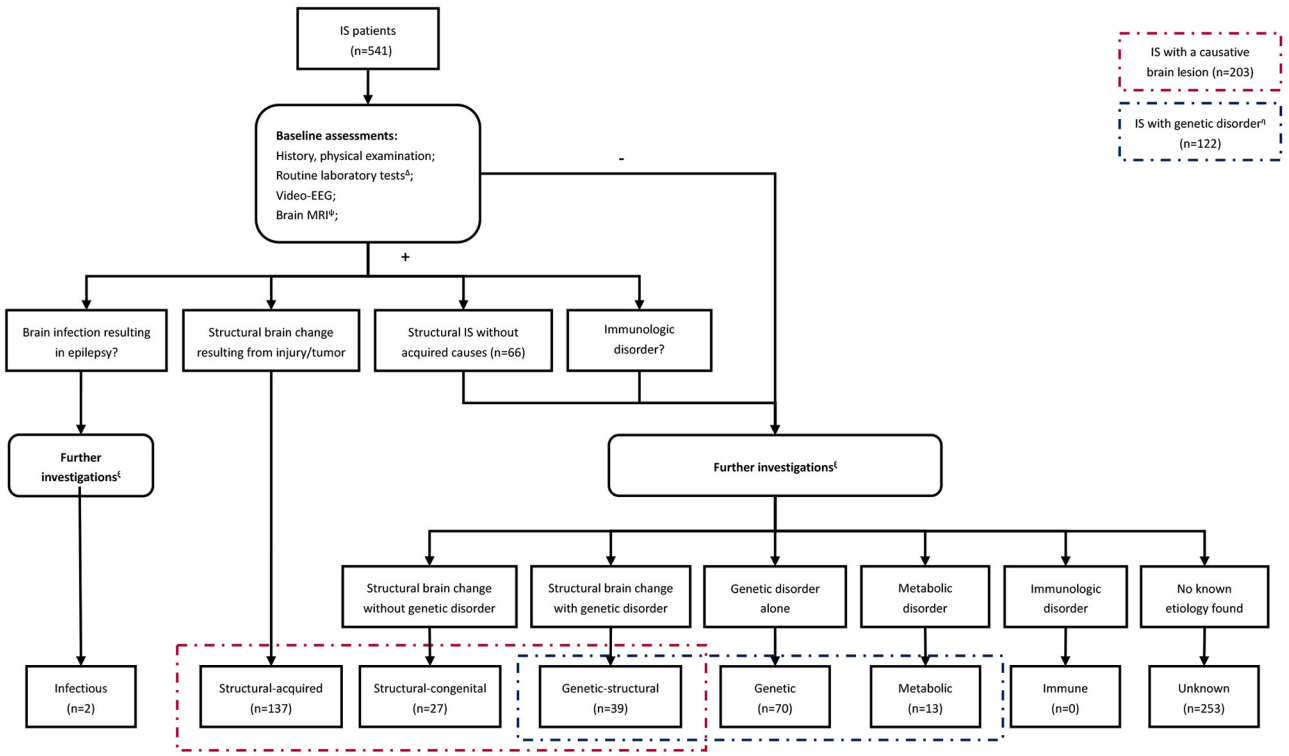

FIGURE 1 | The flow chart summarizing the steps involved in identifying the underlying etiology. Δ Tier1 laboratory investigations included complete blood count, glucose, electrolytes, urea, creatinine, AST, ALT, creatine kinase, ammonia, lactate, homocysteine, and ceruloplasmin. Ψ Brain MRI was rechecked after several months or cranial MRI thin slice scan/positron emission tomography-computed tomography (PET-CT) was performed on patients who had focal clinical seizures or focal abnormalities on EEG. ξ Further investigations included infectious or immunological tests, plasma amino acids, urine organic acids, karyotype analysis, chromosomal microarray analysis, customized multigene panel, whole-exome sequencing, and mitochondrial genome analysis. The performance of these investigations was based on neurologists' judgments of the probable etiology and varied among the patients. Forty-six patients refused any further investigations in this study. η There were 103 cases with available genetic data among those with a genetic disorder, while the remaining 17 met the clinical diagnostic criteria for tuberous sclerosis complex and 2 for neurofibromatosis.

RESULTS

The Demographic Information of the Cohort

A total of 541 patients with IS from 18 provinces were included as shown in **Supplementary Figure S1**. The patient's demographics were presented in **Table 1**.

Diagnostic Yield

Neuroimaging

Brain magnetic resonance images (MRIs) were available for all 541 cases. The causative lesions were identified in 203 (37.5%) cases, of which 137 had structural-acquired lesions, while 66 had non-acquired structural lesions (genetic/congenital-structural). Eight (3.9%) patients with causal structural abnormalities were revealed by repeated brain MRI.

Genetic and Metabolic Tests

After completing baseline assessments, 402 cases without obvious acquired causes (structural-acquired and infectious IS) were transferred for additional genetic and/or metabolic testing. Of the 402 cases, 46 refused any further investigations while the remaining 356 cases underwent genetic and/or metabolic testing. Of the 305 cases who underwent genetic testing, 103 (33.8%) had an established genetic disorder. The diagnostic yield for each test was as follows: plasma amino acids and urine organic acids (1/309, 0.3%), karyotype (2/183, 1.1%), CMA (12/207, 5.8%), customized multigene panels (27/105, 25.7%), WES (63/234, 26.9%; nine of the 63 patients were found through customized multigene panels), and mitochondrial genome analysis (1/34, 2.9%). In addition, 12 candidate genes in 14 patients (14/234, 6%) were identified by WES, of which 5 genes (*ALPL*, *CACNA1C*, *MED12*, *TCF4*, and *TCF20*) might partially explain clinical features and 7 (*CD99L2*, *TAF1*, *CLCN6*, *CYFIP1*, *GPT2*, *ATP2A2*, and *MYO18A*) were identified as relative risk genes.

The Underlying Etiologies Based on the NISC Categories

Overall, 288 (53.2%) cases were identified to have underlying etiology: structural-acquired, 137 (25.3%); genetic, 70 (12.9%); genetic-structural, 39 (7.2%); structural-congenital, 27 (5.0%); metabolic, 13 (2.4%); and infections, 2 (0.4%). None of the cases showed immune etiology (**Table 2**).

TABLE 1 | The baseline characteristics of the group.

Variable	Total patients (*N*) =541	Percentage
Sex		
Male	330	61.0%
Female	211	39.0%
Ethnicity		
Han	514	95.0%
Non-Han	27	5.0%
Classification according to the age at spasms onset (corrected for preterm delivery)		
Early-onset, <3 m	86	15.9%
Classic-onset, ≥3 to <12 m	393	72.6%
Late-onset, ≥12 m	62	11.5%
Preceding seizures		
Yes	133	24.6%
No	408	75.4%
Unequivocally normal development at the onset of spasms		
Yes	161	29.8%
No	380	70.2%
Gestational age		
<32 w	17	3.1%
≥32 to <37 w	33	6.1%
≥37 w	491	90.8%
Birthweight		
<1,500 g	10	1.9%
≥1,500 to <2,500 g	46	8.5%
≥2,500 to <4,000 g	461	85.2%
≥4,000 g	24	4.4%
Presence of hypsarrhythmia in EEG		
Yes	467	86.3%
No	74	13.7%
Presence of spasms in clusters		
Yes	518	95.7%
No	23	4.3%

EEG, electroencephalograph; g, gram; m, month; w, week.

Ninety-six (70.1%) of the 137 structural-acquired cases were caused by perinatal brain injuries, with hypoxic-ischemic encephalopathy (HIE) (51.1%, 70/137) and hypoglycemic brain injuries (13.1%, 18/137) being the most common causes. Of the 66 genetic/congenital-structural patients, 59 (89.4%) exhibited malformations of cortical development and the most common one was tuberous sclerosis complex (TSC) (31/59, 52.5%). The data of genetic tests were available in 20 of the 39 individuals with genetic-structural etiologies, among which *TSC2* (10), *TSC1* (4), and *NF1* (2) were the major associated genes.

Of the 70 genetic IS patients, 59 had monogenic variants and 11 chromosomal aberrations. The common genes were *STXBP1* (12), *CDKL5* (12), and *KCNQ2* (5). Seven of the 13 metabolic IS patients exhibited inborn metabolic errors in organic molecules, including metal metabolism (4/13, 30.7%), amino acid metabolism (1/13, 7.7%), vitamin B6 insufficiency (1/13, 7.7%), and fatty acid oxidation disorder (1/13, 7.7%). Besides, 4 (30.8%) cases had glycosylation disorders and 2

TABLE 2 | The specific causes of the group (*N* = 541).

Etiologic categories (*n*)	Specific causes	*N*
Structural-acquired (*n* = 137)		
	HIE with or without ICH/hypoglycemia	
	Perinatal insult	62
	Postnatal insult	8
	Intracranial infection	
	Bacterial meningitis (perinatal)	9 (1)
	Viral meningoencephalitis	8
	Brain injury secondary to neonatal hypoglycemia	18
	ICH	
	Perinatal insult	13
	Postnatal insult	3
	Encephalomalacia with other causes	
	Indefinite perinatal insult	2
	CVM	2
	Incontinentia pigmenti	1
	Unknown causes	8
	Neuroglioma	2
	Focal brain lesion of unknown cause	1
Genetic-structural (*n* = 39)		
	Tuberous sclerosis complex	
	TSC1 variant	4
	TSC2 variant	10
	NA	17
	Neurofibromatosis	
	NF1 variant	2
	NA	2
	NEDD4L variant (heterotopia, pachygyria-lissencephaly)	1
	DCX variant (pachygyria-lissencephaly, agenesis of the corpus callosum)	1
	NPRL3 variant (pachygyria-lissencephaly)	1
	17p13.3 microdeletion (pachygyria-lissencephaly, heterotopia)	1
Structural-congenital (*n* = 27)		
	Pachygyria-lissencephaly	12
	Focal cortical dysplasia	4
	Heterotopia	2
	Polymicrogyria	1
	Schizencephaly	1
	≥2 Malformations	
	Pachygyria-lissencephaly, heterotopia, schizencephaly, agenesis of the corpus callosum	1
	Heterotopia, focal cortical dysplasia, agenesis of the corpus callosum	1
	Heterotopia, agenesis of the corpus callosum, encephalomalacia with unknown cause (not epileptogenicity)	1
	Focal cortical dysplasia, schizencephaly	1
	Intracranial hemangioma	3

(Continued)

TABLE 2 | Continued

Etiologic categories (*n*)	Specific causes	*N*
Genetic ($n = 70$)		
	STXBP1 variant	12
	CDKL5 variant	12
	KCNQ2 variant	5
	CLCN4 variant	3
	IRF2BPL variant	4
	GNAO1 variant	2
	SCN8A variant	2
	KCNB1 variant	2
	SCN2A variant	2
	SCN10A variant	1
	CYFIP2 + *KMT2D* variant	1
	MECP2 variant	1
	DNM1 variant	1
	ARX variant	1
	GRIN2B variant	1
	AARS variant	1
	NTRK2 variant	1
	SPTAN1 variant	1
	CACNA1A variant	1
	GNB1 variant	1
	GABRE variant	1
	KMT2D variant	1
	UFC1 variant	1
	SMARCA2 variant	1
	Xp22.13 microdeletion (harbors the exon 1 of the *CDKL5* gene)	1
	20q13.33 microdeletion (harbors the *EEF1A2*, *KCNQ2* genes)	1
	9q33.3-34.11 microdeletion (harbors the *STXBP1* gene)	1
	9p24.3-22.3 microdeletion	1
	5p12-11 microduplication (harbors the *HCN1* gene)	1
	3p25.3 microdeletion (harbors the *SETD5* gene)	1
	1p36.33 microdeletion (harbors the *GNB1* gene)	2
	1p36.33-32 microdeletion (harbors the *GNB1* gene)	1
	Xp22.11-21.3 microduplication (harbors the *ARX* gene)	1
	15q11.2 microduplication	1
Metabolic ($n = 13$)		
	Disorder of glycosylation	
	SLC35A2 variant	2
	ALG1 variant	1
	ALG13 variant	1
	Metal metabolism	
	Menkes disease (*ATP7A* variant)	1
	Neurodegeneration with brain iron accumulation (*WDR45* variant)	3
	MMA (*MMACHC* variant)	1
	Pyridoxine-dependent epilepsy (*ALDH7A1* variant)	1
	SCAD deficiency (*ACADS* variant)	1
	Lysosomal storage diseases	
	HEXA variant	1
	Mitochondrial disorders (*MT-ND1* variant)	1
Infection ($n = 2$)		
	Intra-uterine infection	2
Unknown ($n = 253$)		
	Likely genetic	
	CD99L2 variant	2
	TAF1 variant	2
	GPT2 variant	1
	ATP2A2 variant	1
	CYFIP1 variant	1
	MYO18A variant	1
	CLCN6 variant	1
	CACNA1C variant	1
	MED12 variant	1
	TCF4 variant	1
	TCF20 variant	1
	Likely metabolic (supporting evidence)	
	Pyridoxine-dependent epilepsy (resolved with B6 supplementation)	1
	GLUT-1 deficiency syndrome (hypoglycorrhachia)	1
	Leukoencephalopathy (lesions in brain MRI)	1
	Hypophosphatasia (*ALPL* variant)	1
	Others	236

CVM, cerebral venous malformation; GLUT, glucose transporter; HIE, hypoxic-ischemic encephalopathy; ICH, intracranial hemorrhage; MMA, methylmalonic academia; MRI, magnetic resonance imaging; NA, not applicable; SCAD, short-chain Acyl-CoA dehydrogenase.

(15.4%) errors of metabolism in organelles. Ten genes (*WDR45*, *SLC35A2*, *ALG1*, *ALG13*, *ATP7A*, *HEXA*, *MMACHC*, *ALDH7A1*, *ACADS*, and *MT-ND1*) were implicated in metabolic IS.

Secondary Findings

An overview of the available genetic data from 103 patients with genetic disorders (genetic-structural, genetic alone, and metabolic IS) revealed that monogenic variants accounted for 88.3% (91/103) and chromosomal aberrations for 11.6% (12/103) (**Supplementary Tables S1, S2**). Taking into account genes spanned by chromosomal aberrations, a total of 43 causative genes were identified in these 103 individuals (**Supplementary Figure S2**). *STXBP1* (13), *CDKL5* (13), *TSC2* (10), *KCNQ2* (6), *IRF2BPL* (4), and *TSC1* (4) were the most frequently implicated genes, which were responsible for 48.5%

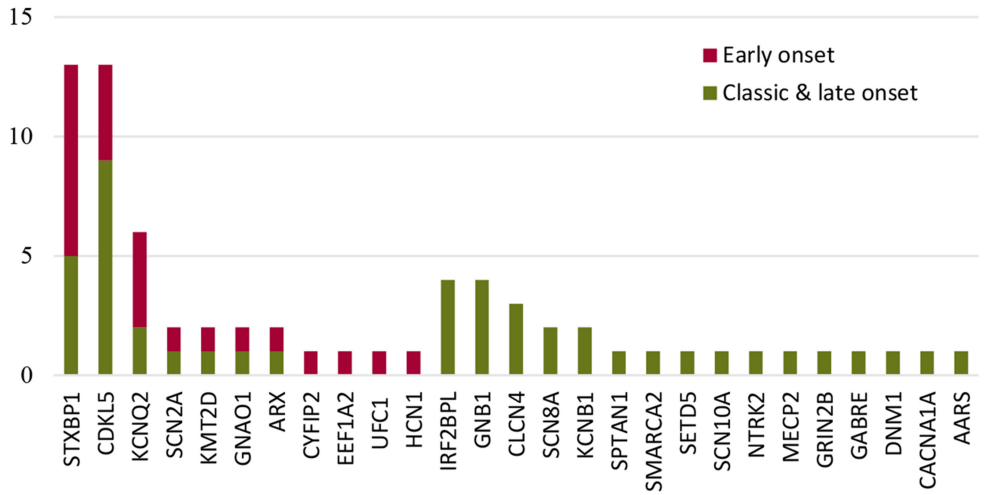

FIGURE 2 | The distribution of the most common genes in the genetic IS group according to the ages of onset.

of the 103 patients with genetic disorders, while other causative genes were found in <3% respectively.

Seventeen (39.5%) of the identified 43 genes were DEE-related genes recorded in the OMIM database, whereas the remaining 26 (60.5%) genes were associated with other diseases. To facilitate an understanding of genetic pathogenesis, the mutated genes were divided into seven groups according to their functions (**Supplementary Table S3**), with genes encoding for ion transmembrane transport being the most prevalent (12/43, 27.9%). In terms of treatability, *TSC1*-, *TSC2*-, *SLC35A2*-, *ATP7A*-, *ALDH7A1*-, and *MMACHC*-related disorders had specific therapeutic regimens and accounted for 18.5% (19/103) of the cases with genetic disorders. Correspondingly, there were preferred drugs for patients with causal variants in *KCNQ2*, *SCN2A*, *SCN8A*, *CLCN4*, such as sodium channel blockers, accounting for 11.7% (12/103) of the 103 cases. In addition, therapies for *STXBP1*-, *CDKL5*-, *NF1*-, *MECP2*-, *DNM1*-, *GRIN2B*-, *HEXA*-related disorders were under investigation.

Etiology and Distribution of IS

In comparison to females, males had a higher proportion of structural-acquired etiologies ($p = 0.006$) and a lower proportion of genetic ($p < 0.001$) and metabolic etiologies ($p = 0.024$). In contrast to term babies, preterm babies had a higher proportion of structural-acquired etiologies ($p = 0.001$). Patients with hypsarrhythmia had a significantly higher ratio of non-acquired structural etiologies (structural-congenital and genetic-structural causes) ($p = 0.022$) compared with those without hypsarrhythmia. Patients with pre-spasm seizures had significantly higher proportions of structural causes ($p = 0.007$), genetic causes ($p < 0.001$), and a lower proportion of unknown causes ($p < 0.001$) compared with cases without pre-spasm seizures. Early-onset IS group had a higher ratio of genetic causes, compared with classic onset IS ($p < 0.001$) and late-onset IS ($p = 0.006$). All other factors showed no significant differences.

Supplementary Table S4 shows the etiological distribution of different IS groups.

The most common causative gene in the early-onset genetic IS group was *STXBP1,* while *CDKL5* was the causative gene for classic and late-onset (**Figure 2**). For neonatal-onset IS ($n = 13$), the leading causes were structural-acquired followed by genetic. Variants in *KCNQ2*, *STXBP1,* and *SCN2A* were responsible for the 3 cases presenting with neonatal-onset spasms, respectively. The distribution of the genes in the genetic IS group was investigated as shown in **Figure 2**. For the 13 cases with *STXBP1* variants, eight cases had spasms onset within the third month of age, and the distribution showed a statistically significant difference when compared with other genes ($p = 0.017$). In contrast, nine of the 13 cases with *CDKL5* variants presented with spasms beyond the early-onset period, however, the distribution showed no statistically significant difference with other genes ($p = 0.736$).

DISCUSSION

To the best of our knowledge, this is the largest cohort study that summarizes the underlying etiologies of IS. The study found that 53.4% of the cases had an underlying etiology. Correspondingly, 64.4 and 58% of the cases in NISC and International Collaborative Infantile Spasms Study (ICISS) studies, respectively, had an identified cause (6, 8). In conjunction with NISC, the leading group was structural acquired followed by genetic IS, and in both studies, there was no immune cause. This study also highlighted the efficacy of brain MRI as a first-line investigation (yield, 37.5%). Furthermore, there are still some differences when comparing the listed cohorts, which might be characteristic of the Chinese population.

Neonatal hypoglycemic brain injury remains an important cause of epilepsy in developing nations, especially for West syndrome, although it has been excluded in studies done in

developed nations. The advanced perinatal care and routinely monitored blood glucose in the Western world might be the explanation for the difference (11). The proportion of hypoglycemic brain injuries in structural-acquired IS in this study was much higher than in previous reports, ranking second only to HIE, indicating the importance of strengthening routine glucose monitoring and hypoglycemia management in perinatal care in China.

Down syndrome is one of the major causes of infantile spasms, contributing to around 10–20% of all known etiologies (6, 8, 12). Surprisingly, Down syndrome was not found in our sample. The disparity might be explained by the introduction of non-invasive prenatal screening for Down syndrome in China, as well as a decrease in the number of live births in infants with Down syndrome (13).

Chromosomal aberrations were only identified in a few of the patients while monogenic variants accounted for a vast majority of the genetic disorders. The most commonly implicated genes were *STXBP1*, *CDKL5*, *TSC2*, *KCNQ2*, *IRF2BPL*, and *TSC1*, which accounted for almost half of the patients with genetic disorders in our study. Except for the genes linked to TSC, the rest were highly consistent with the major genes highlighted in the group with early-onset epilepsies and/or early-onset epileptic encephalopathy with burst suppression (14–16). In retrospect, patients with spasms onset <2 months and those who had previously been diagnosed with Ohtahara syndrome were included in this study. As a result, differences in the major causative genes between studies should be attributed to the broader inclusion criteria used. Differs from the high-frequency genes, other causative genes (ARX, SCN2A, etc.) were only found in <3% of the cases with genetic disorders, respectively. It is evident that IS is highly heterogeneous in terms of genetic etiology. Moreover, the majority of genes involved in our group also showed phenotypic heterogeneity, as it occurs with other neurological disorders in OMIM.

The proportion of metabolic disorders was low in this study, as well as in the NISC study (8). Literature shows that the frequency of inborn errors of metabolism in infants with spasms varies widely (3–22%), depending on the number of patients enrolled and the extent of investigations investigations (4, 8, 17, 18). The fact, that majority of the cases in our cohort showed non-specific changes in routine metabolic screening and were discovered by genetic testing, reflects that metabolic etiologies are likely to be the overlooked contributors of IS, particularly in regions where genetic testing is not available and/or affordable. Genetic testing should, therefore, be considered for cases suspected to have metabolic IS.

As reported, WES explained 28% of cases with IS and also revealed 1–3 *de novo* variants with interesting candidate genes in 64% of the remaining cases (19). In the current study, about 6.0% of the cases that underwent WES revealed 12 candidate genes (*ALPL*, *CACNA1C*, *MED12*, *TCF4*, *TCF20*, *CD99L2*, *TAF1*, *CLCN6*, *CYFIP1*, *GPT2*, *ATP2A2,* and *MYO18A*) which need to be confirmed by reanalyzing the data and functional studies. *CYFIP2* was identified as the causative gene for DEE 65 in OMIM soon after we recognized and reported it as a causative gene for IS (20, 21). Thus, despite the customized multigene panels, WES had much higher diagnostic yields compared to other genetic tests in our study. WES should therefore be considered as the most suitable test when brain MRI fails to detect the underlying etiology. WES is cost-effective, can detect a wide range of genes, and the results can be re-analyzed with time.

The distribution of etiologies is influenced by different factors. Both males and preterm babies independently had higher proportions of structural-acquired etiologies. The male predominance in many acquired diseases such as viral/bacterial meningitis, other uncommon infections, and neonatal stroke (22–25) in children and the association between the low gestational age and high incidence of perinatal brain injury (26, 27) might explain the aforementioned observation in different genders and gestational ages.

Genetic causes play an important role (>20%) in initiating early-onset IS compared with other onset ages. The distribution of *STXBP1* was concentrated in early-onset IS cases ($P = 0.017$). Except for cases with acquired-structural abnormalities, genetic etiology was also a major contributor to neonatal IS and the involved genes were *STXBP1, KCNQ2,* and *SCN2A*. Knowledge of the common causative genes for early-onset IS and neonatal IS might guide clinicians in prescribing precise medication for cases lacking genetic results.

Cases without hypsarrhythmia had a higher ratio of non-acquired structural etiologies, and the reason for this is currently unknown. Patients with previous seizures had a lower proportion of unknown causes and higher proportions of structural-acquired and genetic causes. Consistent with our present study, cases with previous seizures have a high proportion of known etiology (5). The NISC study (2017) indicates that preexisting epilepsy reduces the likelihood of receiving standard therapy, including adrenocorticotropic hormone, prednisolone, or vigabatrin and is associated with low treatment response (7).

LIMITATIONS

This study is the largest cohort study that attempts to investigate the etiology and distribution of IS. However, it is limited by the fact that it was retrospective thus, prone to bias. Besides, the sample size is limited to cases from 18 central-southern Chinese provinces thus, the findings cannot be generalized to other geographical locations.

CONCLUSIONS

More than half of the IS cases in China had an underlying etiology. The most common cause was structural-acquired followed by genetic causes. HIE and hypoglycemic brain injuries were the major causes of structural-acquired IS. Down syndrome was absent in this cohort. Monogenic variants were very heterogeneous. The most commonly implicated genes were *STXBP1*, *CDKL5*, *TSC2*, *KCNQ2*, *IRF2BPL*, and *TSC1*. WES had a diagnostic yield of 26.9% and should be explored when brain MRI fails to find the underlying cause.

The etiological makeup differed in different scenarios. Male and preterm patients were more likely to have a structural-acquired etiology. Early-onset IS cases had a higher ratio of genetic causes. There was a significant proportion of known etiology in those with prior seizures. Cases without hypsarrhythmia had higher ratios of non-acquired structural etiologies compared with those with hypsarrhythmia.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of Xiangya Hospital, Central South University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PP collected, analyzed the data, and drafted the initial manuscript. MK drafted and revised the final manuscript. LM and ZP collected data and carried out the initial analyses. FH, CZ, CC, NP, and FY coordinated and supervised data collection. JP and ZP conceptualized and designed the study, coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content. All authors reviewed the manuscript and approved the submitted version (and any substantially modified version that involves the author's contribution to the study) and have agreed to be personally accountable for the author's contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.774828/full#supplementary-material

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REVIEW
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Synaptopathies in Developmental and Epileptic Encephalopathies: A Focus on Pre-synaptic Dysfunction

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The proper connection between the pre- and post-synaptic nervous cells depends on any element constituting the synapse: the pre- and post-synaptic membranes, the synaptic cleft, and the surrounding glial cells and extracellular matrix. An alteration of the mechanisms regulating the physiological synergy among these synaptic components is defined as "synaptopathy." Mutations in the genes encoding for proteins involved in neuronal transmission are associated with several neuropsychiatric disorders, but only some of them are associated with Developmental and Epileptic Encephalopathies (DEEs). These conditions include a heterogeneous group of epilepsy syndromes associated with cognitive disturbances/intellectual disability, autistic features, and movement disorders. This review aims to elucidate the pathogenesis of these conditions, focusing on mechanisms affecting the neuronal pre-synaptic terminal and its role in the onset of DEEs, including potential therapeutic approaches.

Keywords: synaptopathy, developmental and epileptic encephalopathy (DEE), pre-synaptic mechanisms, drug resistant epilepsy, intellectual disability (ID), SNAREopathies

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INTRODUCTION

Optimal synaptic communication is a complex and finely regulated process that is fundamental for proper nervous system physiology (1–3). The expanding knowledge in the neurobiological field has allowed to increase the accuracy of the etiopathogenesis definition of nervous system diseases: from the macroscopic involvement of anatomical structures and circuitry, the focus shifted to the microscopical elements of this system, including subcellular ones, as transporting proteins, signaling superficial molecules, receptors, and neurotransmitters. The synapse plays a central role in this exchange of information and represents the essential signal transmitting unit of the nervous system (1, 3). The neurotransmitters release requires the availability of synaptic vesicles, which undergo immediate fusion with the pre-synaptic membrane when the action potential arrives (4). The synaptic vesicles undergo repeated recycling, and this process involves the sequential participation of several proteins (see **Figure 1**) (2, 5).

A synaptopathy is defined as an alteration in the functionality of any element constituting the synapse: the pre- and post-synaptic terminals, the synaptic cleft, and all the surrounding components, such as glial cells and extracellular matrix (1, 3, 6). Although the first reference to

the term "synaptopathy" was made in 2003 by Li et al. regarding the Huntington Disease (7), in the last few decades, pathogenic variants in genes encoding synaptic proteins have been demonstrated to determine altered protein levels/function in several neuropsychiatric diseases, such as epilepsy, intellectual disability (ID), and autism spectrum disorder (1, 8).

The term "epileptic encephalopathy" describes a catastrophic form of epilepsy, with a frequent onset in infancy or early childhood, in which the epileptic activity itself significantly contributes to severe developmental delay (DD) and behavioral impairments (9, 10). In those patients presenting with pre-existing DD, the effect of the epileptic activity causes a worsening of the developmental consequences arising directly from the genetic mutation, configuring a clinical phenotype called "developmental and epileptic encephalopathy" (DEE) (10).

Since 2001, when a genetic cause for an epileptic encephalopathy was first reported (11), numerous genes have been associated with DEEs, and several synaptopathies have been described (9). Given that numerous proteins participate in the mechanisms underlying the correct functioning of the synapse, these disorders are generally studied centering the attention on the single affected gene (2). We focused our research on those genes involved in the pathogenesis of DEEs affecting the pre-synaptic compartment, consisting of the axon terminal and the proteins implicated in releasing neurotransmitters. Since these genes are often identified in the context of large cohorts of patients tested with genetic panels, an accurate description is not always available, making it challenging to characterize clinical phenotypes and to identify the DEEs. Therefore, we considered all patients presenting with epilepsy and intellectual disability (ID).

STX1B

Syntaxin1 (Stx1) is a protein widely expressed in the nervous system (12, 13) and, together with Snap25 (encoded by *SNAP25*) and synaptobrevin-2 (encoded by *VAMP*2), form a stable complex called soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complex, made by a four-helix bundle implicated in Ca^{2+}-dependent exocytosis of the synaptic vesicles and neurotransmitter release (14, 15). Vamp2 protein represents the vesicle membrane portion of SNARE neuronal complex (v-SNARE), while the plasma membrane of SNARE (t-SNARE) is constituted by Stx1a and Snap25 (16).

Stx1 is an integral membrane protein composed of three functional domains: (1) the N-terminal peptide, (2) an α-helical domain which is called Habc domain, and (3) the SNARE and transmembrane motif in the C-terminal region (13, 17–20). There are two different protein configurations, namely the "closed" and the "open" Stx1. The first one is characterized by a link between the Habc domain and the N-peptide. Switching from the closed to the open conformation is crucial in regulating the exocytosis mediated by the SNARE complex, and Stx1 plays a crucial role in the initiation of synaptic exocytosis.

The first step that allows the SNARE complex assembly is represented by the binding between Munc18 (encoded by *STXBP1*) and Stx1 in its closed form.

This is the starting point of a process that will end with releasing the neurotransmitter in the synaptic cleft (21–23).

There are two isoforms of Stx1 called 1a and 1b. Even if they share 84% of their amino acid sequence and the basic function as neuronal t-SNAREs, Stx1b is the principal mediator for spontaneous and evoked fast synaptic vesicle exocytosis (24, 25).

The gene encoding for Stx1a is located on chromosome 7 and is one of the genes involved in Williams-Beuren Syndrome (WBS), caused by deletion 7q11.23 (26). In this regard, Gao et al. (27) showed that the level of expression of Stx1a is correlated with the degree of intelligence in patients with WBS. It has also been found that some variants of *STX1A* are associated with an increased chance of developing migraines (28). Moreover, data from studies conducted on many families investigated with Whole Exome Sequencing (WES) suggest that *STX1A* may be a possible candidate gene in the development of neurodevelopmental disorders (29).

On the contrary, less is known about the involvement of pathogenic variants of *STX1A* in the development of epilepsy. However, the influence that Stx1a exerts on glutamate uptake and glutamatergic transmission could partially explain the role of *STX1A* in epileptogenesis. Data from the literature suggest that Stx1a acts by enhancing the internalization of excitatory amino acid transporter 1 (EAAC1) responsible for glutamate re-uptake: increased internalization and reduced expression on the cell surface cause an overall reduction in glutamate uptake (30). Additionally, a Stx1a role in regulating voltage-gated K+ channel determined by physical interaction of this protein with the ion channels has been demonstrated in animal models (31). Moreover, single nucleotide polymorphisms (SNP) of *STX1A* and *VAMP2* have been described in association with cryptogenic epilepsy (32).

Much more has been described regarding the 1b isoform. *STX1B* gene is located on chromosome 16 (16p11.2 region) (33) and studies conducted on mouse models have shown an early death of the animals and an altered function of the neuromuscular junction in KO mice for *STX1B* (34). These findings underline the critical role played by Stx1b in the proper signaling of the nervous system. Therefore, dysfunctions of this protein, whether due to mutations or deletions, are associated with the development of various disorders of the nervous system, including ID, speech disorders, and various forms of epilepsy (35).

To the best of our knowledge, *STX1A*-related DEEs are not reported in the literature. For this reason, we focused on the 1b isoform and identified 20 patients (35–39). Clinical information was collected about the age onset of symptoms, the type of seizures, the presence of febrile seizures (FS), electroencephalographic patterns, and the development of ID

Abbreviations: Abs, Absence; AS, atonic seizure; Atyp Abs, Atypical Absence; CS, clonic seizures; CSE, convulsive status epilepticus; DD, developmental delay; EIMFS, Epilepsy of Infancy with Migrating Focal Seizures; FS, Focal Seizure; GTCS, generalized tonic clonic seizure; ID, intellectual disability; IS, Infantile Spasm; LGS, Lennox Gastaut Syndrome; MA, myoclonic atonic seizure; MAE, myclonic atonic epilepsy; Myo, Myclonic seizure; NA, not available; NCSE, non-convulsive status epilepticus; NV, non-verbal; SE, Status Epilepticus; TS, Tonic Seizure.

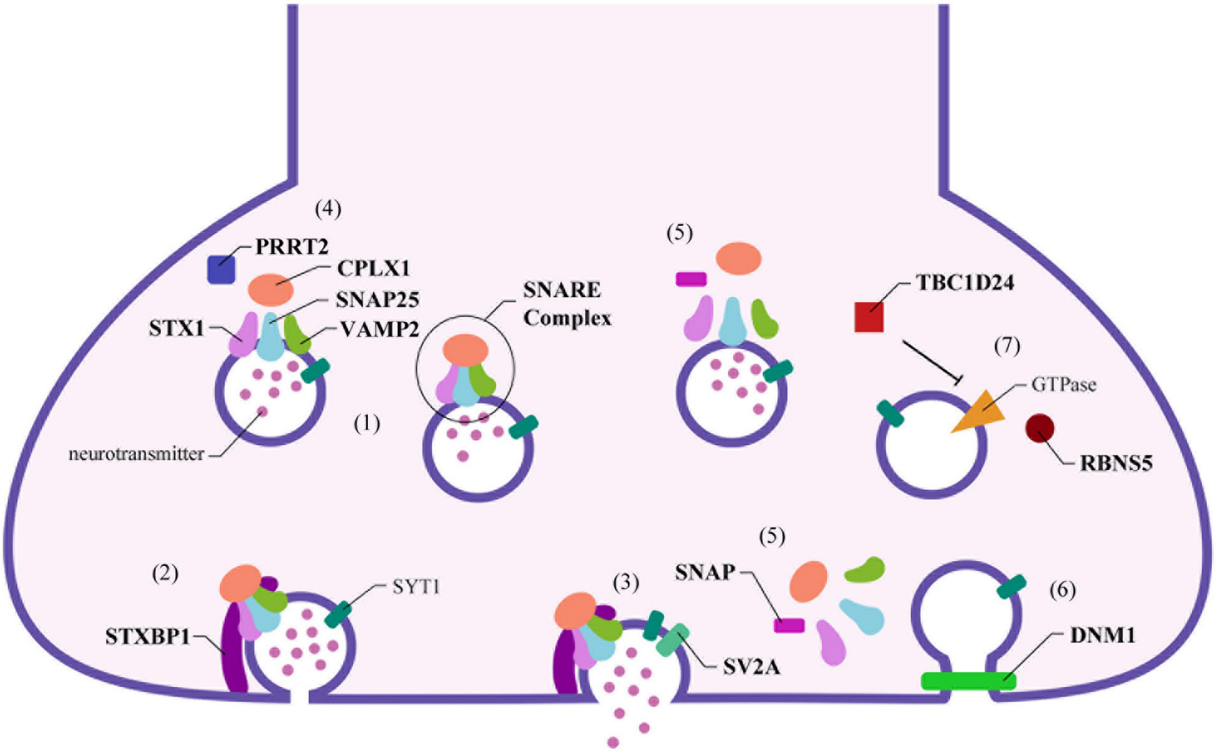

FIGURE 1 | Schematic representation of pre-synaptic proteins localization and their role in the synaptic functioning. Numerous proteins interact together and with the cellular membrane on the pre-synaptic terminal to release the neurotransmitters in the synaptic cleft. (1) Stx1, Snap25, and Vamp2 form the core of the SNARE complex, which is stabilized by Cplx1. (2) When the synaptic vesicle reaches the cellular membrane, Stxbp1 binds Stx1, starting the priming and the rapid fusion of the vesicle. (3) Sv2a, the only specific synaptic protein, interacts with Syt1 and induces neurotransmitter release. (4) On the contrary, Prrt2 inhibits this process by reducing the formation of the SNARE complex. (5) The disassembly of the SNARE complex is mediated by Snap proteins, (6) allowing the subsequent clathrin-mediated endocytosis, with the participation of the Dnm1. (7) This process is coordinated by several GTPases: Tbc1d24 regulates Rab35, while Rbns5 interacts with Rab4 and Rab5 to lead the empty synaptic vesicle toward the endosome for recycling.

and/or movement disorders. Not all clinical information could be traced for all patients.

The age of onset of epilepsy ranged from a few days after birth to a maximum of 4 years (35–39). Most of them present multiple forms of epilepsy: the most represented type of seizures are myoclonic ones (11/20), generalized tonic-clonic ones (10/20), and absences (9/20). Other types of seizures reported are atonic (7/20), tonic (6/20), focal (3/20), spasms (1/20), myoclonic-astatic epilepsy (1/20). FS were also frequent, reported in seven (7/20) patients. The increased susceptibility to develop FS has been reported in previous studies (35, 40, 41), and more recently by Mishima et al. (42). In this cohort of patients, we report a recurrence of ataxia, present in 11 of 20 cases (35, 37, 39).

In addition, seizure control was reached in five patients, but given the large number of drugs administered, often in co-administration, it is difficult to define the most appropriate pharmacological treatment. Clinical data are summarized in **Table 1**.

SNAP25

The *SNAP25* gene is mapped on chromosome 20 (20p12.2 region), and it encodes the synaptosomal-associated protein 25 kDa (Snap25) highly expressed in nerve and neuro-endocrine cells. Snap25 is a crucial component of the SNARE complex and, together with Stx1 and synaptobrevin-2, plays a crucial role in Ca^{2+}-dependent exocytosis of the synaptic vesicles (14).

In mammals, due to the differential splicing of the *SNAP25* gene, two different isoforms (Snap25a and Snap25b) are obtained, which differ only for nine amino acids. However, they show different expression and localization profiles in the various brain regions in humans and mice (43). The most crucial isoform in synaptic transmission is Snap25b, which is mainly expressed in the synapses of the central nervous system and the peripheral motor endplates, and it regulates the exocytosis of neurotransmitters. Given its fundamental role in nervous transmission, *de novo SNAP25* variants are associated with various neurological disorders, such as epilepsy, movement disorders, and psychiatric conditions (39).

We reviewed the recent literature and found the description of 18 cases in which pathogenic variants of *SNAP25* are associated with DEEs (15, 44–47). All clinical data are summarized in **Table 2**.

Most of the patients (13/18) showed seizures onset during the early childhood (ranging from 3 months to 8 years of age); two cases (2/18) presented with neonatal-onset epilepsy,

TABLE 1 | Clinical features of *STX1B*-related patients.

No.	Age of onset	Seizure type at onset	Epilepsy evolution	Eeg	Movement disorders	ID/DD	Genetic variant	Reference
1	1 years	GTCS, FS	MAE	High-amplitude polyspike waves	NA	Moderate to severe ID	Arr[hg19] 16p11.2 (30, 943, 951–32, 151, 753) x1 (*de novo*)	(38)
2	NA	NA	NA	NA	NA	NA	c.140C>A; p.s47* (heterozygous variants)	(35)
3	3 years and 6 months	GTCS, TS, Myo, Abs	NA	Focal sharp wave, generalized sharp wave	Ataxia	Moderate ID	c.657T>A; p.Val216Glu	(35)
4	20 months	GTCS, Myo, Abs, AS, TS	NA	Generalized sharp wave, focal sharp wave	Ataxia	DD	c.676G>C; p.Gly226Arg	(35)
5	13 months	Myo, ats, GTCS	NA	focal sharp wave	Ataxia	DD	Arr[hg19] 16p11.2 (30, 332, 532–31, 104, 012) x1	(35)
6	9 months	Myo, Atyp Abs, GTCS, TS	NA	Generalized sharp wave, focal sharp wave	Ataxia	DD	c.563dupA; p.Asn189Alafs*5	(35)
7	16 months	Myo, Atyp Abs, GTCS, TS	NA	Generalized sharp wave, focal sharp wave	Dystonia	DD	c.563dupA; p.Asn189Alafs*5	(35)
8	2 years	Abs, Myo, as, TS	NA	Generalized polispike sharp wave, generalized sharp wave	Ataxia	DD	c.845T>C; p.Ile282Thr	(35)
9	NA	NA	NA	NA	NA	NA	c.773G>A; p.Ser258Asn	(35)
10	3 years	GTCS, Abs, Myo, AS, FS	NA	Generalized polispike sharp wave, gps	Mild ataxia	DD	c.662T>C; p.leu221Pro	(35)
11	4 years	GTCS, Myo, Abs, AS	NA	Generalized sharp wave	Ataxia	DD	c.155delA; p.q52rfs*2	(35)
12	10 months	Abs	NA	NA	Ataxia, tremor, dysarthria	DD	c.431G>T; p.Cys144Phe	(35)
13	Since birth	IS	NA	Hypsarrhythmia	NA	Severe ID	c.736 G>C; p.Ala246Pro	(35)
14	2 years	AS, Abs, Myo, GTCS	NA	Generalized polispike sharp wave, generalized sharp wave	Ataxia	ID	c. (?_242)_(*3565_?)	(35)
15	2 years	AS, Abs, Myo, GTCS	NA	Generalized polispike sharp wave, generalized sharp wave	Ataxia	ID	c. (?_242)_(*3565_?)	(35)
16	3 months	Myo, apnea and cyanosis	NA	Generalized polispike sharp wave	NA	DD	c.383del; p. Gln128Glyfs*2 (heterozygous variant)	(35)
17	NA	AS, GTCS	NA	Focal sharp wave	/	Mild ID	c.420C>G; p. Tyr140*	(35)
18	13 months	TC, MA, myo, and TS	MAE	Generalized epileptic activity	Ataxia and tremor	Moderate ID	c.676G>C; p.Gly226Arg	(39)
19	NA	Dravet-like	NA	NA	NA	ID	NA	(36)
20	9 months	FS	NA	Focal onset seizure disorder of temporal origin.	Cerebellar ataxia	Mild ID	Deletion of the full coding sequence of stx1b	(37)

while only three patients (3/18) showed epilepsy after the first decade of life (from 13 to 19 years of age). The semiology of the seizures appears to be heterogeneous: although most patients manifested generalized seizures (12/18), also focal seizures (5/18) and epileptic spasms (4/18) have been reported. In one case the description of the crisis was unavailable. Noteworthy, all patients (18/18) developed an intractable severe encephalopathy with moderate to severe ID. When available (3/18), the EEG showed multifocal abnormalities or generalized spike-and-slow wave complex, with a variable response to antiepileptic drugs (AEDs). Half of the patients (9/18) showed a negative MRI, and aspecific neuroradiological anomalies were reported only in three cases (3/18), such as leukoencephalopathy or brain volume loss. Frequent association with movement disorders, such as tremors, dystonia, muscular hypotonia, spasticity, and cerebellar ataxia, is described (13/18), and only in one patient (1/18) the absence of movement disorders was reported. Three patients (3/18) also showed behavioral disorders and autistic features. Response to AEDs was variable, and most of the patients (more than 50%)

TABLE 2 | Clinical features of *SNAP25*-related patients.

No.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID	Genetic variant	Reference
1	Since early childhood	fs	Drug resistant epilepsy	Generalized atypical polyspike and wave discharges and diffuse slowing of the background rhythm	Fatigable weakness, ataxic dysarthria, paretic and ataxic gait	Yes	c. 200 T>A; p.Ile67Asn	(46)
2	18 months	gtcs, fS	DEE	Generalized spike-wave and continuous spike and wave during sleep	Not present	Yes (moderate)	c.496G>T; p.Asp166Tyr	(47)
3	5 months	gTCS, FS	Intractable severe static encephalopathy	Mild generalized slowing, 2–2.5 hz generalized spike-and-slow wave complexes.	Spastic quadriparesis	Yes	c.142G>T; p.Val48Phe	(45)
4	13 yo and 2 months	abs, gs	NA	NA	Muscular hypotonia	Yes, severe	c.118A>G; p.Lys40Glu	(44)
5	8 yo and 1 months	gtcs	NA	NA	Cerebellar ataxia, hand flapping, resting and intention tremor	Yes, mild	c.127G>C; p.Gly43Arg	(44)
6	Infant	gtcs	NA	NA	NA	Yes	c.520C>T; p.Gln174*	(44)
7	5 yo and 19 months	Is, ts	NA	NA	Muscolar hypotonia, spasticity	Yes	c.149T>C; p. (leu50ser)	(15)
8	19 yo and 5 months	NA	NA	NA	Muscolar hypotonia, ataxia, tremor dystonia	Yes (moderate)	c.127G>C; p.Gly43Arg	(15)
9	4 yo and 3 months	gs	NA	NA	Ataxia	Yes (moderate)	c.127G>C; p.Gly43Arg	(15)
10	6 weeks	gs, fs	NA	NA	NA	Yes (profound)	c.170T>G; p.Leu57Arg	(15)
11	8 yo and 3 months	gs, fs	NA	NA	NA	Yes (severe)	c.212T>C; p.Met71Thr	(15)
12	17 yo	gs, fs	NA	NA	NA	Yes (moderate)	c.497A>G; p.Asp166Gly	(15)
13	3 yo and 6 months	IS, gs	NA	NA	Muscolar hypotonia, ataxia, tremor dystonia	Yes (profound)	c.521A>C; p.Gln174Pro	(15)
14	14 yo	Is	NA	NA	NA	Yes (moderate)	c.575T>C; p.Ile192Thr	(15)
15	3 months	IS	NA	NA	Spasticity and muscolar hypotonia	Yes (severe)	c.593G>C; p.Arg198Pro	(15)
16	3 yo and 10 months	Gs, fs	NA	NA	Ataxia and muscolar hypotonia	Yes (moderate)	c.596C>T; p.Ala199Val	(15)
17	2 yo and 6 months	gs	NA	NA	Dystonia and muscolar hypotonia	Yes (mild)	c.114+2T>G	(15)
18	2 yo	IS	NA	NA	Muscolar hypotonia, spasticity	Yes (profound)	c.520C>T; p.Gln174*	(15)

presented with frequent seizures despite being treated with several AEDs. A single patient showed a good response after therapy with valproic acid (VPA) and clonazepam, with a reduction of frequency and severity of seizures. Another case of highly drug-resistant epilepsy was treated with three-drug combinations and trials with a ketogenic diet and intravenous

methylprednisolone. In some cases, information regarding the response to antiepileptic treatments were not reported (15, 44–47).

VAMP2

The VAMP2 gene, mapped on chromosome 17 (17p13.1 region), encodes for Vamp2 (also called synaptobrevin-2) (16, 48). As mentioned, Vamp2 protein constitutes the v-SNARE, and results fundamental to driving synaptic transmission, which is also regulated by Ca^{2+} ions and other proteins (16). Pathogenic variants of VAMP2 gene are associated with neurodevelopmental disorders, such as visual impairment, hyperkinetic movements, autism spectrum disorder and epilepsy (16, 39). More severe neurological phenotypes are described in individuals with nonsynonymous mutations of VAMP2 (39). The importance of a correct mechanism of neuronal trafficking mediated by Vamp2 has been highlighted by a recent study evaluating the essential role of *VAMP2* and *DLG4* in the progression of epilepsy and behavioral disorders, in particular ADHD (49). In these conditions, the expression of Dlg4 and Vamp2 is downregulated, which determines abnormal neurotransmission, presumably the cause of these disorders (49).

To date, just three patients carrying pathogenic variants of VAMP2 associated with DEE are described (see **Table 3**) (16). In particular, Salpietro et al. (16) reported five individuals (from 2 months to 14 years of age) with de novo heterozygous mutations of the VAMP2 gene presenting with various neurodevelopmental phenotypes, such as epilepsy, hypotonia, ID, and autistic features. Two of these patients did not present epileptic manifestations, but they showed EEG anomalies (such as high-voltage delta activity, sharp-and-slow-wave complexes or only a disorganized EEG). Conversely, three patients showed seizures onset within the first months of life: one presented with focal seizures, another one reported generalized tonic-clonic seizures and focal seizures, the last one developed infantile spasms. EEGs showed disorganized activity, generalized and/or multifocal abnormalities, sharp wave-slow wave complexes, or other focal paroxysms. Language is always compromised (1/3) or absent (2/3), and all the patients presented with autistic features and variable motor stereotypies comparable to Rett syndrome (RTT) (3/3) (16). The patients showed highly drug-resistant epilepsy: they trialed several AEDs, such as VPA, vigabatrin, and lamotrigine. VPA has been proved the most beneficial in two individuals, and particularly one of them was reported as seizure-free since the age of 12 years, and his follow-up EEGs were normal (16).

CPLX1

The *CPLX1* gene is located on chromosome 4 (4p16.3), and it encodes for the complexin 1 (Cplx1). The complexin-family is a group of highly conserved cytosolic proteins expressed at the pre-synaptic terminal and interacting with the SNARE complex: Cplx1 and Cplx2 are the two paralogues mainly expressed in the CNS, the former being the most represented isoform, while Cplx3 and Cplx4 are predominantly identified at retinal ribbon synapses (13, 50, 51).

The exact function of the complexins has yet to be fully unraveled, but two mechanisms of action have been proposed. A controversial role as an inhibitor of the spontaneous release of neurotransmitters has been reported, with stronger evidence in invertebrates than in mammals; in fact, an increase in a spontaneous release of neurotransmitter was observed in cultured complexin-1/2 knockdown cortical neurons but not in complexin-1, −2 and double complexin-1/2 knockdown mice (13, 51–53). Secondly, a role as a Ca2+-triggered release promoter has been suggested, given the evidence showing a reduction in synaptic response amplitude after the action potential stimulation (51). Moreover, a reduction in both evoked and spontaneous release of glutamate is described in complexin-1/2/3 null cultured hippocampal neurons (13, 53). To date, the prominent hypothesis for the complexin mechanism of action is represented by its involvement in regulating the vesicle fusogenicity by lowering the energy barrier for primed vesicles to undergo either Ca2+-evoked or spontaneous fusion (13, 51).

To date, only five patients with DEEs associated with pathogenic variants CPLX1 have been described (50, 54), with epilepsy onset from 6 weeks to a maximum of 2 years of life. Infantile spasms (2/5) were the most frequent seizures type at onset, while a single patient showed myoclonic seizures (50). These three patients (3/5) developed myoclonic seizures, and EEG showed generalized epileptiform activity (50). However, all patients presented drug-resistant epilepsy. Conversely, Karaka et al. (54) reported two sisters that developed malignant migrating epilepsy and unspecified ID (54). No association with autistic features were reported in all the subjects. A single patient showed movement disorders and cerebral palsy (50). Brain MRI resulted normal in two patients (2/5), showed cortical atrophy in two patients (2/5), and in a single case (1/5) detected cerebellar abnormality (50). All data are summarized in **Table 4**.

STXBP1

The *STXBP1* (also known as *MUNC18-1*) is a gene located on chromosome 9 (9q34.11 region), which encodes the Syntaxin1a binding protein (Stxbp1), a protein of the SEC1 family that is essential for vesicles trafficking (19, 55). The Stxbp1 is a neural-specific binding protein that organizes the protein complexes that induce secretory vesicle exocytosis (56, 57). Notably, it is crucial to promote the conformation change in Stx1a, allowing the SNARE complex formation (22). This Stxbp1-Stx1a binding serves two purposes: firstly, when Stx1a is in a "closed" conformation, it interacts with the Habc domain and prevents the formation of ectopic and uncontrolled SNARE complexes in the synapses; secondly, when it binds the N-terminal peptide of the "open" Stx1a, Stxbp1 facilitates the synaptic vesicle priming and fusion, allowing the neurotransmitter

TABLE 3 | Clinical features of *VAMP2*-related patients.

No.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Genetic variant	Reference
1	After birth	FS, GTCS	Drug resistant epilepsy	Fast rhytmic activity, sharp wave-slow wave complexes	Generalized chorea	Rett-like	c.233A>C; p.Glu78Ala	(16)
2	1 month	IS	Drug resistant epilepsy-CSE	Disorganized EEG and paroxysms	Choreic movement, myoclonic jerks	Rett-like	c.230T>C; p.Phe77Ser	(16)
3	5 years	FS	ncse	generalized and multifocal abnormalities	Absent	Rett-like	c.128_130delTGG; p.Val43del	(16)

TABLE 4 | Clinical features of *CPLX1*-related patients.

No.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Genetic variant	Neuroimaging	Reference
1	NA	NA	Malignant migrating epilepsy	NA	NA	ID	c.322G>T; p.Glu108* (homozygous non-sense variant)	Cortical atrophy	(54)
2	NA	NA	Malignant migrating epilepsy	NA	NA	ID	c.322G>T; p.Glu108* (homozygous non-sense varia-nt)	Cortical atrophy	(54)
3	6 weeks	IS	Migrating myoclonic epilepsy—deceased	Generalized epileptiform activity and hyperexcitability	NA	DD	c.315C>A; p.Cys105* (homozygous non-sense variant)	Small cleft of lobule VIII of the left cerebellar hemisphere with malorientation of the adjacent cerebellar folia (at 1, 2 and 3 years)	(50)
4	2 ½ months	IS	Myoclonic epilepsy (progressive)	Generalized spikes and waves, hyperexcitability	NA	DD	c.315C>A; p.Cys105* (homozygous non-sense variant)	Normal (at 2 months and 2 years)	(50)
5	2 years	Myo	Myo, TS and GTCS	Marked persistent generalized seizure activity	Cerebral movement disorder and cerebral palsy	DD	c.382C>A; p.Leu128Met (homozygous)	Normal (at 17 months)	(50)

release (58). It has also been demonstrated that Stxbp1 levels correlate with secretion capacity and synaptic strength, making this protein fundamental in synaptic transmission and maintenance of synaptic connections in adulthood (59–61).

Mutations of the *STXBP1* gene determines an alteration in the elaborate mechanism of synaptic exocytosis, leading to an excitatory/inhibitory imbalance which can trigger an increased epileptic activity (60). Additionally, is ubiquitously expressed in the neuron. Stxbp1 also has non-synaptic functions: it regulates the post-Golgi transport of vesicles, it chaperons the α-synuclein, and it is a fundamental element in the development of the brain allowing neurite extension and radial migration of the cortical neurons (58).

Initially, the *STXBP1* gene was associated with Ohtahara syndrome by Saitsu et al. (62). Since this discovery, the development and wide application of genetic testing helped recognize numerous new *STXBP1*-related severe early-onset DEEs (with a median onset of 6 weeks in 85% of the cases). The epileptic phenotypic spectrum includes West syndrome, Lennox-Gastaut syndrome, Dravet syndrome, early myoclonic encephalopathy, and several unclassified DEEs, with the former and the latter representing together half of *STXBP1*-related epilepsy (58, 63). Moreover, patients with disease-causing variants of *STXBP1* have diverse phenotypes, including Rett-like syndrome and non-epileptic presentations, but they always show severe to profound DD/ID (58, 63, 64). In addition, an evolution of *STXBP1*-associated disease with the development of neurologic symptoms similar to early-onset parkinsonism has been recently pointed out (65).

Given the wide clinical spectrum of *STXBP1*-related diseases, a genotype-phenotype correlation is difficult to achieve: haploinsufficiency of the gene associated with a dominant-negative effect has been proposed as the primary pathogenic mechanism behind *STXBP1* encephalopathy, potentially explaining the complex expression of pathogenic variants of this gene (64, 66). Previous reports showed an association between non-sense mutations and early onset DEEs, while missense pathogenic variants are correlated to more various clinical phenotypes (58). Interestingly, most of the pathogenic

variants of *STXBP1* described in the literature are heterozygous mutations, leading to the assumption that they were the only ones tolerated. However, two siblings presenting with Lennox-Gastaut syndrome were recently reported, carrying a homozygous missense mutation of *STXBP1*, causing a gain-of-function (64).

Regarding the treatment options for *STXBP1*-related DEEs, it is noteworthy that the patients carrying a pathogenic variant of *STXBP1* showed seizures refractory to standard AEDs (58). Similarly to most of the neonatal-onset epileptic encephalopathies, Phenobarbital, VPA, vigabatrin, and levetiracetam are the most used drugs, though over half of the patients are treated with more than three AEDs, included ACTH and corticosteroids (58, 63, 67). Novel treatments such as trehalose, sorbitol, and 4-phenylbutyrate have been proven efficacious in restoring Stxbp1 protein levels in primary mouse neurons and *C. elegans* models: these compounds may reverse the deficit caused by the mutant protein and were also able to increase levels of wild-type Stxbp1 (58). Given the above, a clinical trial evaluating the safety and tolerability of Glycerol Phenylbutyrate in a small group of patients began in 2021 and is set to end in 2023 (ClinicalTrials.gov identifier: NCT04937062; accessed on 02 Jan2021).

SV2A

Synaptic vesicle glycoproteins 2 (Sv2) are a transmembrane glycoprotein family located in the synaptic vesicles of neurons. Three isoforms of Sv2 are reported: Sv2a, 2b and 2c (68). The *SV2A* gene, mapped on chromosome 1 (1q21.2 region), encodes for Sv2a, a protein first described by Buckley and Kelly (69) that consists of 12 transmembrane domains and cytoplasmic N- and C-terminal sequences (70). It is widely expressed in GABAergic and glutamatergic neurons of the cerebral cortex, hippocampus and cerebellum, and it controls synaptic transmission through multiple mechanisms: Sv2a promotes the formation of the SNARE complex; also, it intervenes in both immediate synaptic vesicle release and Ca^{2+}-dependent release as it interacts directly with synaptotagmin1 (Syt1). It has also been recognized as an ATP-binding site that would be involved in the process of vesicular priming (71, 72). It also represents the target site of Levetiracetam (LEV) (73). In literature, several studies on animal models showed how *SV2A* missense mutations cause an imbalance in GABAergic and glutamatergic transmission, leading to epilepsy (74–76).

To the best of our knowledge, only three cases of epilepsy associated with mutations in the *SV2A* gene have been reported in humans. Calame et al. (77) described a patient with epilepsy onset at 2 years of age. The administration of LEV caused a worsening of seizures and the development of a status epilepticus. Genetic investigations demonstrated a rare *de novo* variant in heterozygosity in *SV2A*. Subsequently, LEV was suspended, and the patient achieved good seizure control with VPA and a ketogenic diet. Developmental milestones in this patient are described as adequate, ruling out the hypothesis of a DEE.

On the contrary, Wang et al. (78) described a girl with a slight developmental delay and myoclonic seizures. The child was initially treated with levetiracetam, showing a deterioration of the clinical picture with the development of infantile spasms. Nevertheless, epileptic episodes disappeared after the suspension of levetiracetam and the administration of VPA and Clonazepam (78).

Only a case associated with drug-resistant epilepsy and DD/ID was reported by Serajee and Huq (79). This patient showed microcephaly, optic atrophy, epileptic spasms and myoclonus, with onset at 2 months of age (79). The clinical features of these two cases are summarized in **Table 5**.

PRRT2

The *PRRT2* gene is located on chromosome 16 (16p11.2 region) and encodes for proline-rich transmembrane protein 2 (Prrt2), a pre-synaptic protein widely expressed in the cerebellum, the basal nuclei, and the neocortex (80, 81). Its expression is increased at major synaptogenesis stages, and it intervenes in modulating neuronal excitability. Two mechanisms are responsible for this process: Prrt2 regulates Nav1.2/1.6 currents (82) and contributes to controlling the vesicular trafficking and releasing the neurotransmitters. However, the mechanism of action of *PRRT2* still appears controversial: according to Coleman et al. (83), the N-terminal portion of Prrt2 interacts directly with the SNARE complex reducing its formation, thus causing a decrease in the process of vesicular exocytosis (83). On the contrary, Valente et al. (81) proposed an alternative mechanism of action, according to which Prrt2 participates in the regulation of the Ca^{2+} sensing apparatus for the rapid synchronous release of synaptic vesicles by binding Snap25 and Syt1/2. Specifically, they observed a reduced neurotransmitter release in *PRRT2*-silenced primary neurons (81). Therefore, according to these evidences, in both cases Prrt2 influences the vesicular neurotransmitter release but with two apparently opposite effects (81, 83).

Pathogenic variants of *PRRT2* are associated with a large spectrum of familial neurological disorders: *PRRT2* is primarily associated with paroxysmal dyskinesia (PKD) but also hemiplegic migraine (HM), infantile convulsions and choreoathetosis (ICCA) and benign sporadic and familial seizures (BFIS) (84, 85).

Movement disorders are salient features of *PRRT2*-associated conditions, usually with onset in adulthood, and consisting principally in dystonia and choreoathetosis (84). The PKD chorea and dystonia episodes are typically brief (about 1 min) and can be triggered by prolonged exercise (86).

Regarding epilepsy, BIFS usually occurs within the first year of life and has a good response to drug therapy, especially with carbamazepine, phenobarbital, and VPA (87). Usually, seizures consist of brief episodes of psychomotor arrest, accompanied by generalized hypertonia, cyanosis, and limb jerks. In contrast, ICCA is characterized by the precocious onset of seizures and the subsequent development of movement disorders (paroxysmal dyskinesias), which generally occur at 5 years (86).

A genotype-phenotype correlation of *PRRT2*-related disorders is complicated, as it may be inferred by the wide phenotypic

TABLE 5 | Clinical features of *SV2A*-related patients.

No.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Other neurological features	Genetic variant	Neuroimaging	Reference
1	2 months	IS and arching of the back, Myo	Drug resistant epilepsy—TS	Multifocal spikes, diffuse attenuation superimposed by diffuse fast wave activity; spike and waves	NA	DD	Hypotonia, microcephaly, optic atrophy	c.1148G>A; p.Arg383Gln	Diffuse increased T2 signal in the bilateral frontal, parietal and temporal deep cerebral white matter, thin corpus callosum, and mild ventriculomegaly (at 11 months)	(79)
2	2 months	Myo	Epileptic spasms after LEV administration	Burst of poly-spikes bilaterally	NA	DD	NA	c.1708C>T; p.Arg570Cys	NA	(78)

variability of diseases associated with the frameshift mutation c.649dupC (p.R217Pfs*8), which is reported as recurrent in the literature (82, 87, 88).

Generally, the neurological outcome is favorable (87), and there are no clear correlations between *PRRT2* and epileptic encephalopathies (89).

Few cases of pathogenic variants of *PRRT2* associated with clinical phenotypes comparable to DEEs were described: Pavone et al. (90) described a single patient with drug-resistant epilepsy, persistent electroencephalographic abnormalities, and severe DD. Djémié et al. (91) and Jafarpour and Desai (92) reported two cases of West Syndrome; Guerrero-López et al. (93) described a patient with epilepsy and severe ID. Moreover, severe conditions have been associated with homozygosity mutations of *PRRT2* (94).

Although *PRRT2* is known to be responsible for cognitive disorders (95), more studies are needed to assess the frequency of ID and psychiatric symptoms in *PRRT2*-associated syndromes (96).

NAPB

NAPA and *NAPB* genes are mapped respectively on 19 (19q13.33 region) and 20 (20p11.21 region) chromosomes, and encoding for the soluble NSF Attachment Proteins Alpha (αSnap) and Beta (βSnap), ubiquitarious proteins with higher expression in the brain, βSnap only being expressed post-natally (97, 98). They are highly homologous to each other and represent essential components in the vesicular transport, the membrane fusion, and the release of neurotransmitters. In particular, they play a crucial role in dissociating and recycling the SNARE complex, making its components available for subsequent fusion reactions (98). The Snap proteins participate as a co-factor of the NSF ATPase during the SNARE complex disassembly, inducing increased levels of the free SNARE components (5, 98).

Given their role in neuronal regulation and brain development, variants of these genes may be associated with various neurological disorders, yet a clear link to developmental and epileptic encephalopathies has not been reported (97). However, a study conducted on βSnap-KOs mice demonstrated an epileptic phenotype with onset 11 days after birth, consistent with the developmental expression pattern of βSNAP: the mice developed severe recurrent epileptic seizures, occasionally leading to death (98).

To the best of our knowledge, solely four patients with DEEs associated with disease-causing variants of *NAPB* are described (29, 97, 99) (see **Table 6**). Clinical findings of the patient reported by Reuter et al. (29) was unavailable. All the other patients presented a very early onset of seizures (range from 2 to 6 months), and the most frequently reported type of seizures is clonic (3/4), but tonic seizures are also described in association with the clonic ones (97, 99). In the majority of the cases (3/4), the epilepsy evolution is a multifocal epileptic encephalopathy, and all the patients (4/4) presented a severe DD/ID (29, 97, 99). Neuroimaging was reported as normal in 2/4 subjects (2/4 are unavailable) (97, 99). One patient developed movement disorders such as axial and peripheral hypotonia, limb tremulousness and stereotypies (kicking, hand, wrist-twisting, and bringing to the midline) (99). No information about the therapeutic approach was reported.

DNM1

The dynamin (Dmn) is a GTPase involved in vesicular transport and clathrin-dependent endocytosis (100). There are three different isoforms of the protein, called Dnm 1, 2 and 3; variant 1 is the most expressed in neurons (101). Dnm1 is encoded by the DNM1 gene located in 9q34.11, and its levels increase in parallel with synaptogenesis, particularly during the post-natal phase (102, 103). The process of phosphorylation/dephosphorylation of Dnm1, mediated by Cdk5 and Ca^{2+}-dependent calmodulin, respectively, regulates the activation of Dnm1 by facilitating its interaction with other proteins involved in endocytosis. For this reason, pathogenic variants of *DNM1* can cause an impairment of endocytosis of synaptic vesicles with consequent impact on vesicle recycling and synaptic function (104). To date, it has been clarified that DNM1 pathogenic variants can be associated with Lennox-Gastaut syndrome and infantile spasms (105, 106).

TABLE 6 | Clinical features of *NAPB*-related patients.

No.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Other neurological features	Genetic variant	Neuroimaging	Reference
1	5 months	TS and/or CS	Multifocal epileptic encephalopathy	NA	Limb tremulousness and stereotypies (kicking, hand, wrist twisting, and bringing to the midline)	Profound ID, global DD	Axial and peripheral hypotonia	c.565C>A; p.Ser160*	Normal	(99)
2	6 months	Clonic seizures	Multifocal epileptic encephalopathy	Multifocal epileptic discharges in the C-P-O or F-T regions	NA	Profound ID and global DD	NA	c.433-1G>A (homozygous splicing variant)	Normal	(97)
3	2 months	Clonic seizures	Multifocal epileptic encephalopathy	NA	NA	Global DD, profound ID	Microcephaly	c.433-1G>A (homozygous splicing variant)	NA	(97)
4	NA	NA	Epileptic encephalopathy	NA	NA	Profound ID,	Hypotonia, impaired vision	c.173G>A; p.Trp58*	NA	(29)

Our review of the literature has led to the identification of 33 cases with pathogenic variants of DMN1, but only 30 of these are associated with epilepsy and some degree of ID (100, 103, 106–114). Brereton et al. (115) described some cases presenting a milder phenotype, characterized by autistic symptoms and ID without seizure. Clinical features of patients with DNM1-related DEEs are summarized in **Table 7**.

The onset of seizures occurred in almost all cases within the first year of life, and the most frequently reported seizures type is represented by infantile spasms (19/30). Noteworthy is the case of a patient who did not report further epileptic episodes despite presenting an onset with infantile spasms in the first year of life, developing a neurological impairment with profound ID (106).

All patients presented with severe/profound ID, in many cases (17/30) associated with the absence of verbal communication (100, 106, 107, 109). Also, two-thirds of the cases (22/30) showed deep axial and/or diffuse hypotonia and severe involvement of motor skills (100, 106–109, 111, 112).

It is noteworthy mentioning that the genetic variant c.709C>T (p.Arg237Trp) was found in 8 cases out of 30 (27% of patients identified) and that c.1075G>A (p.Gly359Arg) was reported twice (106, 108, 109, 111).

Data regarding the therapy was not available in 7/30 of the patient. Only four patients (4/30) were seizure-free, and in particular, two of them have achieved the absence of seizures following the ketogenic diet (106, 109, 114). In addition, another one obtained the absence of epileptic episodes for a long time following a ketogenic diet, although epilepsy recurred later (109).

ZFYVE20

The *ZFYVE20* gene, mapped on chromosome 3 (3p25 region), encodes for Rabenosyn-5 (Rbsn-5), a large, highly conserved, multidomain protein, which is ubiquitously expressed in mammalian cells. Rbsn-5 is involved in receptor-mediated endocytosis and neurotransmitter recycling (116, 117). Specifically, Rbsn-5 main function is to regulate the intracellular route of internalized neurotransmitters receptors, facilitating their recycling to the plasma membrane through direct interaction with regulatory proteins and lipids, such as the endocytic GTPases Rab4 and Rab5, and phosphatidylinositol-3 phosphate (116–118).

We reviewed recent literature and identified a single case in which a pathogenic variant of *ZFYVE20* is associated with a form of DEE (116). A girl with a homozygous missense mutation of *ZFYVE20*, detected by whole-exome sequencing, showed a severe drug-resistant epileptic encephalopathy and ID (116). She presented seizures onset at 5 months of life with infantile spasms and had marked hypotonus, with the impossibility of sitting and walking independently. The patient also presented facial dysmorphisms, microcephaly, macrocytosis and megaloblastoid erythropoiesis (116). She gained poor seizures control with several anticonvulsive drugs (VPA, Phenobarbital, Levetiracetam, Lamotrigine). At 14 months, a ketogenic diet was started with a report of improvement, and she was clinically seizure-free at 6.5 years of age (116).

TBC1D24

The *TBC1D24* gene, located on chromosome 16 (16p13.3 region), encodes for Tbc1d24, a highly conserved 553 amino acid protein, which consists of two domains, the Tre2/Bub2/Cdc16 domain (TBC) and a TBC/Lysin Motif Domain/Catalytic (TLDc) domain (119). The TBC domain is involved in the regulation of synaptic traffic, while the function of the TLDc domain is less known, but it appears to be involved in oxidative stress processes (119).

Tbc1d24 is involved in vesicle trafficking in the brain, neuronal migration, and somatic cellular development (120, 121).

TABLE 7 | Clinical features of *DNM1*-related patients.

No.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Other neurological features	Genetic variant	Reference
1	7 months	Head dropping	NA	Slow spike-wave, hypsarrhythmia	NA	Severe DD	NA	c.443A>G; p.Gln148Arg	(114)
2	11 months	FS	LGS	Hypsarrhythmia	NA	Severe DD	NA	c.127G>A; p.Gly43Ser	(108)
3	10 months	TS, head dropping	Drug resistant CS and AS, Abs	Hypsarrhythmia	Choreic hand movements and distal limb dystonia	Severe DD	NA	c.709C>T; p.Arg237Trp	(108)
4	7 months	IS	NA	Slow background, multifocal discharges	Ataxia, mild tremor	Severe ID; NV	Hypotonia	c.529G>C; p.Ala177Pro (*de novo*, missense mutation)	(109)
5	6 months	IS	NA	Hypsarrhythmia	NA	Severe ID	General hypotonia	c.618G>C; p.Lys206Asn (*de novo*, missense mutation)	(109)
6	2 months	IS	NA	Bilateral slow spike-wave	na	Severe ID; NV	General hypotonia	c.1076G>C; p.Gly359Ala (*de novo*, missense mutation)	(109)
7	13 months	IS	NA	Hypsarrhythmia	na	Profound ID	Axial hypotonia	c.194C>A; p.Thr65Asn (*de novo*, missense mutation)	(109)
8	12 months	IS	Myo, Atyp Abs, TS, FS, GTCS, obtundation status	Modified hypsarrhythmia	na	Profound ID	Axial hypotonia	c.709C>T; p.Arg237Trp (*de novo*, missense mutation)	(109)
9	NA	IS	LGS	NA	NA	ID	NA	c.618G>C; p.Lys206Asn	(103)
10	NA	IS	LGS	NA	NA	ID	NA	c.529G>C; p.Ala177Pro	(103)
11	7 months	IS	TS	Multifocal epileptiform discharges, hypsarrhythmia	Dyskinesia; Nystagmus	Profound ID		c.865A>T; p.Ile289Phe	(110)
12	4 months	IS	Myo, GTCS, FS	Bitemporal epileptiform activity	NA	Profound ID	Hypotonia	c.709C>T; p.Arg237Trp	(111)
13	12 months	TS	IS, Myo, GTCS, FS	Bilateral occipital epileptiform activity diffuse background slowing multifocal epileptiform discharges	NA	Profound ID	Hypotonia	c.709C>T; p.Arg237Trp	(111)
14	3 weeks	Myo	Abs	Slow background	NA	Profound ID, NV	Hypotonia	c.127G>A; p.Gly43Ser	(106)
15	8 months	GTCS	NA	Multifocal epileptiform discharges, slow background	NA	Profound ID, NV	Hypotonia	c.731G>A; p.Ser238Ile	(106)
16	3 months	NA	NA	Multifocal epileptiform discharges, slow background activity	NA	Profound ID, NV	Hypotonia	c.1075G>A; p.Gly359Arg	(106)
17	3 months	IS, Myo	Seizure freedom	Hypsarrhythmia, multifocal epileptic discharge	NA	Profound ID, NV	Hypotonia	c.1190G>A; p.Gly397Asp	(106)
18	4 months	IS	Abs, TS, GTCS, SE	Hypsarrhythmia, multifocal epileptic discharge, generalized sharp wave	NA	Profound ID, NV	Hypotonia	c.416G>T; p.Gly139Val	(106)
19	2 months	IS	Abs, TS, Myo	Multifocal epileptiform discharges	NA	Severe ID, NV	Hypotonia	c.616A>G; p.Lys206Glu	(106)

(Continued)

TABLE 7 | Continued

No.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Other neurological features	Genetic variant	Reference
20	6 months	IS	AS, GTCS	Hypsarrhythmia, slow spike-wave discharges, focal epileptiform discharges	NA	Severe ID; NV	Hypotonia	c.709C>T; p.Arg237Trp	(106)
21	3 months	IS	AS, GTCS, FS	Multifocal epileptiform discharges, slow spike-wave, slow background	NA	Profound ID, NV	Hypotonia	c.709C>T; p.Arg237Trp	(106)
22	5 months	IS	AS, Abs, GTCS	Hypsarrhythmia, multifocal epileptic discharge, generalized epileptic discharge, slow background	NA	Profound ID, NV	Hypotonia	c.709C>T; p.Arg237Trp	(106)
23	5 months	IS	Abs, Myo, AS, GTCS	Hypsarrhythmia, multifocal epileptic discharge, generalized spike wave	NA	Profound ID, NV	Hypotonia	c.709C>T; p.Arg237Trp	(106)
24	6 months	IS	Myo, TS	Hypsarrhythmia, multifocal epileptic discharge, focal epileptiform discharge, slow background	NA	Profound ID, NV	Hypotonia	c.1037G>T; p.Gly346Val	(106)
25	1 month	IS	Myo, TS, GTCS, FS, SE	Hypsarrhythmia, multifocal epileptic discharge, slow spike wave, generalized spike wave	NA	Profound ID, NV	Hypotonia	c.1075G>A, p.Gly359Arg	(106)
26	4.5 years	Fs	Myo, TS, GTCS, FS, SE	Generalized spike-wave, slow background	NA	Profound ID, NV	NA	c.1117G>A; p.Glu373Lys	(106)
27	1 day	Myo, TS	IS	Normal activity of background, irregular sharp waves and spike and waves complexes followed by attenuation	NA	Profound ID, NV	Hypotonia	Insertion c.1089_1090inscttcca in exon 8; p.asn363_arg364insleupro	(100)
28	5 days	NA	SE	Diffuse slowing and multifocal epileptiform activity	NA	ID, DD	Hypotonia	c.796C>T; p.Arg266Cys	(112)
29	8 months	IS	IS	Sharpe wave, multifocal epileptic discharge	NA	Severe ID, NV	Hypotonia	c.135C>A; p.Ser45Arg	(107)
30	4 months	GTCS	Abs, FS, GTCS	NA	NA	Severe DD	NA	c.431C>T; p.Pro144Leu	(113)

In particular, in the pre-synaptic terminal, it acts as a selective GTPase activating protein for the GTPase Rab35, which allows the endosomal sorting of synaptic vesicle proteins and the replacement of damaged components (122). Moreover, Falace et al. (120) demonstrated that Tbc1d24 binds the GTPase Arf6, proving in mouse models the role of *TBC1D24* in regulating neuronal migration.

Pathogenic variants of this gene are associated with heterogeneous clinical manifestations, including non-syndromic hearing loss and drug-resistant epilepsy, cerebellar alterations, alternating hemiplegia, and symptoms of neurodegeneration (119).

Epilepsy phenotypes associated with pathogenic variants of *TBC1D24* include familial infantile myoclonic epilepsy, epilepsy of infancy with migrating focal seizures (EIMFS) and DOORS (deafness, onychodystrophy, osteodystrophy, intellectual disabilities, and seizures) syndrome (123–125).

In the recent literature, we found the description of 30 cases in which mutations of *TBC1D24* are associated with DEEs (107, 123–131). All clinical features are summarized in **Table 8**.

All patients presented a history of early onset of seizures, ranging from 20 minutes after birth to 8 months of life: in the majority of the patients (28/30), the onset was within 3 months of

TABLE 8 | Clinical features of *TBC1D24*-related patients.

N.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Other neurological features	Genetic variant	Neuroimaging	Reference
1	3 months	GTCS, FS	Spasms in the craniofacial region and all of her limbs, as well as a concurrent, sudden decrease in vision manifested as lethargy and constant crying; NCSE	Theta and delta activity in bilateral hemispheres	Cerebellar ataxia	DD		c.1416_1437del/ c.1499C>T; p.Ala500Val	NA	(107)
2	5 weeks	Migrating CS, FS	EIMFS—deceased at 8 years	First interictal: slow background activity, with slow waves, rare paroxysmal activity; interictal stormy phase: multifocal spikes, slow background activity; ictal: focal theta discharge followed by delta large amplitude hemispheric discharge; interictal late phase: absence of any organization, rare spikes in both temporal regions; myoclonic seizures associated with EEG abnormalities (frequency range 0.25-1 Hz)	Dystonic movements	ID	Severe axial hypotonia	c.468C>A; p.Cys156*/c.686T>C (p.Phe229Ser)	moderate brain atrophy sparing the posterior fossa (at 6 months)	(125)
3	4 weeks	Migrating CS, FS	EIMFS—deceased at 18 months	First interictal: slow background activity, with slow waves, rare paroxysmal activity; interictal stormy phase: multifocal spikes low background activity, rare spindles; ictal: focal migrating discharges; interictal late phase: absence of any organization, rare spikes in both temporal regions; myoclonic seizures associated with EEG abnormalities (frequency range 0.25-1 Hz)	NA	DD	Severe hypotonia	c.468C>A; p.Cys156*/c.686T>C (p.Phe229Ser)	One month old: no structural brain abnormality; 9 months old: global brain atrophy (gray matter) sparing the posterior fossa	(125)

(Continued)

TABLE 8 | Continued

N.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Other neurological features	Genetic variant	Neuroimaging	Reference
4	2 months	CS, Myo	Early-onset epileptic encephalopathy—deceased at 3.5 years	Several waking-sleep EEGs were within normal limits in the early months of the disease; a progressive slowing of the background activity and a gradual regression in the phasic elements of sleep became evident in later records, as periods of waking and sleep became less distinctive, as well as rare and isolated small spikes and multiple spikes that were predominantly in the frontal and central regions	Dystonic episodes (from the second year of life)	ID	NA	chr16:2547714-2547715delGT; p.Ser324Thrfs*3	Diffuse delay in myelination and a thin corpus callosum (at 6 months); diffuse atrophy with dilatation of the cerebral ventricles, subarachnoid space, and brain sulci (at 2 years)	(125)
5	3 weeks	FS, Myo	Early-onset epileptic encephalopathy—deceased at 3.5 years	Monotonous background activity composed of medium voltage and irregular slow waves within theta and delta ranges; amplitude was lower on the right hemisphere	Spastic hemiparesis and dystonia on the left side	ID	NA	chr16:2547714-2547715delGT; p.Ser324Thrfs*3	Diffuse atrophy with right predominance, especially of right hippocampus (at 31 monts); areas of hypoperfusion in right frontal lower and middle, right mesial and lateral temporal, and left mesial temporal areas at brain SPECT	(125)
6	1 months	FS, IS	Early-onset epileptic encephalopathy—deceased at 6.5 years	Early EEGs were reported to have generalized and multifocal multiple spikes as well as spike-waves discharges	NA	ID	NA	chr16:2547714-2547715delGT; p.Ser324Thrfs*3	Progressive, diffuse cerebral and cerebellar atrophy with dilatation of the ventricles, sulci, and subarachnoid space (at 14 and 37 months)	(125)
7	2 months	CS, Myo, FS, GTCS	Early-onset epileptic encephalopathy	Delta rhythm with multifocal paroxysms	NA	ID	NA	c.32A>G; p.Asp11Gly	Brain atrophy (at 8 and 14 months)	(125)
8	2 months	FS, GTCS	Early-onset epileptic encephalopathy	Paroxysmal epileptiform discharges, bouts of intense crying considered ictal on EEG	Choreoatethoid movement, dystonia, spastic quadriplegia	ID	hypotonia	c.731C>T; p.Ala244Val	Elevated glutamine peak (MRI); cerebellar atrophy, volume loss in left frontal lobe, enlargement of temporal horns suggestive of bilateral hippocampal atrophy (CT)	(125)

(Continued)

TABLE 8 | Continued

N.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Other neurological features	Genetic variant	Neuroimaging	Reference
9	3 months	FS, CS, GTCS	Multifocal	Multifocal independent spike waves (at 13 years)	Ataxia, hand tremor, progressive gait deterioration	Clinical deterioration; NV	NA	c.679C>T; p.Arg227Trp/ c.1544C>T p.Ala515Val	Right hippocampal sclerosis, bilateral cerebellar atrophy, hyperintense signal of the cerebellar cortex (at 9 years)	(125)
10	45 min after birth	Myo, TS, CS, IS	Early-onset epileptic encephalopathy—deceased at 20 months	First interictal EEG recording (on first day of life) unremarkable despite frequent seizures; later ictal EEG showed generalized spike-wave and poly-spike discharges with F-C predominance; progression to burst-suppression before death	Dyskinetic movements with upper limb dystonia	ID	Axial hypotonia	c.1008delT; p.His336Glnfs*12/ c.32A>G; p.Asp11Gly	Normal	(125)
11	20 min after birth	Myo, TS, CS	Early-onset epileptic encephalopathy—deceased at 24 months	First interictal EEG recording (on first day of life) unremarkable despite frequent seizures; later generalized spike-wave and multiple spike-wave discharges with F-C predominance, slowing of the baseline activity and multifocal spikes	Dyskinetic movements with upper limb dystonia	ID	Axial hypotonia	c.1008delT; p.His336Glnfs*12/ c.32A>G; p.Asp11Gly	Prominent fronto-temporal atrophy with widening of the subarachnoid spaces and Sylvian fissures (at 1 month)	(125)
12	1 day	FS, Myo	Early-onset epileptic encephalopathy—deceased at 6 months	NA	NA	ID	NA	c.119G>T; p.Arg40Leu	Normal cranial ultrasound after birth	(125)
13	1 day	FS, IS	Early-onset epileptic encephalopathy—deceased at 10 months	Multifocal interictal epileptiform discharges (sharp waves, fast activity, spikes, polyspikes), disorganized and slow background, between 6 weeks and (at 8 months)	Nystagmoid eye movements	ID	NA	c.1460_1461insA; p.His487Glnfs*71/ c.313T>C; p.Cys105Arg	day 7: normal; day 56: increased T2 signal in left hippocampus, prominent extra-axial cerebrospinal fluid spaces	(125)
14	2 months	Apnea attacks	IS; SRSE	Hypsarrhythmia	Myoclonus	ID	NA	c.442G>A; p.Glu148Lys (maternal segmental UPiD of chromosome 16)	NA	(128)
15	2 weeks	FS	Deceased at 3 months	Multifocal seizure activity	Myoclonus	DD	NA	c.338C>A;p.Ala113Asp/ c.476T>C;p.Leu159Pro	Mild volume loss with minimal progression of myelination	(129)

(Continued)

TABLE 8 | Continued

N.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG	Movement disorders	ID/DD	Other neurological features	Genetic variant	Neuroimaging	Reference
16	first week	FS	Multiple SE–deceased at 4 years	NA	NA	severe DD	NA	c.338C>A;p.Ala113Asp/ c.476T>C;p.Leu159Pro	Mild brain atrophy	(129)
17	3 weeks	NA	Multiple SE—decease at 1 year	NA	NA	DD	NA	NA (sibling of patients 15 and 16)	NA	(129)
18	3 months	Myo, CS	NA	Slow background activity and rare sharp waves over the C regions of the left hemisphere (from 3 months); numerous spikes over the vertex and the C regions of both hemispheres prevalent on the left side (at 5 years).	NA	DD	NA	c.457G>A; p.Glu153Lys/ c.1142+1G>A	Hypotrophy of the posteroinferior regions of the cerebellum with mild cortical signal hyperintensities, and delayed myelination over the periventricular and temporal regions	(130)
19	15 days	Myo	Multifocal Myo	FAST RHYTHMS OVER THE FRONTAL REGION	NA	DD	NA	c.1499C > T; p.Ala500Val	Cranial magnetic resonance imaging at the age of 5 months revealed prominent sulci, subarachnoid enlargement, and a cavum septum pellucidum.	(131)
20	2 days	FS	Drug resistant epilepsy—SRSE which resulted in her death—deceased at 9.5 months	Increased delta rhythmic activity on the left hemisphere and infrequent multifocal acute waves	NA	DD	NA	c.121C>T; p.Gln41*	NA	(124)
21	2 months	FS	SRSE; drug-resistant epilepsy	Multifocal spikes	NA	DD	NA	c.121C>T; p.Gln41*/c.321T>A; p.Asn107Lys	NA	(124)
22	3 months	FS	Drug-resistant epilepsy	Rhythmic left-T theta activity with evolution to delta activity	NA	DD	NA	c.845C>G; p.Pro282Arg/c.919A>G	NA	(124)
23	3 months	FS	Drug-resistant epilepsy	Interictal diffuse background slowing without epileptiform discharges	NA	DD	NA	c.845C>G; p.Pro282Arg/ c.919A>G	NA	(124)
24	3 months	FS	Myoclonic epilepsy	Diffuse mild background slowing	Fatigue and gait ataxia; Parkinsonism	mIld ID	NA	c.404C>T; p.Pro135Leu/c.1078C>T; p.Arg360Cys	NA	(127)
25	1 month	FS, migrating CS/TS, IS	SE; frequent Myo	Multiple independent ictal foci in different regions	NA	DD	NA	c.404C>T; p.Pro135Leu/c.457G>T; p.Glu153*	NA	(126)
26	2.5 months	NA	EIMFS	NA	NA	DD	NA	c.116C > T; p.Ala39Val/c.1499C > T; p.Ala500Val	NA	(123)

(Continued)

TABLE 8 | Continued

N.	Age of onset	Seizure type at onset	Epilepsy evolution	EEG		Movement disorders	ID/DD	Other neurological features	Genetic variant	Neuroimaging	Reference
27	1.5 months	NA	EIMFS—deceased at 19 months	NA		NA	DD	NA	c.116C > T; p.Ala39Val/c.1499C > T; p.Ala500Val	NA	(123)
28	8 months	NA	Epileptic encephalopathy	NA		NA	DD	NA	c.116C > T; p.Ala39Val/ c.241_252del; p.Ile81_Lys84del	NA	(123)
29	7 months	NA	Progressive myoclonic epilepsy	NA		NA	DD	NA	c.241_252del; p.Ile81_Lys84del/ c.1153C > T; p.Gln385*	NA	(123)
30	3 months	NA	Progressive myoclonic epilepsy	NA		NA	DD	NA	c.241_252del; p.Ile81_Lys84del/ c.139A > G; p.Ser47Gly	NA	(123)

life, and, among them, 6/30 individuals developed seizures within the first week (107, 123–131).

The most commonly reported type of seizures is migrating focal ones (17/30), followed by migrating clonic ones (9/30), myoclonic ones (9/30), generalized tonic-clonic ones (4/30), epileptic spasm (4/30), tonic ones (3/30), and apnea attacks (1/30) (107, 123–126, 128–131). In six patients, the description of the seizures was not available (123, 129).

All patients developed a drug-resistant epileptic encephalopathy: five patients (5/30) showed epilepsy of infancy with migrating focal seizures (EIMFS) (123, 125, 126). In two cases, an evolution in progressive myoclonic epilepsy has been described, poorly controlled by drug therapy and often triggered by fever (123).

A non-convulsive super-refractory status epilepticus treated with midazolam, ketamine, and pentobarbital was reported in two cases (124). One of these patients was also treated with hypothermia and died at 9 years (124). Another patient presented with super-refractory status epilepticus treated with midazolam or thiamylal (128). Moreover, the patient described by Li et al. (107) presented with non-convulsive status epilepticus treated with intravenous injection of diazepam, while the patient reported by Lozano et al. (129) developed multiple status epilepticus and died at 1 year of age.

Overall, 13 patients (13/30) died at a very young age (ranging from 3 months to 9.5 years of age) (123–125, 129).

The EEGs did not show a typical pattern, and various abnormalities were described: focal epileptiform discharges in different regions of cerebral hemispheres were reported in seven cases (7/30) (124–126), while 8/30 patients showed generalized or and/or multifocal discharges (125). A diffuse background slowing was described in nine patients (9/30), despite the presence or absence of focal/multifocal discharges (124, 125, 127), while 3/30 initially presented EEGs within the normal limits (125). Lately, 5/30 patients showed a total absence of organization (125). One patient (1/33) developed a burst-suppression pattern (125) and one (1/30) presented with hypsarrhythmia at 4 months of age (128). In 8/30 cases, EEG data was not available (123, 125, 129).

All of them showed DD/ID, and in many cases, the expressive language was inadequate or dysarthric (107, 123–126, 128–131).

In one case, a young woman with an early onset of myoclonic epilepsy showed movement disorders (1/30) with cerebellar ataxia and fatigue, parkinsonism and symptoms of psychosis with hallucinations and depression (127). Other reported movement disorders were Dystonic movements (6/30), cerebellar ataxia (2/30), dyskinetic movements (2/30), non-epileptic myoclonus (2/30), spastic hemiparesis (1/30), choreoatethoid movements (1/30), spastic quadriplegia (1/30), nystagmoid eye movements (1/30) (107, 124–126, 128, 129). In 7/30 cases, the description of movement disorders and other additional features was not available (123, 130, 131).

Most of the patients (29/30) developed an epileptic encephalopathy with frequent seizures non-responsive to various therapeutic approaches and ketogenic diet therapy (107, 123–125, 128–131). Fang et al. (126) described a single patient that experienced >50 % seizure reduction after diazepam treatment. The ketogenic diet was adopted in two

cases (123, 124), causing a reduction of seizures and cognitive improvement after a year and a half of diet in one patient (123). In one case, a description of the therapeutic response was not available (127).

DISCUSSION

DEEs are a heterogeneous group of conditions with onset in infancy or early childhood, in which the epileptic activity significantly interferes with the development, determining severe DD/ID and other neuropsychiatric disorders (9, 10). These diseases may be caused by an alteration of the synapse, the fundamental unit of signal transmission in the nervous system (1, 3). Numerous genes contribute to the synaptic transmission's proper functioning, and alterations of this complex mechanism may result in synaptopathy (1, 8).

We reviewed the literature, focusing on those genes involved in the correct operation of the pre-synaptic terminal, and analyzed the clinical features of 119 patients that showed a clinical presentation resembling a DEE.

A valid genotype-phenotype correlation is difficult to deduce especially in those reports including only few patients with DEEs (50, 79, 116). On the contrary, when many patients with pathogenic variants of the same gene present with different phenotypes, a clear correlation is difficult to achieve: this is even truer in those cases in which the same mutation is related to various clinical presentations (58, 63, 64, 82, 87, 88). This phenotypic heterogeneity has been long studied, and it is related to several factors intervening during the development, including epigenetic factors, timing and location of physiological gene expression and modifier genes (9).

Nevertheless, some noteworthy features may be underlined regarding the clinical phenotypes of DEEs related to the genes we reviewed, which can lead the clinician to a genetic suspect.

For instance, patients with *VAMP2* pathogenic variants showed a neurodevelopmental disorder characterized by ID, central visual impairment, movement disorders, epilepsy or electroencephalographic abnormalities, autistic features, and loss of purposeful hand movements resembling Rett syndrome (16).

STX1B-related DEE must be hypothesized when a patient presents with myoclonic seizures at onset (35, 40–42). Subsequently, these patients may manifest ataxia (35, 37, 39).

Patients carrying *DNM1* mutations may present with infantile spasms in more than a half of the cases, usually associated with axial and/or diffuse hypotonia with severe impairment of motor skills (100, 106–109, 111, 112).

Almost the totality of the patients with a pathogenic variant of TBC1D24 present the first seizure within 3 months after birth, with severe progression frequently leading to death within the first decade of life (107, 123–131). Although EIMFS was reported as a typical epileptic phenotype related to *TBC1D24*, in our review, we could identify 5/30 (16%) patients that developed this type of DEE (123–126). Instead, noteworthy mentioning is the association with status epilepticus (107, 124, 128, 129).

Usually, patients with *SNAP25*-DEEs show seizure's onset after 2 years of age, with generalized seizures and a frequent association to movement disorders (15, 44–47), while *NAPB*-associated DEEs are characterized by a high frequency of clonic seizures and an evolution in multifocal epileptic encephalopathy (97, 99).

Overall, when a patient presents with some degree of DD, an early epilepsy onset with drug-resistant seizures, possibly associated with a movement disorder, the suspect of a synaptopathy must be taken into account when approaching the differential diagnosis.

Concerning the therapeutic approaches, an individually-tailored treatment is desirable to intervene directly on the altered mechanism determining the DEE, improving the seizure control and the developmental outcome. However, in most severe epilepsies, a gene-specific therapy is not available, and the treatment options are represented by the usual AEDs, that do not address the underlying causative mechanism (9). Our literature review regarding DEEs related to genes involved in pre-synaptic mechanisms confirms these data: most patients showed a bad prognosis with highly drug-resistant seizures, despite the multiple therapeutic combinations. Moreover, literature data suggested the efficacy of the ketogenic diet in part of the patients with DEEs related to *DNM1*, *TBC1D24*, and *ZFYVE2O*. In particular, two subjects with pathogenic variants of *DNM1* achieved the absence of seizures, and another one obtained the absence of epileptic episodes for a long time, although epilepsy recurred later (106, 109). The ketogenic diet was also considered as a valid therapeutic approach for seizure control in two cases related to *TBC1D24*, and especially one of these patients showed a decrease in the frequency of seizures and cognitive improvement after a year and a half of diet (123, 124). Finally, the girl with a disease-causing variant of *ZFYVE2O*, after several AEDs trials, started a ketogenic diet, obtaining a gradual improvement and seizure freedom at 6.5 years of age (116).

This work's limitation is the paucity of a complete description of the patients, making it difficult to obtain homogenous information and, therefore, to deduce a clear genotype-phenotype correlation. A more detailed clinical description of the patients may be desirable to improve the genotype-phenotype correlation and better guide the choice of the genetic testing, allowing to obtain an early diagnosis and to develop individually-tailored therapies.

AUTHOR CONTRIBUTIONS

AN and GD conceived planned and supervised the study. GS, GV, and MS wrote the first draft of the manuscript and prepared the tables. GS prepared the figure. AB, GA, and VS helped supervise the project. All authors contributed to manuscript revision, read, and approved the submitted version.

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A Treatable Genetic Disease Caused by CAD Mutation

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Type 50 early infantile epileptic encephalopathy, or EIEE-50 for short, is an autosomal recessive genetic disorder resulting from CAD mutations. So far, little has been reported on the disease. In this article, we will discuss the case of a male infant who is 8 years and 5 months old. A whole-exome sequencing of the boy revealed CAD compound heterozygous mutations. He suffered from global developmental delay and regression, refractory epilepsy, and anemia. After his diagnosis, we used uridine treatment and gained encouraging results. In this article, we will analyze our case studies in the context of the literature, so as to improve pediatricians' understanding of the disease.

Keywords: early infantile epileptic encephalopathies, CAD, treatment, uridine, genetic disease

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INTRODUCTION

With the continuous development of precision medicine, we can reach the stage of molecular diagnosis of disease. Sadly, however, progress in identifying the molecular basis has not always translated into effective treatments (except in a few cases including EIEE-50). EIEE-50 is an autosomal invisible genetic disorder caused by a CAD mutation that encodes a multifunctional enzyme complex involved in *de novo* pyrimidine biosynthesis (1). When a CAD gene mutates, it will cause a nascent barrier to pyrimidine synthesis. In normal cells, the activation of the *de novo* pyrimidine synthesis pathway is essential for satisfying the nucleotides required for DNA and RNA replication. When this pathway is destroyed, not only will the biosynthesis of pyrimidine be impaired, but the level of glycosylated precursor will also be reduced, which will seriously affect the normal proliferation and metabolism of cells (2).

The clinical manifestations of EIEE-50 can be categorized into three main signs: global growth retardation or degeneration, refractory epilepsy, and anemia with anisopoikilocytosis (3, 4). In addition, some patients may suffer from optic nerve damage (5). This disease can be cured at an early stage with oral uridine. Otherwise, if left untreated, the disease is usually fatal.

CASE REPORT

An 8-year-plus-5-month-old boy was hospitalized with refractory epilepsy. He was born at full term without perinatal abnormalities. His parents and little sisters were healthy, and the parents did not marry close relatives. Unluckily, the boy had slow motor function development. It was not until 12 months before he could sit alone and 30 months before he could walk. Later, when he was 3 months old, it was discovered that he had anemia and his hemoglobin levels fluctuated between 69 and 110 g/L without a definite diagnosis. He was treated with red blood cell transfusions and immunoglobulin. After 9 months, he underwent surgery for left hydronephrosis.

The seizures began with a 2-year-old febrile seizure. When he was 7 years and 4 months old, the seizures gradually developed into afebrile seizures, namely, generalized tonic–clonic convulsion

lasting 1–2 min and occurring two-to-three times a day. Two months later, he was treated with an antiepileptic drug (valproic acid). After relapse, the level of valproic acid increased to 25 mg/kg/day. However, there was no significant remission, so another antiepileptic drug was added (perampanel). When he was 8 years and 4 months old, he tried two antiepileptic drugs, but the frequency of epileptic seizures did not decrease—up to 20 attacks a month. Meanwhile, his cognitive and motor functions were under observation. He could not eat, sit, stand alone, or communicate with others, and he had hypersomnia. As a result, he had to accept further examination.

When the boy was 8 years and 4 months old, he began to suffer from electroencephalogram (EEG) with multiple spikes and spike-and-wave discharges, and persistent low voltages were also detected at some electrodes (**Figures 1A,B**). Due to developmental delay and corpus callosum aplasia, his first magnetic resonance imaging (MRI) was taken when he was 10 months old. Therefore, a combination of mNGF (mouse nerve growth factor) and rehabilitation was used. mNGF was extracted from the mouse submandibular gland, which could promote the survival, growth, differentiation, and regeneration of peripheral nerve and central neuron (6). An MRI examination 7 months later revealed mild enlargement of the left lateral ventricle and abnormal signal in the bilateral ventricles, suggesting a delayed formation of the myelin sheath in the periventricular white matter. Also, when the boy was 8 years and 4 months old, the MRI showed a large cisterna occipitalis with an enlargement of the left lateral ventricle (**Figures 1C,D**). Routine blood tests indicated the anemia that persisted during his hospitalization.

Family-based whole-exome sequencing (WES) and Sanger sequencing were given to the boy and all his family members; compound heterozygous variants in the CAD gene were identified in the proband. A missense (c.2342G>C: p.R781P) variant came from the proband's mother, and the other missense (c.5998T>A: p.S2000T) variant came from the proband's father (**Figures 2A,B**). According to the guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology, both variations were interpreted as potentially pathogenic variants associated with EEIE-50.

During hospitalization, the boy received nasogastric tube feeding, red blood cell transfusion, intravenous potassium, and other supportive therapy. Furthermore, he was treated with an oral uridine dose of 100 mg/kg/day. After jabbing oral uridine, the boy was surprisingly better; he was able to sit alone for a few seconds, his mental status improved, and no more seizures were observed. Things were not always good; he was still unable to eat, walk alone, or communicate with others. A follow-up EEG examination after uridine treatment was rejected.

DISCUSSION

EIEE is a refractory form of epilepsy that begins in infancy and is thought to be associated with a variety of causes, including hereditary, metabolic disorders, brain abnormalities, and brain damage. So far, nearly 100 genes related to EIEE have been identified, which can be roughly divided into ion channel genes, neurotransmitter receptor genes, and other genes related to cell metabolism and signal transduction. Most of these diseases cannot be cured with several exceptions. Recently, a new treatable category of EIEE has attracted public attention, namely, EIEE-50, caused by mutations in the CAD gene (7). Without timely treatment, this disease is progressive and fatal. However, a growing number of reports claim that there are several patients who have successfully relieved their symptoms thanks to oral uridine (3, 4).

CAD, which is located on chromosome 2 (2p23.3), encodes a highly conserved multifunctional enzyme complex related to the synthesis of *de novo* pyrimidine. This complex consists of three parts, including carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (1) (**Figure 3**). The more conserved the amino acids are in the evolution of the species, the more important amino acid sites are for maintaining protein function. A comparison between the multi-sequence alignment of CAD and amino acid sequences revealed that amino acid site 129 was conserved in these species, including *Homo sapiens*, *Macaca mulatta*, *Canis lupus familiaris*, *Bos taurus*, *Mus musculus*, and *Xenopus tropicalis*. Mutations in conserved proteins make them vulnerable to disease.

EIEE-50 is a newly discovered disease in recent years since 2015, when Ng et al. reported the first one. In that case, the patient was diagnosed with pandisylase deficiency at 6 months and with renal tubular acidosis at 1 year. When he was 1 year and 5 months old, he had seizures and fine motor and language problems, and laboratory auxiliary tests suggested that he had anemia with anisopoikilocytosis. Finally, at the age of 4, he was admitted to the National Institute of Health (NIH) Medical Center and diagnosed with EIEE-50 by WES (8). In 2017, four children with EIEE-50 from three different families were introduced by Koch and his colleagues. Of these four patients, two were siblings and their parents were close relatives. The four children showed similar clinical features, including global developmental delay or regression, refractory epilepsy, and anemia with ischemic polycythemia. In addition, one of them had strabismus, but the Koch study did not directly connect strabismus to EIEE-50 (3). In 2019, a team led by Zhou presented the first Chinese patient with EIEE-50, whose clinical manifestations were consistent with previous studies. Later, this team reported another two cases of EIEE-50 in mainland China. On the basis of confirming the original clinical findings, they found that the children with EIEE-50 also had optic nerve damage caused by optic nerve transmission block. This finding was consistent with Koch's observations and had improved the clinical features of EIEE-50 (4, 5). Subsequent reports on the clinical manifestations of this disease were coherent with previous studies (9). In our case, the child's clinical features were in line with those previously published. The child endured anemia when he was 3 months old and febrile convulsion when he was 2 and gradually suffered from non-febrile convulsion that could not be well controlled by two antiepileptic drugs. The child had a history of backwardness. Prior to admission, he showed significant developmental regression. In our case, however, we did not find any visual impairment in the child, nor did we do any tests to confirm whether the child had optic nerve damage.

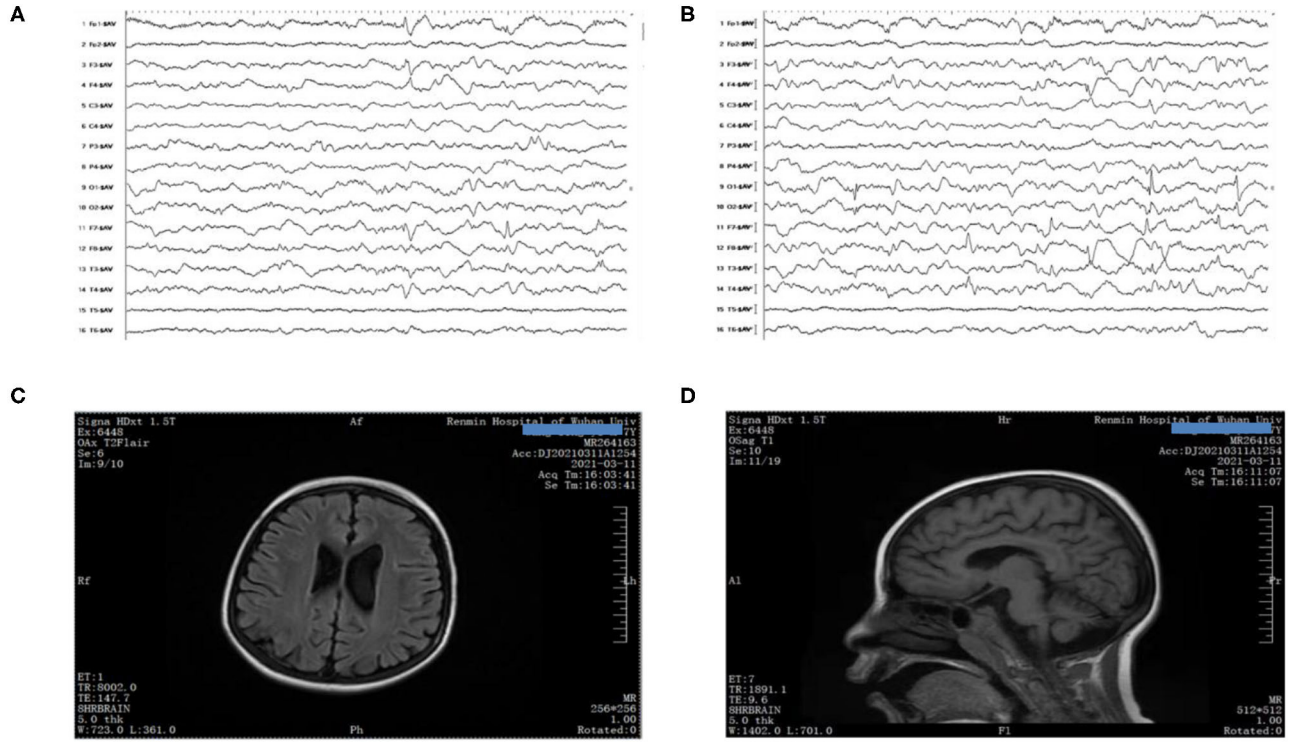

FIGURE 1 | (A,B) EEG showed poor background with multiple spikes and spike-and-wave discharges. **(C,D)** Brain MRI presented mega cisterna magna, together with enlarged left lateral ventricle.

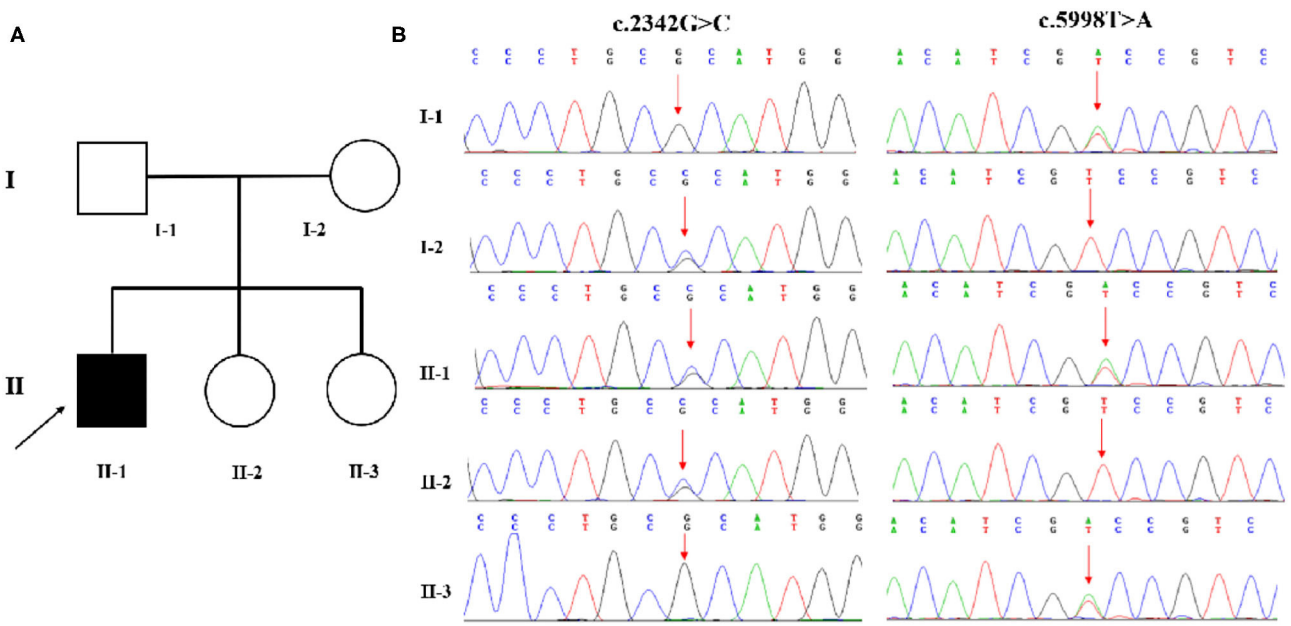

FIGURE 2 | (A,B) WES identified two variations in CAD. A missense (c.2342G>C: p.R781P) variant was inherited from the proband's mother, and the other (c.5998T>A: p.S2000T) variant came from his father.

EEG, brain MRI, and blood routine examinations are the auxiliary examinations of the essence of EIEE-50. They have some regularity, but they are far from enough to locate EIEE-50. Previous research found that an abnormal background rhythm with epileptic-like discharge was a common feature of EEG (3, 10). In our case, the situation was the same. Our patient

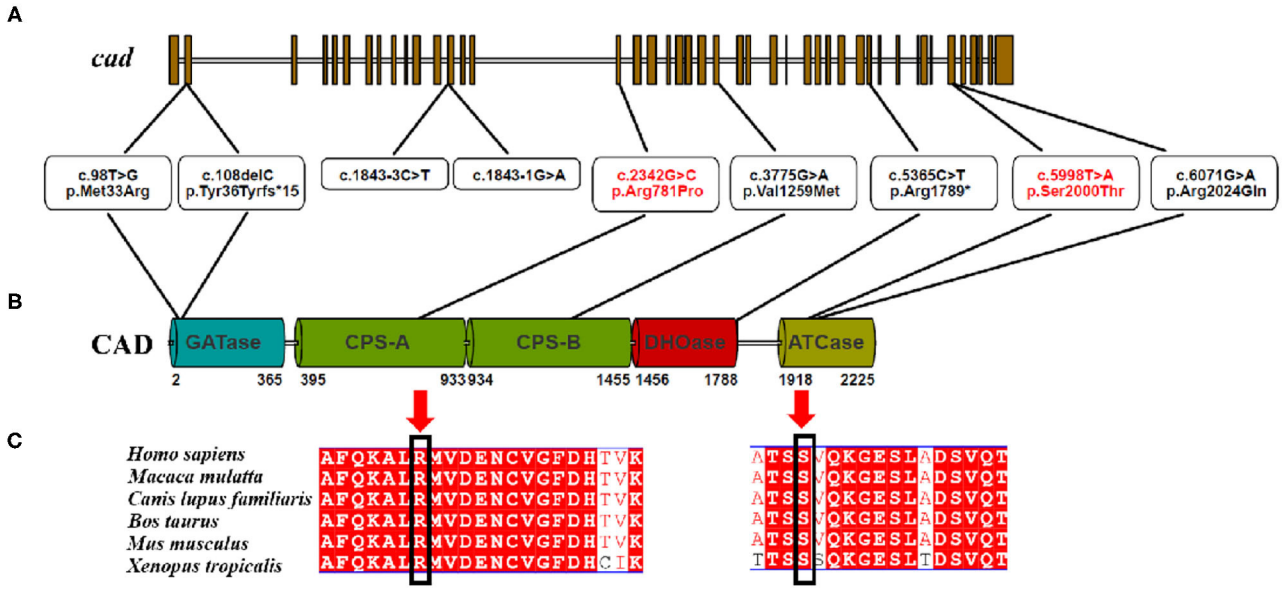

FIGURE 3 | (A–C) CAD is evolutionally conserved among different species.

showed persistent low voltage on some electrodes. Beyond that, multifocal spikes and spike-and-wave were also detected. Based on our understanding, such a kind of EEG reflects the state of epileptic encephalopathy, which is not the unique feature of EIEE-50. As for craniocerebral MRI, it could be normal in the early stages. With the development of the disease, many cases appeared with global brain atrophy, suggesting that the lesions in the brain may be continuous. Although previous articles have shown that brain atrophy occurs in children as early as 3.5 years of age, our case did not show the sign. A routine blood examination ordinarily shows moderate anemia with anisopoikilocytosis. In our report, anemia was the predominant symptom in this child.

The above-mentioned means are important detecting procedures, though the specificity is not enough. This reminds us that we need to consider the possibility of EIEE-50 combined with the results of auxiliary examination in clinical cases of children with refractory epilepsy, developmental delay, and anemia. Undoubtedly, the definite diagnosis of this disease depends on WES.

CAD mutation is the pathogenesis of this disease, and the main function of CAD is to participate in the biological synthesis of pyrimidine, serving as compensatory substances to promote the formation of pyrimidine in a therapeutic approach. Uridine can be ingested and phosphorylated by cells, which provides a mechanism to overcome the defects of pyrimidine nucleoside, hence serving as an irreplaceable drug for the treatment of EIEE-50. On the basis of the information available, uridine has been successfully applied to deal with EIEE-50. Uridine was first used for EIEE-50 in 2015, in which case the child was treated with oral uridine but the results were not described in detail (1). It was not until 2017 that Koch and his teammates elaborated on the effects of uridine on EIEE-50. Of the four children according to his article, two died at 4 and 5, respectively, because they did not receive timely treatment. The other two who received oral uridine had their seizures stopped, and their conditions improved significantly (3). Since 2019, Chinese researchers have begun to recognize and treat EIEE-50, as favored by the following results (4, 5). Since then, clinicians around the globe have become more aware of EIEE-50 and have increased the use of uridine. In our case, uridine was given at a dose of 100 mg/kg immediately after diagnosis, and no significant side effects were observed. A week later, the seizures stopped, and the boy made more eye contact with others. He could sit alone for a few seconds, though he could not stand, eat alone, or talk to others. After a month of taking oral uridine, his mother told us that he could eat and sit alone but still could not walk alone.

CONCLUSION

EIEE-50, caused by a CAD mutation, will lead to intractable epilepsy, developmental delay or regression, and anemia in children. Some patients may also suffer from impaired optic nerves. Normally, EEG shows the change of background rhythm and epileptiform discharge. Early craniocerebral MRI may show normal or mild abnormalities. As the disease progresses, brain atrophy may occur. Uridine (with a dosage of 100 mg/kg/day) is an effective drug for the treatment. The children who were treated with uridine clearly improved, and the remission of symptoms was closely related to the course of the disease. Genetic testing is a tool to confirm this disease. EIEE-50 is one of the

few genetic diseases that can be treated, and the results are favorable, highlighting the importance and value of precision medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XP and BY drafted the article. L-pX and H-jZ helped in correcting the mistakes. XP, JZ, S-qY, SW, Y-mX, and BY were responsible for collecting the dates. JY did great jobs in the revision process. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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KCNQ2-Related Neonatal Epilepsy Treated With Vitamin B6: A Report of Two Cases and Literature Review

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Potassium Voltage-Gated Channel Subfamily Q Member 2 (KCNQ2) gene has been initially associated with "Benign familial neonatal epilepsy" (BFNE). Amounting evidence arising by next-generation sequencing techniques have led to the definition of new phenotypes, such as neonatal epileptic encephalopathy (NEE), expanding the spectrum of KCNQ2-related epilepsies. Pyridoxine (PN) dependent epilepsies (PDE) are a heterogeneous group of autosomal recessive disorders associated with neonatal-onset seizures responsive to treatment with vitamin B6 (VitB6). Few cases of neonatal seizures due to KCNQ2 pathogenic variants have been reported as successfully responding to VitB6. We reported two cases of KCNQ2-related neonatal epilepsies involving a 5-year-old male with a paternally inherited heterozygous mutation (*c.1639C>T*; p.Arg547Trp), and a 10-year-old female with a *de novo* heterozygous mutation (*c.740C>T*; p.Ser247Leu). Both children benefited from VitB6 treatment. Although the mechanisms explaining the efficacy of VitB6 in such patients remain unclear, this treatment option in neonatal-onset seizures is easily taken into account in Neonatal Intensive Care Units (NICUs). Further studies should be conducted to better define clinical guidelines and treatment protocols.

Keywords: KCNQ2, neonatal epilepsy, vitamin B6, pyridoxine, pyridoxal 5 phosphate, pyridoxine-dependent epilepsy (PDE), pyridoxine-responsive epilepsy

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INTRODUCTION

Potassium Voltage-Gated Channel Subfamily Q Member 2 (KCNQ2) gene, first described by Singh et al. (1), is located on chromosome 20q13.3 and encodes for the voltage-gated potassium channel subunit Kv7.2. It has been traditionally related to "Benign familial neonatal epilepsy" (BFNE). This clinical entity often occurs within the first 2 weeks of life and is characterized by tonic/clonic early-onset seizures with apneic episodes and autonomic manifestations. Generally, it presents a self-limiting course for weeks/months with a regular neurodevelopmental outcome (2–4).

Amounting evidence arising from innovative genetic testing, and particularly next-generation sequencing (NGS) techniques, has revealed that different pathogenic variants of the same gene are responsible for several epileptic phenotypes; in particular, KCNQ2-related neonatal epileptic

encephalopathy (NEE) contributed to expanding the spectrum of KCNQ2-related epilepsies (5). This is a severe phenotype, with an onset during the first days of life of intractable seizures (usually focal tonic), variably evolving into seizure freedom or a worsening/relapsing of the epileptic attacks over time, and an inevitable, though variable, adverse neurodevelopmental outcome (4–6). The electroencephalogram (EEG) may show a burst-suppression pattern or multifocal epileptiform abnormalities with background attenuation. This phenotype is mostly due to *de novo* missense variants of KCNQ2 causing a dominant-negative effect leading to a loss or a reduction of the M-current (a slow activating non-inactivating potassium current modulating the resting membrane potential) and less commonly to a gain of function effect, but not to a loss of function (haplo-insufficiency) (4, 7–9).

First described by Hunt et al. (10), pyridoxine (PN) dependent epilepsies (PDE) are a heterogeneous group of autosomal recessive disorders associated with neonatal-onset seizures not well-controlled with anti-epileptic drugs (AEDs) but responsive to large daily doses of vitamin B6 (VitB6) (11). PDE are caused by inborn errors of PN metabolism, usually due to pathogenic variants of ALDH7A1 gene (antiquitin). ALDH7A1 encodes for the alpha-aminoadipic semialdehyde (a-AASA) dehydrogenase, a key enzyme in the lysine metabolism, eventually implicated in the activation of VitB6 (12). Moreover, pyridoxamine5'-phosphate oxidase (PNPO) and pyridoxal 5′-phosphate binding protein (PLPBP) genes [both involved in the homeostasis of the active form of PN, namely pyridoxal 5′-phosphate (PLP)], have recently been related to variants of PDE more responsive to PLP than PN (13, 14).

The phenotypic spectrum associated with ALDHE mutation include: (1) Classic PDE-ALDH7A1 (with a dramatic early onset of prolonged or recurrent seizures of different types); (2) Atypical PDE-ALDH7A1 (including a late-onset, or seizures initially responding only to AEDs and eventually controllable only with PN/PLP several months later, or folic acid-responsive seizures, anyhow associated with variable degree of neurocognitive outcomes) (15). Clinical diagnosis is based on the demonstration of seizures ceasing after PN treatment, even after the elimination of all AEDs, and re-occurrence after its withdrawal (11, 16).

In addition to genetic testing, elevated levels of a-AASA in plasma and urine, and of pipecolic acid in plasma, urine and cerebrospinal fluid (CSF), as well as a high plasma-to-CSF PLP ratio, can be suggestive for VitB6 disorders (17, 18). However, a clear interpretation of these results is complicated; in fact, to date, no studies on CSF PLP levels in healthy newborns or infants are available, hence reference ranges derive from neurologically abnormal children undergoing CSF metabolite measurement for diagnostic workup (18).

Interestingly, PN and PLP have been proven to be effective in several early-onset seizures, not meeting the diagnosis of PDE, but instead falling within the category of "Pyridoxine-responsive epilepsy" (PRE) (19).

In this regard, they have been increasingly used in the clinical practice as add-on treatments in early-onset epilepsies, and it should be noted that few reports are today available specifically on KCNQ2-related neonatal epilepsies trialed with VitB6 (either in its inactive or active form) with variable outcomes (**Table 1**). Herein, we reported two new cases of KCNQ2-related neonatal epilepsy who benefited from VitB6 treatment.

CASE REPORTS

Case 1

A 6-year-old Caucasian boy was born at term by eutocic vaginal delivery from an uneventful pregnancy. Birth weight was 2,870 gr. Perinatal period was unremarkable and physical examination at birth normal. He was the second child of healthy related parents with common ancestors. The patient's family history was positive for epilepsy both in the maternal and paternal line; in particular, the father was diagnosed with a KCNQ2-related-epilepsy treated with Valproate (VPA) and Phenobarbital (Pb). It is to note that the father referred this data only at the end of our patient's genetic investigation.

On the second day of life, the patient presented clonic seizures at the limbs. The episodes resolved spontaneously. On the fourth day, he was hospitalized for the re-occurrence of seizures associated with perioral cyanosis. An electroencephalogram (EEG) showed polyspike wave complexes. Given the persistence of continuous clonic seizures, associated with perioral cyanosis, revolving eyes and buccal automatisms (sucking), midazolam by continuous intravenous infusion and intravenous boluses of Pb were administered. Brain magnetic resonance imaging (MRI) was normal. On the 10th day of life, oral Pb treatment was started, with seizure control for almost 3 months. Meanwhile, metabolic tests and the sequencing of KCNQ2 gene were performed.

At 3 months of life, recurrent convulsive seizures/status epilepticus occurred; thus, VPA was started at 10 mg/kg/bid, with subsequent titration up to 30 mg/kg/bid with transient remission of seizures. About a month later, focal clonic seizures at the limbs appeared, mainly with deviation of the eyes and cyanosis, lasting 60 s. An EEG showed "slight abnormalities of electrical brain activity in the left posterior areas." Further brain MRI scans were normal. Given the age of the patient and the partial response to stabilized treatment with VPA (100 mg/bid) and intravenous Pb (7.5 mg/bid), we decided to start PN supplementation as an intramuscular formulation of the vitamin B complex, containing 100 mg/day of PN. Seizure cessation and disappearance of the EEG abnormalities were gained after few days of treatment. Maintenance treatment was carried on, in agreement with the patient's parents (who were hesitant about PN withdrawal, as initially planned), with an oral formulation of multivitamin B complex at a very low dosage of 5 ml/day, equivalent to 0.45 mg of PN, with a seizure-free interval of about a month. In the meanwhile, a Developmental Quotient (DQ) of 70 was measured by using the Brunet-Lezine developmental scale, detecting a neurodevelopmental delay. The next month, in concomitance to an infectious episode, he presented with a further epileptic seizure characterized by clonic movements of the limbs, revolving eyes, perioral cyanosis, lasting about 60 s and resolving spontaneously. Antiepileptic treatment was unchanged. No further seizures occurred thereafter, either during infectious episodes.

TABLE 1 | Patients described in the literature who carry a KCNQ2 mutation and have been trialed with vitamin B6.

References	Patient	Phenotype	Mutation	Sex	Perinatal and early history	Seizure onset	Seizure features	AEDs administered (response)	Type of vitamin B6/Response	EEG	Neuroimaging	Seizure and clinical outcome	Additional information
Our patients	1	Pyridoxine responsive epilepsy	c.1639C>Tp.Arg547Trp Paternally inherited heterozygous mutation	M	Normal	2 d	Clonic szs, perioral cyanosis, revolving eyes and buccal automatisms	MDL (acutely effective) PB, VPA (partially effective)	PN i.m.100 mg/d (later switched to oral maintenance therapy)/ Successful electro-clinical response	Initial EEG: Abnormal After PN initiation: Normal	MRI (4 d and 4 mo): Normal	Sz-free after PN start Today (6 ys): still sz-free; global DD; mild autism-like features	ALDH7A1 sequencing: negative. No further genetic testing was performed
	2	Pyridoxine responsive epilepsy	c.740C>Tp.Ser247Leu *De novo* heterozygous mutation in exon 5	F	Normal	2 d	Myoclonic szs with rolling eye movements	PB (acutely ineffective), Several AEDS at onset (ineffective), VGB, FA (in addition to PLP—effective), CBZ (maintenance therapy)	PN/ Unsuccessful PLP (start at 36 d, p.o. 500 mg/d in 4–6 doses)/Immediate sz control up to 18 mo	Initial EEG: Abnormal After PN initiation: Abnormal	MRI (2 d): Normal	Sz-freedom for 18 mo (after PLP start at 36 d) Today (10 ys): discrete sz-control	ALDH7A1 and PNPO sequencing: normal
Millichap et al. (20)	3	EOEE	c.1009G>A p.Ala337Thr	NR	NR	5 mo	NR	LEV, CBZ, CLB, CLZ, FLB, PB, VPA (NR) EZO (szs began to decrease by the second week on 13 mg/kg/d)	PN/ Unsuccessful	Initial EEG: Abnormal After EZO initiation: Normal	NR	Sz decrease after EZO initiation Abnormal development	Sz proved refractory to multiple AEDs and PN as well
Sands et al. (21)	4–5 (twins)	BFNE	c.1057C>G p.Arg353Gl	F	Born at 34 wks	2 d	Focal asymmetric tonic posturing, associated with apnea and desaturation	PB i.v. 40 mg/kg, CLN p.o., DZP i.v. (NR) CBZ p.o. 10 mg/kg/day (effective)	PN i.v.100 mg/ Unsuccessful	EEG (patient 4): Normal EEG (patient 5): Abnormal	MRI: Normal	Both sz-free off meds at 16 ys Normal development	Family history positive for neonatal szs
Mulkey et al. (22)	6	NEE	c.601C>T p.R201C	NR	Normal	1 d	Exaggerated and sustained startle reaction to touch	EZO, VGB, CLZ, FA (NR)	PN, PLP/ NR	Initial and 1 mo EEG: Abnormal	MRI (1 wk and 3 mo): Abnormal	Sz outcome NR Profound DD Died at 13 mo for cardio-pulmonary arrest	Early neurologic exam: Abnormal
	7	NEE	c.601C>T p.R201C	NR	Normal	1 d	Exaggerated and sustained startle reaction to noise/touch, apnea Infantile spasms at 4 mo	EZO, VGB, PB, VPA, PHT, FA, TPM, LEV, ZNS, LOC, CBZ, STM, KD, CBD enriched cannabis (NR)	PN, PLP/ NR	Initial and 1 mo EEG: Abnormal	MRI (1 wk): Abnormal	Sz outcome NR Profound DD	Early neurologic exam: Abnormal
	8	NEE	c.601C>T p.R201C	NR	Normal	2 d	Stiffening events	VGB, PB, TPM, ACTH, KD, LEV, CLZ, FA (NR)	PN, PLP/NR	Initial and 1 mo EEG: Abnormal	MRI (1 wk): Normal MRI (1 y): Signal anomalies	Sz outcome NR Profound DD	Early neurologic exam: Abnormal
	9	NEE	c.601C>T p.R201C	NR	Born at term: bradycardia prior to delivery via caesarian section	1 d	Exaggerated startle response, apnea	EZO, VGB, CBZ, CLZ, PB, FA, PHT (NR)	PLP/NR	Initial and 1 mo EEG: Abnormal	MRI (2 and 4 mo): Abnormal	Sz outcome NR Profound DD Deceased	Early neurologic exam: Abnormal
	10	NEE	c.602G>A p.R201H	NR	Normal	1 d	Myoclonic spontaneous movements, exaggerated startle to noise/ touch Infantile Spasms at 2 months	VGB, CBZ, ZNS, PB, FA (NR)	PLP/NR	3 mo: Abnormal	MRI (3 mo): Abnormal	Sz outcome NR Profound DD	Early neurologic exam: Abnormal
Sharawat et al. (23)	11	NEE	c.835G >A p.Gly279Ser Likely pathogenic heterozygous Missense variant in exon 6	M	Normal	7 d	Repeated szs with up-rolling of eyeballs, generalized stiffening of body and cry	PB (sz-free for 3 months in combination with PN) CBZ 20 mg/kg/d (sz-free within a week)	PN started at 15 mg/kg/d, later increased to 50 mg/kg/d/Partial response	Initial EEG: Abnormal Repeat EEG: Normal	MRI (28 d): Normal	Sz-free at 3 mo (after PN start) 5 mo: re-occurrence of 1–2 sz/mo 1 y: re-occurrence of 8–10 sz/d Sz-free at last follow-up and mild global DD	Partial and temporary response to PN. Sz-freedom achieved with CBZ and PN together
Spagnoli et al. (24)	12	NEE	c.913_915del p.Phe305del *De novo*	M	Born full-term Apgar Score 3/10 Urgent cesarean section (cardio-tocographic abnormalities)	10 hs	10 hs: versive tonic spasms, ± flushing and desaturation ± focal clonic components	PB, PHT, LEV, MDL (ineffective) CBZ (sz-free)	PN, PLP/ Unsuccessful	Initial EEG: Abnormal After plural AEDS (9 mo): Abnormal	MRI (1 d): Minor abnormalities	Sz-free at 9 mo (after CBZ start) Severe DD; Spastic-dystonic tetraplegia	The patient was trialed with FA as well, unsuccessfully
Vilan et al. (25)	13	BFNE	c.1076C>A p.Thr359Lys Maternally inherited	F	Normal	1 d	Tonic with cyanosis	PB, CZP, PHT, VPA (NR) Lidocaine (partially effective)	PN/ Unsuccessful	Ictal aEEG: Abnormal Interictal: Normal	MRI: Normal	Sz-free at 1.5 mo Recurrence of frequent Sz after 4 years; Mild ID, ADHD (13 y)	Pyridoxine was administered to 8 infants without any beneficial effect. All infants needed ≥2 AEDs to control their szs

(Continued)

TABLE 1 | Continued

References	Patient	Phenotype	Mutation	Sex	Perinatal and early history	Seizure onset	Seizure features	AEDs administered (response)	Type of vitamin B6/Response	EEG	Neuroimaging	Seizure and clinical outcome	Additional information
	14	NR	c.1955dupC p.Pro652fs *De novo*	M	Normal	2 d	Tonic with cyanosis	PB, MDL, CZP (NR) Lidocaine (partially effective)	PN/Unsuccessful	Ictal aEEG: Abnormal Interictal: Normal	MRI: Normal	Sz-free at 21 d Recurrence of 2 Sz at 4 ys. Mild delay in MD (5 ys)	
	15	NR	c.1065C>G p.Asp355Glu *De novo*	M	Normal	2 d	Tonic with cyanosis	PB, MDL (NR) Lidocaine (acutely effective) PHT (effective)	PN/Unsuccessful	Ictal aEEG: Abnormal Interictal: Normal	MRI: Normal	Sz-free at 12 d Normal outcome (2 ys)	
	16	NR	c.2296delC p.Leu766fs *De novo*	M	Normal	3 d	Tonic with cyanosis	PB, MDL (NR) Lidocaine (partially effective)	PN/Unsuccessful	Ictal aEEG: Normal Interictal: Discontinuous normal voltage	MRI: Normal	Sz-free at 14 d Further outcome: NR	
	17	NR	c.1527delA p.Glu509fs *De novo*	M	Normal	24 d	Tonic with cyanosis	PB (not effective)	PN/Unsuccessful	Ictal aEEG: Abnormal Interictal: Normal	MRI: Normal	Sz-free at 14 d Further outcome: NR	
	18	NEE	c.830C>T p.Thr277Ile *De novo*	M	Normal	2 d	Tonic with cyanosis followed by focal clonic activity	PB, MDL, CZP, TPM (NR) LEV and lidocaine (acutely effective) VPA (effective)	PN/Unsuccessful	Ictal aEEG: Abnormal Interictal: Discontinuous normal voltage	MRI: Normal	Sz-free at 1.5 mo Non-verbal, autistic features, delay in MD (3 ys)	
	19	NR	c.1657C>T p.Arg553Trp *De novo*	F	Normal	1 d	Tonic with cyanosis followed by focal clonic activity	PB, LEV, MDL (NR) CBZ (effective)	PN/Unsuccessful	Ictal aEEG: Abnormal Interictal: Normal	MRI: Normal	Sz-free at 15 d Normal outcome (16 mo)	
	20	NEE	c.901G>A p.Gly301Ser *De novo*	F	Normal	1 d	Tonic with cyanosis	PB, MDL, LEV (NR) Lidocaine (acutely effective) CBZ (effective)	PN/Unsuccessful	Ictal aEEG: Abnormal Interictal: Normal	MRI: Signal anomalies	Sz-free at 15 d	
Pisano et al. (26)	21–32	NEE	12 patients were trialed with adequate dose of pyridoxine, 7 received pyridoxal-phosphate. At the onset, all patients showed axial hypotonia. During follow-up, cognitive impairment										
Klotz et al. (27)	33	NEE	c.1023G>C p.Gln341His *De novo*	F	Born at term but SGA	7–8 d	Tonic with cyanosis.	PB, LEV (partially effective, in combination with PN)	PN trial: 30 mg/kg over 3 d/ No immediate effect on sz frequency or EEG Consequent PLP trial: 30 mg/kg/Improvement of sz frequency shortly after its start (in combination with PB and LEV)	Initial EEG: Abnormal Last EEG (date NR): Abnormal	MRI: Normal	Sz reduction (once-twice every few wks) Neuro-cognitive sequelae: abnormal MD and DD	No detected mutations in ALDH7A1 or PNPO After PLP discontinuation: re-occurrence of 20 szs/d After PLP reintroduction seizures resolved Further switch to PN: no change in seizure frequency
Reid et al. (18)	34	NEE?	c.629G>A p.Arg210His *De novo*	F	Born at 38 wks + 6 by spontaneous labor, after an uneventful pregnancy	4 d	Episodes of choking and cyanosis, associated with stiffening after which she became floppy; "cycling" movements of arms; desaturation	PB, LZP (not effective) PHT, CBZ (partially effective in combination with PN)	PN, PLP/Successful 2 mo: sz control after 6 d of 0.25 mg of PN (oral drops), at a time when she was also receiving 6.7 mg/kg/day of PHT and 11 mg/kg/day of CBZ	9 d: Abnormal 7 ys: Abnormal	MRI (28 d); Minor abnormalities	Sz outcome at 7 years: szs presenting only during intercurrent illness 7 y: DD with minimal expressive language	No mutations detected in ALDH7A1 or PNPO
Allen et al. (3)	35	BFNE	c.419_430dupp.Val143_Arg144insGlnTyrPheVal Maternally inherited (affected mother)		Normal	4 d	Clonic and tonic szs. Clusters multiple/day or days sz-free. Minor cyanosis subsequently, mainly tonic. Multiple/day, then weeks and months sz–free	LEV (some response, required dose increases) Other drugs used but ineffective: PB, MDZ, LZP	PLP (used acutely)/ NR	Initial EEG: Abnormal 3.5 mo: Normal	MRI (3 wks): Normal	Sz outcome: sporadic breakthrough minor szs Normal developmental outcome (1 y)	
Mefford et al. (16)	36	Pyridoxine-dependent epilepsy?	1.5 Mb terminal deletion of the long arm of chromosome 20	M	Precipitous after a 36 week pregnancy complicated by frequent Braxton-Hicks contractions.	2 wks	Reddening and tonic stiffening of arms, lasting approximately 1 min Initially sporadic but, by 8 wks of age, occurring 4–6/day	PB 15 mg/day (partial response)	PN 100 mg/d, later increased to 200 mg/d and eventually reduced to 150 mg/d/ Good electroclinical response (so that PB was discontinued at 11 months)	Initial EEG: Abnormal 5 ys: Abnormal	First MRI: Minor abnormalities MRI (11 mo): Normal	Sz-free (7 ys) ID and delay in MD	Sequencing of the ALDH7A1 gene did not detect mutations

(Continued)

TABLE 1 | Continued

References	Patient	Phenotype	Mutation	Sex	Perinatal and early history	Seizure onset	Seizure features	AEDs administered (response)	Type of vitamin B6/Response	EEG	Neuroimaging	Seizure and clinical outcome	Additional information
Weckhuysen et al. (5)	37	NEE	c.613A>G p.Ile205Val	M	During the last 2 months of pregnancy rhythmical jerking similar to szs Subsequent normal perinatal and early development	2 d	Generalized tonic with clonic components, lip smacking, back arching, and apnoea. Multiple szs daily	VGB (initially reduced szs and normalized EEG with 7 wks sz freedom) MDZ (partially effective) PB, FA, betamethasone, VPA (all ineffective) TPM, VGB (effective in combination with PN)	PN/The combination of TPM, VGB, and PN controlled szs	7 d: Abnormal 9 mo: Normal	CT scan (2 d) and MRI (11 d and 3.5 ys): Signal anomalies	Status epilepticus at 3 mo; Sz -free from 9 mo until 8 ys ID and delay in MD	
Borgatti et al. (28)	38	BFNE, epileptic encephalopathy and profound mental retardation?	c.1620G>A p.K526N Maternally inherited (Affected mother and two younger sisters)	F	Born at 40 wks by cesarean section due to podalic presentation	3 d	Clonic szs. Subsequently right sided clonic and tonic-clonic szs with oro-alimentary automatism	ACTH (partially effective) PB, VGB, benzodiazepines, PHT, VPA, CZP, immune-globulin (ineffective)	PN/NR	Initial ictal EEG: Abnormal Last EEG (date NR): Abnormal	MRI (around 4 mo): Abnormal	Not achieved complete sz control. Many polymorphic szs/d Severe spastic tetraparesis and profound ID	
Dedek et al. (29)	39	BNFE	p.Ser247Trp	M	Born by cesarean section due to prolonged delivery period and symptoms of fetal distress	3 d	Left or right head deviation, and upper and lower limb involvement	ACTH (effective) PB, PHT,VGB (ineffective)	PN /Unsuccessful	8 d: Normal 2.5 ys: Abnormal	CT (7 d): Normal MRI (41 d): Normal	Sz-free (szs stopped at 13 wks) Immediate improvement of EEG after ACTH initiation DD (2 ys and 5 mo)	
Martin et al. (30)	40	NEE (Ohtahara syndrome)	c.827C>T p.T276I	M	Born at 41 wks by emergency cesarean section due to failure to progress	1 d	Cyanotic episodes, then more obvious szs; tonic spasms	TPM, DZP, and NZP (fits initially continued, but at 5 months were less severe, ceasing by 17 months) CZP, VGB, FA (ineffective)	PLP/ Unsuccessful	1 d: Abnormal 4 ys: Abnormal	MRI: Abnormal	Sz-free Severe DD (4 ys)	
Numis et al. (31)	41	NEE	c.1734 G>Cp.Met578Ile	NR	Born at 34 wks. Lack of visual fixation, decreased spontaneous movements, and axial hypotonia	4 d	Tonic head, conjugate eye, mouth deviation, unilateral tonic abduction of the limbs. Apnoea and desaturation	CBZ (effective—sz free within 2 wks) PB, LEV, TPM, VGB, CLB, CZP, KD, FA (ineffective)	PN, PLP/ Unsuccessful	Interictal and Ictal EEG: Abnormal	MRI (20 and 33 d): Abnormal	Sz-free DD and delay in MD	
Saitsu et al. (32)	42	NEE (Ohtahara syndrome)	c.1010C>G p.A337G	M	NR	7 d	Tonic szs, vomiting. Complex partial szs since age 5	High dose PB (sz-free and burst-suppression disappeared) ZNS (ineffective)	PN/ Unsuccessful	Initial EEG: Abnormal	NR	Sz-free after high dose PB DD and delay in MD	
	43	NEE (Ohtahara syndrome)	c.341C>T p.T114I	F	NR	0 d	Tremor of the upper extremities then generalized convulsions with cyanosis Complex partial szs since age 5	ZNS (sz free) CZP, PHT (ineffective)	PN/ Unsuccessful	Initial EEG: Abnormal	NR	Sz-free after ZNS Profound DD and abnormal MD	
	44	NEE (Ohtahara syndrome)	c.794C>T p.A265V	M	NR	1 d	Apnoeic spell, then tonic spasms with right opsoclonus like movement.	ZNS, VPA, CZP, CBZ (ineffective)	PN/ Unsuccessful	Initial EEG: Abnormal	NR	Intractable szs DD Myoclonus at the bilateral upper extremities	
Kato et al. (7)	45	NEE (Ohtahara syndrome)	c.650C>Ap.Thr217Asn *De novo*	F	NR	0 d	At onset: Pale face for tens of seconds 1 d: Eye deviation to left followed by tonic szs (0.5–1/h)	High dose PB (sz-free) ZNS (ineffective)	PLP/ Unsuccessful	1 d: Abnormal	MRI (2 d, 1 and 6 mo): Signal anomalies MRI (2 ys): Normal	Sz-free after high dose of PB Profound DD and abnormal MD	
	46	NEE?	c.794C>T p.Ala265Val *De novo*	M	NR	2 d	At onset: Facial flushing and eye fixation 3 d: Tonic szs (daily)	DZP, MDL, high-dose PB (partially effective) VPA (ineffective) CBZ (successful)	PLP/ Unsuccessful	5 d:Abnormal	MRI (7 mo and 2 y): Abnormal	Sz-free after CBZ. No szs since 16 mo Moderate DD and abnormal MD	
	47	NEE (Ohtahara syndrome)	c.794C>T p.Ala265Val *De novo*	M	NR	2 d	At onset: No cry, poor suck, stiffening, and arching with eye rolling 5 d: left-sided szs	PB, CLB, MDZ, VGB (ineffective)	PLP/ Unsuccessful	5 d:Abnormal	MRI (0 mo): Normal	Intractable szs DD and abnormal MD Died at 3 mo	

(Continued)

TABLE 1 | Continued

References	Patient	Phenotype	Mutation	Sex	Perinatal and early history	Seizure onset	Seizure features	AEDs administered (response)	Type of vitamin B6/Response	EEG	Neuroimaging	Seizure and clinical outcome	Additional information
	48	NEE (Ohtahara syndrome)	c.854C>A p.Pro285His Maternally inherited	F	NR	0 d	At onset: poor feeding followed irritability with hypoxia and tonic szs (1–4/d).	VPA (sz-free) PB (ineffective)	PLP/ Unsuccessful	12 d:Abnormal	MRI (12 d and 3 mo): Signal anomalies	Sz-free after VPA (since 3 mo) DQ 35. Moderate DD and abnormal MD	
	49	NEE (Ohtahara syndrome)	c.881C>T p.Ala294Val *De novo* Domain in protein: Transmembrane domain (S4)	M	NR	1 w	Convulsion-like movements followed by asymmetric tonic szs	TPM (sz-free) ZNS, VPA (partially effective) CZP, PB (ineffective)	PLP/ Unsuccessful	<1 mo: Abnormal 3 mo: Abnormal	MRI (3 mo and 9 mo): Abnormal	Sz-free since 6 mo Profound DD and abnormal MD	
	50	NEE (Ohtahara syndrome)	c.997C>Tp.Arg333Trp *De novo* C-terminal region	M	NR	2 d	Tonic szs followed by partial ones (eyes rolling up)	ZNS (almost sz-free) VPA, lidocaine (partially effective) DZP, PB, PHT (partially effective)	PLP/ Unsuccessful	42 d:Abnormal	NR	Sz-free. Only one sz in 10 ys. Severe DD	
	51	NEE (Ohtahara syndrome)	c.1669C>Gp.Asp563Glu C-terminal region	F	NR	1 d	Poor feeding with cyanosis, followed by tonic szs, facial clonic sz, and generalized tonic-clonic convulsions	CBZ and CZP (sz-free) PHT, PB (partially effective) VPA, NZP (ineffective)	PLP/ Unsuccessful	4 d: Abnormal	CT (0 m): Normal	No szs since 10 y, but relapsed at 24 ys after a y of drug withdrawal Moderate DD with autistic features	
Mills et al. (6)	52–57	Six patients described that have been stated	treated with vitamin B_6 during the first month of life. Responses to each AED not										

NR, not reported; BFNE, Benign familial neonatal epilepsy; EIEE, early infantile epileptic encephalopathy; EOEE, early onset epileptic encephalopathy; NEE, neonatal epileptic encephalopathy; F, female; M, male; IV, intravenous; P.O., per os; S, second(s); Mn, minute(s); H(s), hour(s); D, day's; Wk(s), week(s); Mo, month(s); Y(s), year(s); CBZ, carbamazepine; CLB, clobazam; CF, calcium folinate; CLZ, clonazepam; EZO, ezogabine; FA, folinic acid; FLB, felbamate; KD, ketogenic diet; LEV, levetiracetam; LOC, lacosamide; LZP, lorazepam; MDL, midazolam; NZP, nitrazepam; PB, phenobarbital; PHT, phenytoin; PN, pyridoxine; PLP, pyridoxal 5' phosphate; TPM, topiramate; STM, sulthiame; VB6, vitamin B6; VGB, Vigabatrin; VPA, valproic acid; ZNS, zonisamide; Sz(s), seizure(s); DD, developmental delay; ID, intellectual disability; MD, motor development.

Throughout the following years, VPA first, and Pb later, were discontinued. Today the child is seizure-free and takes daily multivitamin B complex, also containing PN. Despite the global neurodevelopmental delay, he is able to walk alone, jump and climb; his expressive language is limited to simple and short phrases and characterized by dyslalia, whereas the comprehension is adequate. The child presents mild autism-like features (such as self-stimulatory, repetitive and stereotyped behaviors, and relational difficulties). He has also gained sphincteric control.

Direct sequencing of the KCNQ2 (NM_172107.4; NP_742105.1) gene demonstrated a paternally inherited heterozygous mutation (c.1639C>T; p.Arg547Trp), leading to the replacement of Arginine with Tryptophan at position 547 of the protein and involving a conserved amino-acid of the protein predicted to have functional consequences on the protein (**Figure 1**). This variant had already been reported in the literature by Zara et al. (2) (as causative of a BFNE in a female patient with a maternal inheritance) and Lindy et al. (33) (no clinical data available). *In silico* analysis performed with bioinformatic tools predicted that mutation has a potential strong damaging effect on the structure/function of the KCNQ2 protein. ACMG standard criteria for assessment of pathogenicity of variants (Varsome) (34) should be considered as "Pathogenic" (**Table 2**).

In the case of our patient, ALDH7A1 sequencing was later reported to be negative, and no further genetic testing was performed. This data prevented us the possibility to explain his positive response to PN and his clinical phenotype (that, despite the clinical continuum existing between BFNE and NEE, seems closer to the NEE phenotype), in which a possible role of other genes on his impaired neurodevelopment cannot be ruled out.

Case 2

A 10-year-old Caucasian female was born at term by eutocic vaginal delivery from an uneventful pregnancy. Birth weight was 3,400 gr. Prenatal and perinatal history was unremarkable. On the second day of life, the baby showed myoclonic seizures associated with sudden loss of muscle tone and rolling eye movements. The patient started treatment with Pb, without effects. The EEG showed a suppression-burst pattern. Brain MRI and routine metabolic investigations resulted to be normal. At day 36 of life, after unsuccessful therapeutic attempts with Pb and VPA, and the start of PN administration, she was admitted to the NICU without considerable response. Herein, she was started on oral PLP therapy (up to a dose of 500 mg/day divided in 4–6 administrations), achieving immediate seizure control. An extended metabolic workup was performed highlighting an elevation of pipecolic acid both in serum and urine, albeit the sequencing of ALDH7A1 and PNPO genes was normal. Given the good clinical response to PLP and the poor neurodevelopment performance (no achievement of motor milestones, marked muscular hypotonia, and visual disturbances), she received a diagnosis of "Focal epilepsy with pyridoxal phosphate-responsive seizures and global neurodevelopmental delay."

Feature key	Position(s)	Description	Mutation	Graphical view
Topological domain	1 – 91	Cytoplasmic		
Transmembrane	92 – 112	Helical; Segment S1		
Topological domain	113 – 122	Extracellular		
Transmembrane	123 – 143	Helical; Segment S2		
Topological domain	144 – 166	Cytoplasmic		
Transmembrane	167 – 187	Helical; Segment S3		
Topological domain	188 – 195	Extracellular		
Transmembrane	196 – 218	Helical; Voltage-sensor; Segment S4		
Topological domain	219 – 231	Cytoplasmic		
Transmembrane	232 – 252	Helical; Segment S5	c.740C>T (p.S247L)	
Topological domain	253 – 264	Extracellular		
Intramembrane	265 – 285	Pore-forming; Segment H5		
Topological domain	286 – 291	Extracellular		
Transmembrane	292 – 312	Helical; Segment S6		
Topological domain	313 – 872	Cytoplasmic	c.1639C>T (p.R547W)	

FIGURE 1 | Functional domain position of p.R547W and p.S247L amino acid changes of KCNQ2 protein (adapted from https://www.uniprot.org). The figure shows the KCNQ2 functional domain position of the variants found in our two cases (p.R547W and p.S247L). *In silico* analysis (**Table 2**) predicted that the two mutations have potential strong damaging effect on the structure/function of the KCNQ2 protein.

TABLE 2 | *In silico* prediction of the KCNQ2 missense mutations*.

NM_172107.4 (NP_742105.1) (GRCh38)	c.1639C>T (p.R547W)	c.740C>T (p.S247L)
PolyPhen2 prediction: B (benign), P (possibly damaging), D (probably damaging)	D	Variable: P;P;B;B;B;B
SIFT prediction	Damaging	Damaging
LRT prediction	Deleterious	Neutral
MutationTaster prediction	Disease causing	Disease causing
MutationAssessor prediction	Medium impact	High impact
FATHMM	Damaging	Damaging
fathmm-MKL	Damaging	Damaging
M-CAP	Damaging	Damaging
CADD [the larger the score the more likely the SNP is damaging (PHRED-like)]	27.2	25.9
MetaSVM	Damaging	Damaging
MetaLR	Damaging	Damaging
PhyloP 20way the larger the score, the more conserved the site (max 1.199000)	−0.409000	0.982000
PhyloP 100way the larger the score, the more conserved the site (max 10.003000)	0.097000	6,62,8000
GERP RS the larger the score, the more conserved the site (max 6.17).	−1.15	3.25
1000 Genomes	No data	No data
gnomAD	No data	No data
Interpro domain	Potassium channel, voltage dependent, KCNQ, C-terminal	Ion transport domain
ClinVar interpretation	Pathogenic/Likely_pathogenic	Pathogenic/Likely_pathogenic
ACMG classification (Varsome**)	Pathogenic	Pathogenic

**Data from HGMD professional database [www.hgmd.cf.ac.uk; (35)].*
***Kopanos et al. (34).*

After 18 months of seizure-free interval, epileptic tonic spasms occurred, both during sleep and wakefulness. The EEG showed multifocal epileptic discharges.

Low CSF folic acid levels were revealed; hence, folic acid was started (7.5 mg/bid, later increased at 7.5 mg/tid), in add-on to her previous therapy,

with benefits in terms of number and intensity of epileptic seizures.

Despite the overall discrete seizure control, subsequent interictal EEG recordings showed marked diffuse abnormalities up to a *quasi*-periodic epileptiform pattern. The patient showed severe global neurodevelopmental delay at the follow-up.

Further genetic testing was performed. Array-CGH was not informative. Whereas, trios NGS (epilepsy panel) revealed a *de novo* heterozygous mutation in Exon 5 of the KCNQ2 gene (NM_172107.4; NP_742105.1): c.740C>T/(p.Ser247Leu), affecting a highly conserved aminoacidic region and predicted to be deleterious *in silico* programs (**Figure 1**). However, using the standard procedures for assessment of pathogenicity of variants (ACMG criteria—Varsome) (34), should be considered as "Pathogenic" (**Table 2**). This variant has already been reported in the literature and related to early-onset epileptic phenotypes with a variable degree of neurocognitive impairment (36–41). However, none of these reports shows evidence of VitB6 supplementation. Our patient was maintained on Vigabatrin, PLP, and Folic acid for several years. By the age of 10 years, given the occurrence of severe behavioral and sleep disorders, PN, Folic acid, and Vigabatrin were withdrawn, and maintenance therapy with carbamazepine, in add-on to Lorazepam and Promazine, was started.

To date, the child failed to achieve trunk control, independent standing or walking. She shows axial hypotonia and limbs hypertonia. Language is limited to vocalizations and she presents severe intellectual disability. In addition, behavioral disturbances are evident, with aggressive manifestations and sleep disorders. She displays a good clinical control, with occurrence of rare seizures (one episode/1–2 years).

DISCUSSION

Herein, we reported two cases of KCNQ2-related epilepsy, a 5-year-old male with a paternally inherited heterozygous mutation (c.1639C>T; p.Arg547Trp), and a 10-year-old female with a *de novo* heterozygous mutation (c.740C>T; p.Ser247Leu), who benefited from PN treatment.

The first patient presented with neonatal epilepsy with an onset during the first week of life, which was later ascribed to a KCNQ2 pathogenic variant paternally inherited.

The same variant had already been described in the literature (2, 33) and reported as causative of a BFNE phenotype in a female patient with maternal inheritance (2).

Our patient, with his overall good seizure-responsivity despite impaired neurodevelopment, does not show all the typical characteristics of BFNE or NEE. Even if no evidence of an inborn error of VitB6 was available for our patient (ruling out the diagnosis of a PDE), his seizures, scarcely controlled by AEDs, revealed a good and immediate electro-clinical response to the initiation of intramuscular PN. Interestingly, this effect did not cease after switching to PN oral administration, neither after the discontinuation of AEDs, strengthening the diagnostic hypothesis of PN-responsive epilepsy (PRE).

The second patient, after a sudden and severe onset of neonatal seizures, requiring plural hospitalizations, and failed attempts with AEDs, such as Pb and VPA, and with PN too, demonstrated an immediate clinical benefit from the initiation of PLP, and, over time, to folic acid as well.

In this case, a *de novo* disease-causing KCNQ2 variant was detected (c.740C>T; p.Ser247Leu), and the good clinical response to PLP and folic acid were supported, respectively, by elevated levels of pipecolic acid both in serum and urine and by reduced CSF folates level. Moreover, genetic testing resulted negative for ALDH7A1 and PNPO pathological variants. These data supported the possibility to classify this clinical phenotype into the PRE spectrum.

Moreover, even though the clear effect of PLP reduced over time, other AEDs (i.e., Pb and VPA) proved to be ineffective, and the neurocognitive outcome of the patient is quite poor, she still presents an overall good clinical control, with seizures being years apart.

Summarizing, regardless of the several divergences between these 2 cases, both of them may fit into the category of PRE, particularly within the subgroup of KCNQ2-related ones.

Data from the literature have demonstrated the usefulness of VitB6, either as PN or, even better, as PLP, in certain types of neonatal-onset epilepsies (13, 19). In addition, recent reports have been unraveling a potential correlation between KCNQ2-related epilepsies and VitB6 responsiveness (18). To date, several cases of patients carrying a pathogenic variant of KCNQ2 trialed with VitB6 (either in the form of PN or PLP) have been reported in the literature. Unfortunately, the effects of VitB6 have not been accurately described for all of them. Additionally, several cases have been extracted from studies focusing on other than the KCNQ2-PN correlation, making it arduous to infer the exact impact of VitB6 on the clinical picture (6, 22, 26). Five reports have openly disclosed various degrees of a successful response to PN/PLP in KCNQ2-related epilepsies (5, 16, 18, 23, 27). Interestingly, despite their small number, all patients share a neuro-cognitive impairment in the lack of genetic data confirming/denying the PN-dependency/responsiveness, in line with ours. All cases are reported in **Table 1** (and detailed in the complete version of the table—see **Supplementary Material**).

Currently, the etiopathogenic mechanism underlying the PN responsiveness of certain neonatal seizures, and particularly of those KCNQ2-related, remains unclear. However, some hypotheses have been proposed [(18); **Figure 2**].

First of all, VitB6, particularly as PLP, is notably implicated as a cofactor of hundreds of enzymatic reactions, having a role in several functions, such as in the metabolism of amino-acids and the synthesis of neurotransmitters (42). In this regard, PLP is notably a cofactor of glutamic acid decarboxylase (GAD), an enzyme involved in the synthesis of gamma-amino butyric acid (GABA) from glutamate (GLUT). These two are, respectively, the key inhibitory and excitatory neurotransmitters of the CNS, and an imbalance between them has long been considered among the molecular mechanisms underlying epilepsy (43). The reduction of GABA deriving from low PLP levels has been proposed as a potential mechanism in the genesis of PDE, but it is

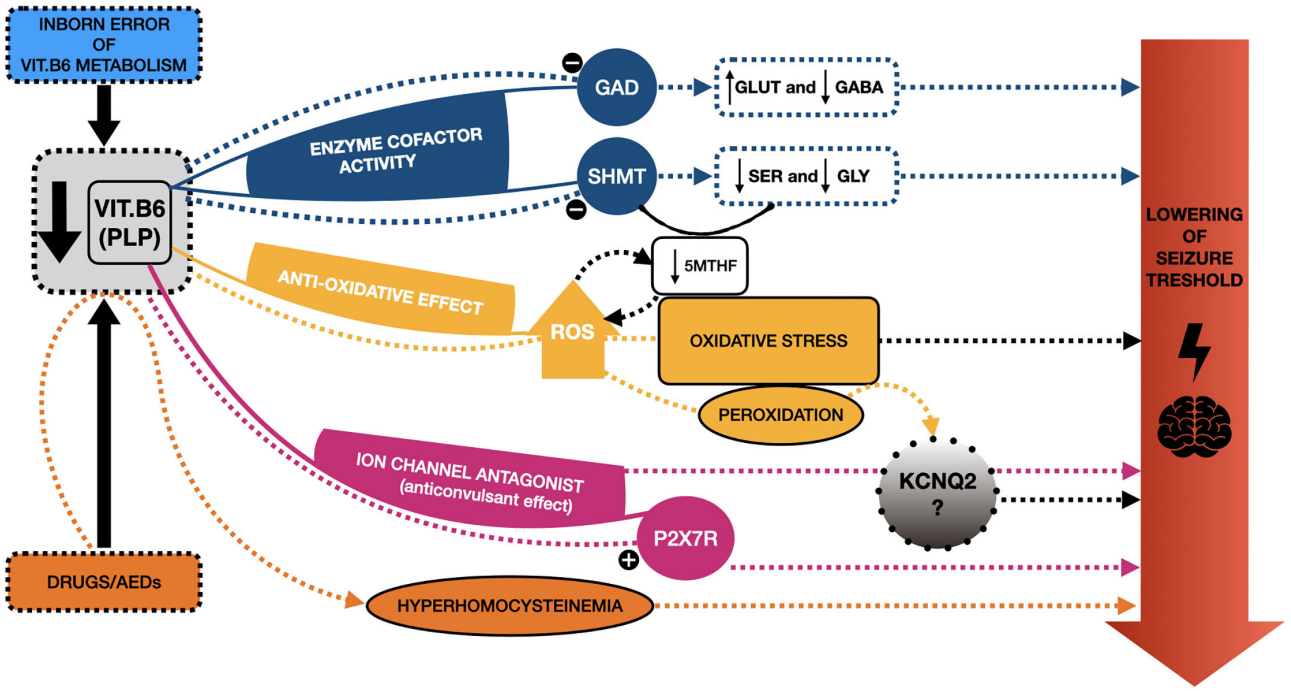

FIGURE 2 | Schematic representation of the mechanisms proposed to explain seizure responsiveness to vitamin B6 and the potential link with KCNQ2. Several etiopathogenic mechanisms have been proposed to explain the responsiveness of certain neonatal seizures to vitamin B6 (VitB6), and particularly to its active form, pyridoxal 5′ phosphate (PLP). PLP acts as a cofactor of several enzymes, such as Glutamic acid decarboxylase (GAD), regulating the synthesis of gamma-amino butyric acid (GABA) from Glutamate (GLUT). A reduction of PLP may determine an imbalance between these two (in favor of GLUT), hence decreasing the seizure threshold. PLP is also involved in the synthesis of serine (SER) and glycine (GLY), modulating the serine hydroxymethyltransferase (SHMT) activity; thus, a reduction of PLP may determine lower levels of these two amino-acids, whose reduction has been related to epileptic phenotypes. SHMT is involved in the metabolic pathway, eventually leading to the formation of the main circulating form of folates, the 5-methyltetrahydrofolate (5-mTHF), a reduction of which has been reported in some patients suffering from pyridoxine-dependent epilepsies (PDE). Furthermore, the reduction of 5-mTHF may fall into another proposed mechanism underlying epilepsy, namely oxidative stress, with excessive production of reactive oxygen species (ROS). This may be a consequence of low levels of VitB6 (anti-oxidative agent) and, at the same time, a cause of folates depletion. Additionally, ROS contribute to the peroxidation of several molecules, also affecting ion channels, hence potentially KCNQ2. PLP also acts as an antagonist of specific ion channels, as P2X7R, with a possible anticonvulsant effect, which could not occur when PLP levels are low. A similar antagonist action on KCNQ2, with possible analogous effects, has been proposed. Finally, some drugs, especially antiepileptic ones (AEDs), may be responsible for the depletion of VitB6 and, indirectly, hyperhomocysteinemia, which is known to lower the seizure threshold. Overall, these mechanisms may explain the responsiveness of some neonatal seizures to VitB6 treatment and help shed light on the potential link with KCNQ2-related epilepsies.

widespread among the authors the opinion that this cannot be the only one (43–45). Ramos et al. (45) have recently highlighted that conflicting data have been reported on GLUT and GABA levels in the CSF of patients suffering from VitB6 deficiency, supporting this hypothesis (45). In this regard, they carried out a study on a model system of VitB6-deficient Neuro-2a cells, revealing a significant reduction in the *de novo* synthesis of serine and, consequently, glycine, whose reduction has been related to epileptic phenotypes, suggesting a potential role of these two (45, 46).

Additionally, it is noteworthy to mention that glycine synthesis from serine depends on the enzyme serine hydroxymethyltransferase (SHMT), which requires PLP as a cofactor. SHMT is involved in the metabolic pathway which encompasses the enzyme methylenetetrahydrofolate reductase (MTHFR) as well, eventually leading to 5-methyltetrahydrofolate (5-mTHF) formation, the main circulating form of folate (45). This is particularly interesting since low levels of 5-mTHF in CSF have already been reported in our second patient and some PDE patients (18, 47).

In particular, the patient described by Reid et al. (18) and ours reported a positive response to VitB6 treatment, both in the presence of low folates and of different isolated lab findings suggestive for VitB6 disorders (respectively, a high plasma-to-CSF PLP ratio in the former, and an elevation of pipecolic acid both in serum and urine in the latter), though in the absence of a genetic confirmation of inborn errors of VitB6 metabolism. Although these findings cannot be fully explained, and no clear evidence on the topic is yet available, all this suggests not only a potential role of folates in PREs and PDEs, but also that there is much more to find out.

Besides, when it comes to folates, it is not possible to state whether their involvement in PDEs/PREs, and, in general, in epilepsy may be a causative factor, a consequence, or both. Their involvement in these conditions may be mutually related to another proposed mechanism underlying epilepsy, namely the

excessive production of reactive oxygen species (ROS) (17, 18). Several studies have demonstrated an excessive ROS production in epilepsy, so they outgrow the capability of endogenous antioxidants to contrast their effect (43). Besides, the excess of ROS may determine a depletion of folates, which are known to exhibit antioxidant functions acting as ROS scavengers (18, 48).

Overall, high levels of ROS may have detrimental effects, including the peroxidation of structures and molecules (i.e., enzymes and components of cell membranes), potentially affecting ion channels indirectly as well (43). Moreover, given the demonstrated anti-oxidative effects of VitB6 (49) and the potential effect of ROS on ion channels, one could speculate that there may be a close, albeit yet unknown, relation between VitB6 and channelopathies, such as KCNQ2-related ones.

Anyhow, when ROS production is excessive, the result is a strong oxidative stress and an imbalance favoring the excitotoxicity (43).

Another possible mechanism may involve the recently demonstrated antagonist action of PLP toward P2X-receptors (and particularly the subtype P2X7R) (50). These receptors are a class of ligand-gated ion channels, activated by ATP, contributing to neuro- and glio-transmission and lately associated with epileptic conditions, such as status epilepticus (51). Since P2X7R antagonists have recently been reported as having anticonvulsant effects, this may apply as well to PLP, explaining at least partly its role in controlling seizures (52).

Given the above, Reid et al. (18) proposed that PLP may as well have a direct antagonist effect on ion channels, including the one encoded by KCNQ2. However, further evidence is needed to confirm this hypothesis.

Other potential mechanisms explaining PDE and PRE may relate to secondary defects of VitB6, for instance drug-induced ones. Several drugs, such as carbamazepine, phenytoin, and PB, may reduce VitB6 levels and indirectly lead to hyperhomocysteinemia (which is known to lower seizure threshold) (53, 54). It is likely that the same effect may apply to other AEDs and that it may partly explain the VitB6 responsiveness of certain patients, even when temporary. Unfortunately, it is not possible to confirm or deny this hypothesis for our patients.

Our study presents some limitations, such as its retrospective nature, with consequent possible information biases and lack of specific data (such as details about treatments and diagnostic assessment taking place in different centers from ours, or the correlation between AEDs modification and concomitant levels of VitB6 and homocysteine), as well as the small sample of patients, making it difficult to generalize our findings.

In the light of the above, further studies focusing on the correlation between types of AEDs used, treatment duration, concomitant plasma levels of VitB6, homocysteine and folates, and possibly considering the eventual disease-causing variants associated (and in particular KCNQ2 ones), may help unravel new evidence on the topic.

Prospective studies are needed to analyze and compare the effects of standardized treatment protocols with VItB6 in neonatal epilepsies in general and in those KCNQ2-related in particular.

All this is particularly important when considering the widespread resort to VitB6 trials in neonatal epilepsies in NICUs, where this treatment is quickly taken into account, though not devoid of risks. In fact, high doses of PN and PLP have been reported as causative of peripheral neuropathy and liver toxicity, respectively (14, 18). In our opinion, this sets some limits on the advisability of resorting to a blanket VitB6 treatment. Therefore, our suggestion would be to initiate it as soon as possible in all those cases in which clinical features and/or genetic testing and lab findings suggest, or, even better, clearly demonstrate a VitB6 disorder (preferably after excluding liver diseases and nerve conduction defects). Otherwise, we would instead reserve VitB6 treatment for peculiar situations, such as drug-resistant NEEs, including those with an already proven pathogenic KCNQ2 variant.

CONCLUSIONS

Despite the limits of our study, our data contribute to adding new evidence on the potential beneficial effect of VitB6 treatment in KCNQ2-neonatal epilepsies, in apparent lack of inborn errors of VitB6 metabolism. Further studies should be conducted to elucidate the mechanisms underlying the variability of VitB6 effects in these patients, to help discriminate whether to include or not KCNQ2 (besides ALDH7A1 and PNPO) in the genetic testing of neonatal-onset seizures responsive to PN/PLP, and, finally, to define appropriate clinical guidelines and treatment protocols.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available due to ethical and privacy restrictions. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant for the publication of this case report.

AUTHOR CONTRIBUTIONS

AN and GD conceived planned and supervised the study. GA and AB wrote the first draft of the

manuscript. GA, AB, and FC prepared the tables. GA and FC designed the figures. GS, GV, MS, and VS helped supervise the project. All authors contributed to manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.826225/full#supplementary-material

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Presenting Patterns of Genetically Determined Developmental Encephalopathies With Epilepsy and Movement Disorders: A Single Tertiary Center Retrospective Cohort Study

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Background: This paper aimed to evaluate the frequency of observation of genetically determined developmental encephalopathies with epilepsy and movement disorders in a specialistic center, the distribution of etiologies and presenting clinical hallmarks, and the mean times for the achievement of molecular genetic diagnosis.

Patients and Methods: Retrospective data about clinical phenotypes, etiology, and diagnostic pathways were collected in all the genetically confirmed patients with developmental encephalopathies with epilepsy and movement disorders referred to our institution between 2010 and 2020. The cohort was divided into two groups according to the predominant movement disorder type: 1) Group A: patients with hyperkinetic movement disorders; 2) Group B: patients with hypokinetic movement disorders. Both groups were analyzed in terms of developmental, epileptic, and movement disorder phenotypes.

Results: The cohort included 69 patients (Group A = 53; Group B = 16). The etiological spectrum was heterogeneous with a predominance of Rett and Angelman syndrome in Group A and neurodegenerative disorders in Group B. A moderate/severe intellectual disability was assessed in 58/69 patients (mean age at the first signs of developmental impairment = 1,87 ± 1,72 years). Group A included patients with an earlier onset of epileptic seizures (2,63 ± 3,15 vs. 4,45 ± 5,55 years of group B) and a predominant generalized motor semiology of seizures at the onset. Focal seizures were the main initial epileptic manifestations in Group B. Seizures were noticed earlier than movement disorders in Group A while the opposite occurred in Group B. A higher increase in molecular genetic diagnosis was obtained in the last five years. Mean diagnostic delay was longer in Group B than in Group A (12,26 ± 13,32 vs. 5.66 ± 6.41 years). Chorea as an initial movement disorder was associated with a significantly longer diagnostic delay and a higher age at etiological diagnosis.

Conclusions: This study suggested: (a) a higher frequency of genetic defects involving neurotransmission, neuronal excitability, or neural development in patients with hyperkinetic movement disorders; (b) a higher frequency of neurodegenerative courses and a longer diagnostic delay in patients with hypokinetic movement disorders.

Keywords: developmental and epileptic encephalopathies, movement disorders, neurogenetic disorders, next generation sequencing-NGS, phenotypes

INTRODUCTION

The spectrum of genetic developmental encephalopathies presenting with epilepsy and movement disorders has significantly expanded in the last decade with the characterization of more than 100 monogenic disorders, and several genotype-phenotype correlation studies prominently focusing on single genes, specific pathogenic copy number variants, or non-mendelian conditions (1, 2).

The increasing availability of next generation sequencing techniques resulted in a higher number of etiological diagnoses, but also highlighted the importance of a careful evaluation of phenotypes for the interpretation of molecular data (3, 4).

The present single tertiary center study retrospectively analyzed the distribution of etiologies and presented phenotypes in a cohort of patients with these complex pediatric-onset disorders. The study aimed to evaluate the frequency of observation of these conditions in a specialistic center, their most frequent clinical hallmarks, and the mean times for the achievement of molecular genetic diagnosis in the last decade (during the age of introduction and diffusion of next generation sequencing methods).

PATIENTS AND METHODS

We retrospectively collected 69 patients (35 females and 34 males) with genetically confirmed developmental encephalopathies with epilepsy and movement disorders, who were referred to our Institution and received a genetic diagnosis between January 2010 and January 2020.

Medical records and video recordings for movement disorder characterization were reviewed and data were collected in a standardized digital form to include demographic information, perinatal history, developmental milestones, predominant seizure, and movement disorder subtypes, onset and evolution of seizures and movement disorders during the follow-up, the severity of developmental impairment, age at the diagnosis, diagnostic delay (time between the onset of symptoms and the molecular genetic diagnosis), EEG patterns at onset and during the follow-up, neuroimaging features, relevant neurophysiologic and laboratory investigations, pathogenic or likely pathogenic gene or copy number variants.

Seizure types and epilepsy syndromes were classified according to the 2017 classification by ILAE Commission for Classification and Terminology (5, 6).

Patients were subdivided into two groups, patients with hyperkinetic (Group A) and patients with hypokinetic (Group B) movement disorders according to the same criteria that are currently adopted for adults (7). Hyperkinetic movements (i.e., unwanted and excess movements) included dystonia, chorea, athetosis, myoclonus, tremor, tics, ataxia, and stereotypies. Hypokinetic movements (i.e., decrease in the number of movements) included hypokinetic-rigid syndrome or parkinsonism (8, 9).

In both groups, we evaluated the timing for the achievement of the etiological diagnosis through a comparison between temporal parameters (age at the onset of seizures, age at the onset of movement disorders, age at the molecular genetic diagnosis and diagnostic delay) and clinical manifestations (seizure types and type of movement disorder).

We also analyzed the distribution of the diagnostic delay during the years in which the molecular diagnoses were made. The temporal periods were divided in 4 intervals: PERIOD 1: 2016-2020; PERIOD 2: 2011- 2015; PERIOD 3 = 2006-2010; PERIOD 4 = before 2005. This last temporal clustering was introduced to minimize the burden of the different diagnostic yields associated with the various molecular genetic technologies that were available over the years. These different methods included Sanger sequencing of specific single genes, array CGH, targeted gene panels for epilepsy and/or movement disorders, clinical exome, or whole-exome sequencing. Single gene variants were classified according to the American College of Medical Genetics criteria (3, 10).

Statistical analysis was performed using SPSS 26.0 statistical software package for Windows. Data were reported as frequencies and percentages for discrete variables, as well as means and standard deviations for continuous variables. Chi-squared test ($\chi 2$) was used to reveal differences between and within groups in the discrete variables investigated, while a one-way analysis of variance (ANOVA) was carried out to test between and within groups differences in continuous variables. Bonferroni correction was applied to the *post-hoc* tests. The significance level for all statistical tests was set a priori to $\alpha = 0.05$.

RESULTS COHORT COMPOSITION

Sixty-nine patients (35 females and 34 males), with a mean age of 16.26 ± 9.18 years, with a confirmed molecular genetic diagnosis, were selected. Group A included 53 patients (29 females and 24 males) while 16 patients (6 females and 10 males) belonged to Group B.

The mean age at the molecular genetics diagnosis was 8.75 ± 8.743 years with a mean diagnostic delay of 6.21 ± 8.05 years.

TABLE 1 | Demographic, clinical, and molecular genetics features of patients with developmental encephalopathies presenting with epilepsy and hyperkinetic movement disorders (Group A).

Predominant movement disorder	Demographic data		Etiology			Age at onset of symptoms and time to diagnosis					Symptoms at the onset			Symptoms during the follow-up		
	Patient and sex	Age	Diagnosis	Gene	Gene variant	Age at the diagnosis	Age at the onset of seizures	Age at the onset of movement disorders	Age at onset of neuro-developmental disorder	Diagnostic delay	Seizure type at the onset	Movement disorder at the onset	Neuro-developmental disorder signs at onset	Seizure type during the follow-up	Movement disorder during the follow-up	Neuro-developmental disorder during the follow-up
Chorea	1 M	34	GNB1 encephalopathy	*GNB1*	c.357C> G (p.Asn119Lys)	34	1	8	NA	33	FIA,FA,M, A,TC	Ch, D (p)	NA	SF	Ch,D,P	SP-ID
	2 M	26	KCNA2 encephalopathy	*KCNA2*	c.890G > A (de novo)	23	2	1	24	21	FS, TC	Ch, Ata	Clu	SF	Ata, D	Mo-ID
	3 M	16,58	MeCP2 duplication syndrome	MeCP2	dup Xq28	2,1	1,08	11	10	1,18	TC	Ch, S	HCD	TC, A, T, C	S	SP-ID
	4 M	15,16	MeCp2 duplication syndrome	MeCP2	dup Xq28	4	9	12	24	5	T, A	Ch	W	T	S	SP-ID
	5 M	9,7	MEF2C encephalopathy	*MEF2C*	microdel 5q14.3	10,83	1,4	1,5	17	9,4	FTBTC	Ch	TCD	FA, T	Ch	SP-ID
	6 M	9,3	GNAO1 encephalopathy	GNAO	c139 A > G	2,75	4	5	9	2,5	FIA	Ch, D, B (p)	HCD	FIA, FTBC	D, S, B	SP-ID
Ataxia	7 M	27,6	Succinic semialdehyde dehydrogenase deficiency	ALDH5A1	0.526G > A /c.278>T	5	0,58	11	8	4,4	A	Ata	HCD	A, C, TC	Ata	Mo-ID
	8 F	21,25	BRAT 1-related syndrome	BRAT1	c.638_639insA/ c.1395G>A	19	13,9	13,9	36	5,08	T	Ata	LDD	FTBC	Ata	Borderline
	9 F	20,3	KCTD7-related progressive myoclonus epilepsy	KCTD7	c.533C>T	6,4	0,8	1,6	18	5,5	M	Ata	R	TC, A, AA	Ata	SP-ID
	10 F	18,1	Ceroid lipofuscinosis type V	CLN5	c.595 C > T	9,6	5	5	NA	4,6	C	Ata	R	C	D	SP-ID
	11 F	18	Angelman syndrome	15q11-q13	PWS-AS maternal allele absence	2	1	10,83	24	1	FIA	Ata	LD	A, FIA	Ata	SP-ID
	12 F	14,4	Angelman syndrome	15q11.2-12	altered methylation fragments SNRPN 04103, 04106,04104,11181	10,8	10	1,5	12	9,3	AA	Ata	LWD	My	Ata	SP-ID
	13 F	14,3	GLUT1-deficiency	SLC2A1	c.470dup	11	3	0,1	24	8	AA	Ata (p)	LWD	AA	Ata, D	M-ID
	14 M	13,5	GLUT 1 deficiency	SLC2A1	c.631 C > T	9	2	5	36	7	AA	Ata (p)	AD	M, TC	Ata	Mo-ID
	15 F	13	SCN1A encephalopathy	*SCN1A*	c.4907G > A; p.Arg1636Gln	8	0,2	7	36	8	C, T, TC	Ata	W	SF	Tr,P,Ata	Mo-ID
	16 F	12,3	GLUT1 deficiency	SLC2A1	c.274 > T	3	0,33	0,66	NA	2,6	FIA	Ata (p)	LDD	FIA	Ata	Mo-ID
	17 M	12,1	Angelman syndrome	15q11q13	15q11q13del	12,4	2	2	24	10,4	AA	Ata	LDD	AC, C	Ata	SP-ID

(Continued)

TABLE 1 | Continued

Predominant movement disorder	Demographic data		Etiology			Age at onset of symptoms and time to diagnosis					Symptoms at the onset			Symptoms during the follow-up		
	Patient and sex	Age	Diagnosis	Gene	Gene variant	Age at the diagnosis	Age at the onset of seizures	Age at the onset of movement disorders	Age at onset of neuro-developmental disorder	Diagnostic delay	Seizure type at the onset	Movement disorder at the onset	Neuro-developmental disorder signs at onset	Seizure type during the follow-up	Movement disorder during the follow-up	Neuro-developmental disorder during the follow-up
	18 M	11,3	Ceroid lipofuscinosis type II	TPP1	c.225A > G/c.1542A > T	4,25	3	3	18	1,25	FTBTC	Ata	LWD	FTBC	Ch	SP-ID
	19 F	5,3	Ceroid lipofuscinosis type II	TPP1	c.1644G > A	4	3	4	40	1	A	Ata	LR	A	Ata	ASD
Dystonia	20 M	32,16	Succinic semialdehyde dehydrogenase deficiency	ALDH5A1	c.526G > A /c.278 > T	11	7	3	36	4	C	D, S	LDD	AA, TC	D, S	ASD
	21 F	17,25	Niemann Pik type C	NPC1	c.349 G > A/c2795+a G > C	4	4	2,3	60	1,6	TC	D. S	AD, R	M, T, FA	D, Br	SP-ID
	22 F	14,9	Rett syndrome	CDKL5	Xp22.13 DEL	6	0,08	1,4	11	5,9	FA	D	R	TC, FTBC, T, C	D	SP-ID
	23 M	14,6	MFF encephalopathy	MFF	c.892 C > T	11	1,5	1,5	12	9,5	M	D	TCD	FA	D	SP-ID
	24 M	14	SCN8A paroxysmal dyskinesia with epilepsy	*SCN8A*	c.4447G > A; p.E1483K	14	9 months	13	84	12	FIA,FA	D (p)	Clu	SF	D, P	Borderline, DCD
	25 M	13,9	Niemann Pick type C	NPC1	c.1211G > A/c.3493G > A	10	10	10,25	84	0	FIA	D, S	AD, R	AA, C, A, FTBC	D	SP-ID
	26 F	13,8	PRRT2-related syndrome	PRRT2	c.649dup	10	0,4	1,1	18	9,5	T	D	LWD	AA, T, M	Ata	SP-ID
	27 M	13,6	KDM5C-encephalopathy	KDM5C	c.1592C > T	8,6	10	7	4	2	TC	D	LD	TC	D	M-ID
	28 M	12,6	ATP1A3 encephalopathy	ATP1A3	c.2324C > G	7	0,25	0,25	24	6,75	T, AA	D (p)	LWD	AA, T	D, Ata	SP-ID
	29 F	10,5	CHD2 encephalopathy	CHD2	c.561del	9	0,66	4	24	8,3	FIA	D	LDD	FTBC, A, C	D	M-ID
	30 F	9,7	PMM2 encephalopathy	PMM2	c.323C>T/ c.710C>G	9	0,4	0,4	20	8,5	FIA	D	TCD	FA	Ata,	SP-ID
	31 M	9,7	Canavan disease	ASPA	G503	1,8	0	1,8	6	1,8	T	D	HCD	C	D, R, My	SP-ID
	32 F	8,2	AP4M1-related syndrome	AP4M1	c.10 C > T/ 498del	6	0,58	0,58	6	5,4	A	D	HCD	AA	D	SP-ID
	33 F	4,6	FOXG1 encephalopathy	FOXG1	c.946del	1,3	0,58	0	24	0,75	FIA	D	TCD	FTBC	D	GDD
	34 M	4	Sodium cluster channel deletion developmental encephalopathy with epilepsy	2q24.3q31.1	Microdeletion on 2q24.3q31.1, (164375953-	1,5	0,5	0,33	8	1	TC	D	TCD	AA, C	D, Ch	GDD
	35 F	2,5	PRRT2-related syndrome	PRRT2	microdel 16p11.2	0,6	0,4	0,66	24	0,25	FTBTC	D	LDD	FTBC, A	D	GDD

(Continued)

TABLE 1 | Continued

Predominant movement disorder	Demographic data		Etiology			Age at onset of symptoms and time to diagnosis					Symptoms at the onset			Symptoms during the follow-up		
	Patient and sex	Age	Diagnosis	Gene	Gene variant	Age at the diagnosis	Age at the onset of seizures	Age at the onset of movement disorders	Age at onset of neuro-developmental disorder	Diagnostic delay	Seizure type at the onset	Movement disorder at the onset	Neuro-developmental disorder signs at onset	Seizure type during the follow-up	Movement disorder during the follow-up	Neuro-developmental disorder during the follow-up
Myoclonus	36 M	28	ARGHEF9	*ARGHEF9*	c.1300G > C (de novo)	24	1,6	3	18	22,4	FS,TC	Hy, My	LDD	SF	Hy,Tr,D	Mo-ID
Stereotypes	37 F	29,5	Rett syndrome	MeCP2	803delG	7	7	2	20	5	A	S	R	TC	S	SP-ID
	38 F	19,5	FOXG1-encephalopathy	FOXG1	c.969delC	7	0,58	0,33	4	6,4	T	S, D	LWD	T	Ch	SP-ID
	39 F	18,08	Rett syndrome	CDKL5	c.587C > T	5	0,08	1	15	4,9	C	S	TCD	T, TC	S	SP-ID
	40 M	16,3	SCN1B-related epilepsy	SCN1B	c.574G > A	11,1	0,8	0,8	12	10,3	FTBTC	S	LDD	FTBC, TC	S	ASD
	41 F	14,6	Angelman syndrome	15q11-q13	paternal uniparental disomy (15q11-q13), altered methylation of maternal allele	3	3	5	21	0,12	M	S	LWD	AA	S	SP-ID
	42 F	14	PURA encephalopathy	PURA	c.768 dup	11	0,9	0,8	3	10,1	T	S	LH	AA, T, A	s	SP-ID
	43 F	13	Rett syndrome	CDKL5	c.551 T > A	2,3	0	2,08	NA	2,33	T	S, A	W	T, IS, TC	D, Ata, S	SP-ID
	44 F	12,25	Rett syndrome	MeCP2	c.502C > T	1,6	1,5	1,75	22	0,16	FIA	S	TCD	FA	S	SP-ID
	45 F	12	Angelman syndrome	15q11.2-12	15q11.2-12 del	3,8	3,8	4	5	0	TC	S	HCD	C	S, My	SP-ID
	46 M	11	Angelman syndrome	15q11-q13	del15q11-q13	1,5	0,91	1,25	12	0,58	C	S	TCD	C, A, TC	S, Tr	SP-ID
	47 F	10,6	Rett syndrome	MeCP2	c.808C > T	4,5	1,5	1	18	0,25	T	S	W	SF	S	SP-ID
	48 F	10,5	IQSEC2-encephalopathy	IQSEC2	c.4110_4111del	6	3	2	24	2,75	T	S	LDD	FA	S	ASD
	49 M	10,4	IQSEC2 encephalopathy	IQSEC2	c.854del	6	2	3	15	4	FTBTC, A	S	TCD	AA, FIA	S, D	SP-ID
	50 F	7	Rett syndrome	MeCP2	c.445.C > G	4	6,3	1,6	18	2,3	T	S	LH	T	S, Ata	SP-ID
	51 F	7	SYNGAP1 encephalopathy	*SYNGAP1*	c.3706C > T	3,6	4	1	15	none	AA, FIA, M	S	TCD	AA, A	S,D,Ata	ASD
	52 M	5,08	PRICKLE1 encephalopathy	PRICKLE1	c.820G > A	1,1	0,83	1	12	0,33	C	S	W	T, FA	S	SP-ID
	53 M	3,1	Williams syndrome	7q11.23	microdeletion 7q11.23	1,6	1,4	1,25	10	0,16	A	S	HCD	T	S, My	SP-ID

T, tonic; A, atonic; AA, Atypical absences; Br, bradykinesia; FTBTC, FA to bilateral tonic clonic; M, myoclonic; FIA, focal seizures with impaired awareness; FA, focal seizures with preserved awareness; C, clonic; D, Dystonia; Ata, ataxia; S, stereotypies; B, ballismus; Ch, chorea; My, myoclonus; p, paroxysmal; R, rigidity; IS, infantile spasms; Tr, Tremor; TC, tonic-clonic; Hy, hyperekplexia; FS, febrile seizures; SF, seizure free; HCD, head control delay; TCD, trunk control delay; LDD, language development delay; LD, language disorder; LR, language regression; AD, academic difficulties; R, psychomotor regression; Clu, clumsiness; LWD, language and walking delay; W, walking delay; LH, language halt; DCD, developmental coordination disorder; Borderline, Borderline cognitive impairment; M-ID, Mild intellectual disability; Mo-ID, moderate intellectual disability; SP- ID, severe/profound intellectual disability; ASD, Autism Spectrum Disorder; GDD, global developmental delay; NA, not available.

TABLE 2 | Demographic, clinical, and molecular genetics features of patients with developmental encephalopathies presenting with epilepsy and hypokinetic movement disorders (Group B).

Predominant movement disorder	Demographic data		Etiology			Age at diagnosis and age at onset of symptoms					Symptoms at the onset			Symptoms during the follow-up		
	Patients - sex	Age (years)	Diagnosis	Gene or cromosomal region Gene variant o cnv		Age at diagnosis (years)	Age at the onset of seizures	Age at the onset of movement disorder	Age at the onset of neuro-developmental disorder	Diagnostic delay	Seizure type at the onset	Movement disorder at the onset	Neuro-developmental disorder signs at onset	Seizure type during the follow-up	Movement disorder during the follow-up	Neuro-developmental disorder during the follow - up
Hypokinesia	1 F	36,91	WDR45 deficiency	*WDR45*	c.439+5G > A	35	5	5	NA	30	TC	H, P	NA	SF	P, D	SP-ID
	2 M	29,73	HIBCH deficiency	*HIBCH*	C.777T > A	25	6	0	6	19	M	H, Ata, My	H	SF	D, H, Tr	SP-ID
	3 F	17,9	DHPR deficiency	*QPDR*	c.41T > C	11,8	3	2	36	8,3	AA	H, Ata, D	LD	FIA, C, TC	P, D	SP-ID
	4 M	13	Menkes disease	*ATP7A*	c.3561G > A	0,5	0,41	0,41	NA	0,08	FIA	H	NA	AA	H	SP-ID
	5 M	8,4	Menkes syndrome	*ATP7A*	c.2938C > T	1	1	0,66	6	0	FIA	H	R	FIA	P	SP-ID
Parkinsonism	6 F	43	Rett syndrome	*MECP2*	c.547A > T	2,08	1,5	1	NA	43	T	S	NA	AA	S, H	SP-ID
	7 M	36,9	KCND3 encephalopathy	*KCND3*	c.901T > C	17	7	5	36	10	FIA	T, D	Clu	FIA	P, D	SP-ID
	8 M	35,15	DHDDS deficiency	*DHDDS*	c.632G > A	35	10	3	18	25	FIA	T, My	TCD	M	P, My	ASD
	9 F	30	KCNQ2 encephalopathy	*KCNQ2*	c.629G > A	30	0	2	NA	30	FIA	S, H	NA	FIA, TC	D, S, H	SP-ID
	10 M	22,9	Neuronal ceroid lipofuscinosis type 6	*CLN6*	c.700 T > C	13,5	13	13	NA	0,5	FIA	My, P	NA	TC	P	SP-ID
	11 F	22	Rett syndrome	*MeCP2*	c.473 C > T	8	4	2	18	44	FIA	S, P	LR	TC	S, H	SP-ID
	12 M	21	Dravet Syndrome	*SCN1A*	C.4814A > T	5	0.5	2.6	120	4.6	C	Tr	AD	T, C, TC	P,Tr	M-ID
	13 F	17	SYNGAP1 encephalopathy	*SYNGAP1*	c.1352 T > A	16	14	15	36	3	AA, M	Tr	LDD	AA, M	P, D, T, Ata	Mo-ID
	14 M	13	WARS2- early onset parkinsonism	*WARS2*	WARS2 c.37T > G and c.679A > G	12,5	5	0,91	13	11,5	FIA	P, T, My, D	R	FIA	P, My, D, Tr	SP-ID
	15 M	9,9	Menkes disease	*ATP7A*	del c.467 delA	0,6	0,66	0	6	0	IS	P	H	SF	P	SP-ID
	16 M	8,75	Adenyl-succinate lyase deficiency	*ADSL*	c.65C > T and c.340 T > C	6,4	0,25	0	6	6,16	FIA	P	H	T, M, TC	P	SP-ID

FIA, focal seziures with impaired awareness; S, Stereotypes; TC, Tonic-clonic; H, hypokinesia; M, Myoclonic; AA, Atypical absences; IS, Infantile spasms; Ata, Ataxia; C, clonic; SF, seizure free; D, dystonia; P, Parkinsonism; Tr, Tremor; My, myoclonus; NA, not available; HCD, head control delay; TCD, trunk control delay; LDD, language development delay; LD, language disorder; LR, language regression; AD, academic difficulties; R, psychomotor regression; Clu, clumsiness; LWD, language and walking delay; W, walking delay; LH, language halt; DCD, developmental coordination disorder; Border, Borderline cognitive impairment; M-ID, Mild intellectual disability; Mo-ID, moderate intellectual disability; SP- ID, severe/profound intellectual disability; ASD, Autism Spectrum Disorder; GDD, global developmental delay.

Tables 1, **2** summarize the demographic, genetic, and clinical data of patients with hyperkinetic (Group A) and hypokinetic movement disorders (Group B).

Table 3 summarizes both groups: (a) demographic data; (b) temporal distribution of clinical presentations (e.g., age at the onset of each cluster of symptoms) and diagnostic steps (e.g., age at the diagnosis and diagnostic delay); (c) distribution of seizure and movement disorder types.

In Group B the mean patient age was almost double compared to that of Group A (**Table 3**).

A neurodegenerative course was observed in 30/53 patients of Group A and 14/16 patients of Group B.

ETIOLOGICAL SPECTRUM

The distribution of genetic etiologies according to the different presenting seizure and movement disorder types in the whole cohort is summarized in **Figure 1**, **Table 4**.

Almost half of the cases carried pathogenic variants of genes involved in the regulation of the cellular cycle and intracellular metabolism (**Table 4**).

In Group A the most frequent diseases were Angelman Syndrome (6/53) and typical (5/53 patients harboring pathogenic MeCP2 variants) or Atypical Rett Syndrome (8/53 patients with pathogenic variants in *CDKL5*, *FOXG1*, *IQSEC2*, *PURA* or *MEF2C* genes), accounting for 35% of diagnosis (19/53).

TABLE 3 | Differences between groups in continuous and discrete variables.

	Group A ***N*** **= 53 (29 F; 24 M)**		**Group B** ***N*** **= 16 (6F; 10M)**		**F**	***p*-value**
	M	**SD**	**M**	**SD**		
Age (years)	14,04	7,11	23,62	11,92	15.849	0.001
Age at the diagnosis (years)	7,48	6,34	16,32	13,61	13.203	0.001
Age at the onset of seizures (years)	2,63	3,15	4,45	4,55	3.280	0.075
Age at the onset of movement disorders (years)	3,50	3,75	3,55	4,55	0.01	0.974
Age at the diagnosis of neurodevelopmental disorder (years)	2,28	2,76	1,78	1,41	0.744	0.392
Diagnostic delay (years)	5,66	6,41	12,26	13,32	7,467	0.008
	N	**%**	***n***	**%**		
Gender					1.458	0.265
M	24	45.25	10	62.5		
F	29	54.75	6	37.5		
Seizure types					15,353	0.004
Generalized motor	27	50,9	5	31,25		
Generalized non motor	5	9.4	-	-		
Focal to bilateral tonic clonic	5	9.4	-	-		
Focal motor	10	18,8	11	68,75		
Focal non-motor	6	11,3	-	-		
Movement disorder type					44.167	0.001
Ataxia	13	24,5	1	6,25		
Dystonia	17	32,07	1	6,25		
Parkinsonism	-	-	5	31.25		
Stereotypies	17	32,07	3	18,75		
Hypokinesia	-	-	3	18,75		
Tremors	-	-	3	18,75		
Chorea	4	7.5	-	-		
Neurodevelopment disorder type						
Developmental coordination disorder	1	1	-	-		
Borderline cognitive impairment	3	6	-			
Global developmental delay	3	6	-	-		
Mild intellectual disability	3	6	1	3		
Moderate intellectual disability	5	9	1	3		
Severe and profound intellectual disability	33	62	13	91		
Autism spectrum disorder	5	9	1	3		

Significance = $p < 0.01$.

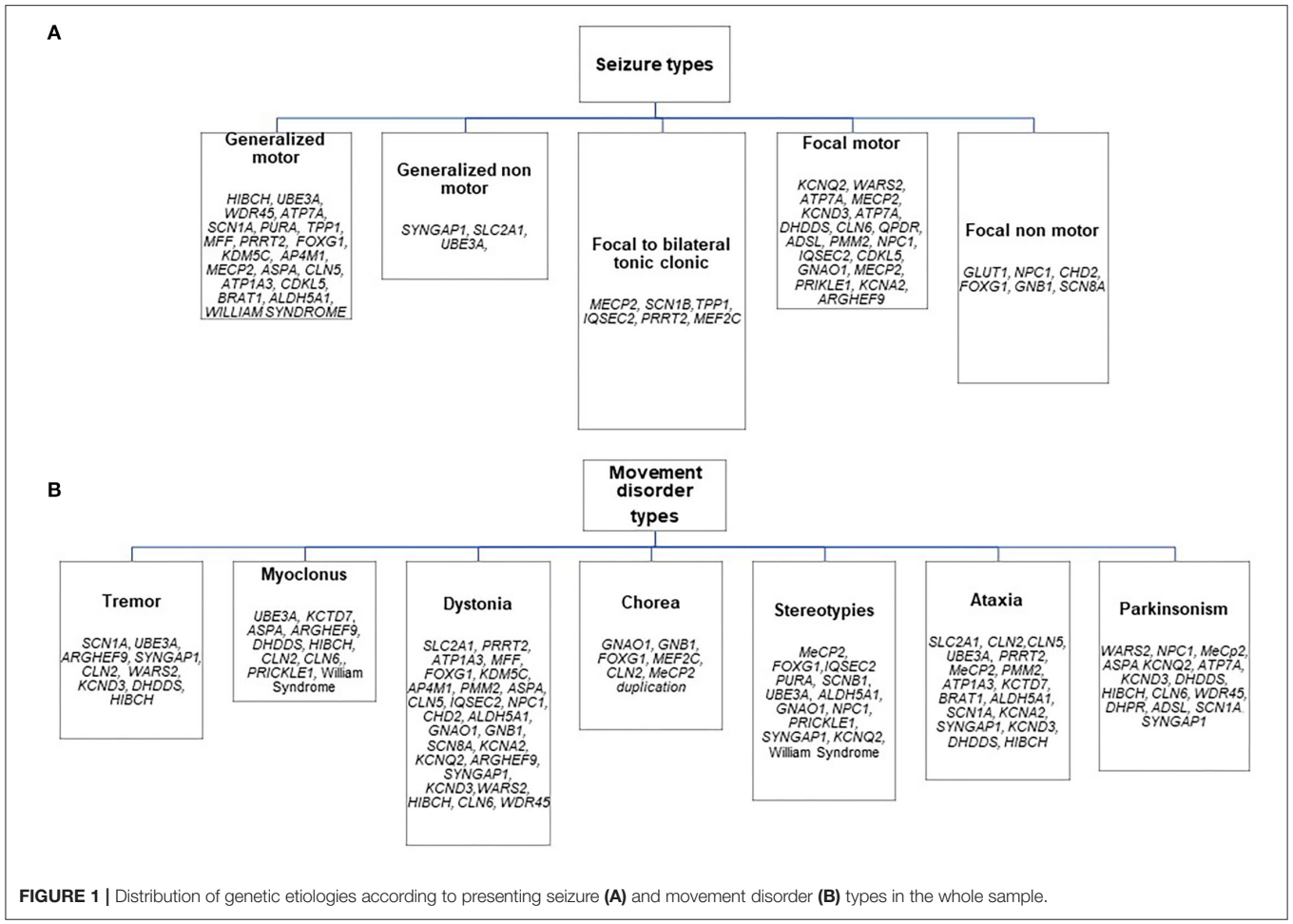

FIGURE 1 | Distribution of genetic etiologies according to presenting seizure **(A)** and movement disorder **(B)** types in the whole sample.

A metabolic disorder was diagnosed in 22% of patients (13/53). Sodium and potassium channelopathies (5/23 patients), synaptopathies (3/53), and postsynaptic signaling disorders (3/53) accounted for most of the remaining cases.

In Group B 10/16 patients had a metabolic disorder (3/9 with Menkes disease), 3/16 a sodium or potassium channelopathy (*SCN1A*, *KCNQ2*, *KCND3*), 2/16 Rett syndrome, and 1/16 a synaptopathy (**Table 2**). All metabolic disorders described in this group, except for DHPR deficiency, had a neurodegenerative course and 5/16 were associated to brand new genes or genes described in the last decade (e.g., *WARS2*, *KCND3*, *DHDDS*, *HIBCH*, *WDR45*).

A subset of genes including MeCP2, *SCN1A*, and *SYNGAP1* was identified both in Group A and B in patients with different ages and disease stages (**Tables 1**, **2**). Coherently hypokinetic features have been reported as late features in these conditions, preceded by hyperkinetic movement disorders.

DEVELOPMENTAL HISTORY

A complete developmental history was available for 62/69 patients. The mean age at the first signs of neurodevelopmental impairment was lower than the onset of seizures and movement disorders without significant differences between Group A and Group B (**Table 3**).

No developmental milestones were achieved in 16/69 patients while 48/69 patients experienced different degrees of delay in one or more developmental milestones (**Table 3**). Motor impairment was variable with an autonomous walking that was achieved in 34/69 patients while 18/69 were wheelchair bounded and 17/69 were bedridden.

A profound-severe to moderate cognitive impairment was diagnosed in 58/69 patients (71% of patients in Group A vs. 94% of patients in Group B). Developmental regression was observed in 8 patients (Patients 9, 10, 19, 21, 22, 25, 37 in **Table 1** and patient 14 in **Table 2**). An autism spectrum disorder was diagnosed in 12% of patients.

The developmental impairment was borderline in 2 patients belonging to group A (patients 8 and 24 in **Table 1**). Patient 8 in **Table 1** was a female carrying a pathogenic variant of *BRAT1* gene who had also a less severe epilepsy phenotype compared to other cases previously reported in the literature harboring the same variant (11). Patient 24 in **Table 1** presented with a previously reported *SCN8A* variant associated with a benign childhood focal epilepsy, paroxysmal dyskinesia, and borderline cognitive functioning with minor coordination issues (12).

TABLE 4 | Distribution of epilepsy and movement disorder phenotypes at onset and during the follow-up in the different functional groups of genetic etiologies.

Etiological category	Genes	N of patients	Movement disorder phenotype at onset (n of patient)	Movement disorder phenotype on follow up	Seizures phenotype at onset (n of patient)	Seizures phenotype on follow up
Channellopathies	*KCTD7, KCNQ2, KCNA2, KCND3, SCN1A, SCN1B, SCN8A, 2q24.3q31.1 deletion*	9	Dystonia (3/9), Ataxia (2/9), Stereotypies (2/9), Chorea(1/9), Tremors (1/9)	Dystonia 4/9, Ataxia (2/9), Parkinsonism (1/9), Tremors (1/9), Stereotypies (1/9)	Generalized motor (4/9), Focal motor (3/9), Focal to bilateral tonic-clonic (1/9), Focal non motor (1/9)	Generalized motor (5/9), Focal motor (2/9), Focal to bilateral tonic-clonic (1/9), Focal non motor (1/9)
Transportopathies	*SLC2A1, ATP1A3, ATP7A*	7	Ataxia (3/7), Hypokinesias (2/7), Dystonia (1/7), Parkinsonism (1/7)	Ataxia (2/7), Dystonia (2/7), Parkinsonism (1/7), Hypokinesias (1/7) Stereotypies (1/7)	Generalized motor (2/7), Generalized non motor (2/7), Focal Motor (2/7), Focal non motor (1/7)	Generalized non motor (4/7), Focal motor (1/7), Focal non motor (1/7), seizure free (1/7)
Synapthopathies	*PRRT2*	2	Dystonia (2/2)	Chorea (1), Dystonia (1)	Generalized motor (1/2), Focal to bilateral tonic-clonic (1/2)	Generalized non motor (1/2), Focal to bilateral tonic-clonic (1/2)
Disorders of intermediate metabolism	*DHPR, ALDH5A1, HIBCH, ASPA, ADSL, WARS2*	7	Dystonia (2/7), Ataxia (2/7), Parkinsonism (2/7), Hypokinesias (1/7)	Parkinsonism (3/7), Dystonia (3/7), Ataxia (1/7)	Generalized motor (4/7), Focal motor (3/7)	Generalized motor (3/7), Generalized non motor (1/7), Focal to bilateral tonic-clonic (1/7), Focal motor (1/7), seizure free (1/7)
Disorders of complex molecule and organelle metabolism	*CLN2, CLN5, PMM2, MFF, WDR45, AP4M1, NPC1, DHDDS*	11	Dystonia (5/11), Ataxia (3/11), Parkinsonism (3/11), Tremors (1/11)	Dystonia (5/11), Parkinsonism (3/11), Stereotypies (2/11), Ataxia (1/11)	Generalized motor (5/11), Focal motor (4/11), Focal non motor (1/9), Focal to bilateral tonic-clonic (1/9)	Generalized motor (5/11), Generalized non motor (2/11), Focal motor (2/11), Focal non motor (1/9), Focal to bilateral tonic-clonic (1/9)
Disorders of post-synaptic cellular signaling	*IQSEC2, GNAO1, GNB1, ARGHEF9, SYNGAP1*	7	Stereotypies (4/7), Tremors (1/7), Chorea (1/7), Myoclonus (1/7)	Stereotypies (3/7), Parkinsonism (1/7), Dystonia (1/7) Chorea (1/7), Hyperklepsia (1/7)	Generalized motor (3/7), Generalized non motor (2/7), Focal non motor (1/7), Focal to bilateral tonic-clonic (1/7)	Generalized motor (2/7), Generalized non motor (3/7), Focal non motor (2/7)
Disorders of cellular cycle's regulation	*FOXG1, PURA, CDKL5, MECP2, CH2D, BRAT1, KDM5C, MEF2C, PRIKLE1*	19	Stereotypies (10/19), Dystonia (4/19), Ataxia (1/19), Chorea (3/19)	Stereotypies (10/19), Dystonia (4/19), Ataxia (2/19), Chorea (2/19)	Generalized motor (10/19), Focal motor (5/19), Focal to bilateral tonic-clonic (2/19), Focal non motor (2/19)	Generalized motor (9/19), Generalized non motor (3/19), Focal motor (2/19), Focal to bilateral tonic-clonic (1/19), Focal non motor (3/19)
Disorders of degradation/turnover of intra and extracellular components	*UBE3A*	6	Ataxia (3/6), Stereotypies (3/6)	Ataxia (4/6), dystonia (1/6), Stereotypies (1/6)	Generalized motor (3/6), Generalized non motor (2/6), Focal motor (1/6)	Generalized motor (4/6), Generalized non motor (1/6), Focal non motor (1/6)

EPILEPSY PHENOTYPE

Seizure onset occurred during infancy or early childhood in 82,7% of patients. Motor seizures accounted for 82,61% of initial epileptic manifestations with almost half of the patients presenting with a generalized semiology while the frequency of non-motor seizures tended to double on follow-up (**Table 4**).

Patients belonging to Group A experienced an earlier onset of seizures than the ones of group B even if the difference in terms of age at the onset was not statistically significant (**Table 3**).

The initial epileptic manifestations mainly included generalized motor seizures in Group A (with a predominance of tonic seizures accounting for 21% of cases) and focal motor seizures with impaired awareness in Group B (56% of the cases) (**Tables 1**–**3**). In Group A, seven patients became seizure-free (13,2%), and none in Group B (**Tables 1**, **2**).

These clinical patterns were associated with a concurrent predominance of multifocal (58,5% of patients in Group A) and focal (43% of patients in Group B) interictal EEG abnormalities. EEG was generally non-specific in terms of consistency with the epilepsy phenotype reported in the literature for the considered gene, with two significant exceptions in Group A (**Table 1**: Patients 9 and 19 who had early onset photosensitivity at low frequencies in association, respectively, with a neuronal ceroid lipofuscinosis type 2 and KCTD7-related progressive myoclonus epilepsy) and in two patients of Group B (**Table 2**: Patient 9 who had focal abnormalities during neonatal age as frequently reported in KCNQ2 encephalopathy and Patient 15 with Menkes disease who experienced a West syndrome at the age of 8 months).

The response to antiepileptic treatments was poor in most patients with the best response to valproate (27.65% of patients in Group A and 28,57% in Group B). A ketogenic diet resulted in a dramatic seizure reduction in 3 patients with Glut1 deficiency (**Table 1**: patients 13, 14, and 16) and one patient with adenyl-succinate lyase deficiency (**Table 2**: patient 16). The treatment with carbamazepine resulted in a mild improvement of paroxysmal dystonia in one patient with ATP1A3 encephalopathy (**Table 1**: patient 28) while a ketogenic diet was associated with an improvement of ataxic gait in 3 patients with Glut1 deficiency (**Table 1**: patients 13, 14, and 16). Valproate induced a worsening of tremors in 5 patients (**Table 1**: patients 13, 36, and 46; **Table 2**: patients 2 and 13).

MOVEMENT DISORDER PHENOTYPE

The onset of movement disorders was noticed in the age-range 1–6 years in 57,97% of cases and before the age of 12 months in 24,63% of patients. A relevant number of patients presented with more than one subtype of hyperkinetic movement disorder (11 out of 53 in Group A) or hyper and hypokinetic features observed simultaneously at disease onset or sequentially during the follow-up (7 out of 16 in Group B). In 7 out of 16 patients belonging to Group B, parkinsonism was preceded by hyperkinetic movement disorders (Patients 2, 4, 6, 7, 9, 11, 13 in **Table 2**).

Stereotypes and dystonia were the most frequent hyperkinetic movement disorders at the onset in Group A (17 patients for both symptoms: **Tables 1**, **3**). The first ones were predominant in the disorders of the cellular cycle's regulation while dystonia was the presenting signs of different groups of disorders involving all the steps of cellular signaling and metabolism (**Figure 1**; **Table 4**). Paroxysmal movement disorders were observed at the onset in 3 patients with GLUT1 deficiency who presented with episodic ataxias and in 1 patient with ATP1A3-related dystonic attacks (Patients 13, 14, 16, and 28 in **Table 1**).

In Group A, only 2 patients (Patients 6 and 18 in **Table 1**) received pharmacological treatments for movement disorders with a limited response, while a few therapeutic strategies were attempted in Group B in which the most relevant positive effects were observed in 2 patients (Patient 3 and 14 in **Table 2**) who received dopaminergic agents to treat, respectively, WARS2-related parkinsonism and a DHPR deficiency.

TIME TO ACHIEVE A MOLECULAR GENETIC DIAGNOSIS

The present study failed to demonstrate that specific combinations of seizure or movement disorder types might have been associated with an earlier etiological diagnosis (**Tables 3**, **5**, **6**). Diagnostic delay was longer in Group B than in Group A (**Tables 3**, **5**). The analysis of the distribution of genetic diagnosis per year and diagnostic delay showed a higher gain in diagnostic yield of molecular genetic investigations in the last 5 years (**Figures 2A,B**).

Analysis of variance and *post-hoc* tests with Bonferroni correction for multiple comparisons evidenced, in Group A, a significantly longer diagnostic delay and higher age at molecular genetic diagnosis in patients who presented with chorea at the onset if compared with the ones presenting with ataxia, dystonia, and stereotypies (**Table 6**).

DISCUSSION

The literature provides few epidemiological data about the distribution of molecular genetic diagnosis of developmental encephalopathies with epilepsy and movement disorders in the pediatric populations while several recent studies reported detailed and updated genotype-phenotype correlations for about 60 single-gene related disorders other than historically well-known patterns such as the ones of Rett or Angelman syndrome (1, 13–18). The studies including epidemiological data were often hardly comparable because of several methodological differences in terms of analyzed cohorts, inclusion and exclusion criteria, and modalities of data collection (1, 2). Most of these data were not statistically significant because of the rare or ultrarare prevalence of the analyzed diseases.

A recent systematic review of 49 papers identified 27 neonatal-onset single gene-related diseases presenting with severe epileptic and developmental encephalopathies and a predominance of

TABLE 5 | Differences between Group A and Group B in terms of diagnostic delay and temporal periods in which the molecular genetic diagnosis was made.

	Period 1		Period 2		Period 3		Period 4		F	*p*-value
	M	sd	M	sd	M	sd	M	sd		
Group A (*N* = 53)										
Diagnostic delay (years)	6.86	7.52	5.26	6.22	3.18	2.28	3.60	1.73	0.933	0.432
$^{*}p < 0.01$										
Group B (*N* = 16)										
Diagnostic delay (years)	23.15	13.32	3.1	4.13	4.67	4.07	-	-	8,126	0.005

Period 1, 2016-2020; Period 2, 2011- 2015; Period 3, 2006-2010; Period 4, before 2005.

TABLE 6 | Differences in terms of age at diagnosis, age at the onset of movement disorders, and diagnostic delay according to the different hyperkinetic movement disorders that were observed in patients of Group A.

	Ataxia		Dystonia		Stereotypies		Chorea		F	*p*-value
	M	sd	M	sd	M	sd	M	sd		
Group A (*N* = 53)										
Age at the diagnosis (years)	8.04	4.68	6.69	48,96	4.08	3.04	16.33	12,63	6,792	,001
Age at the onset of the movement disorders (years)	5.05	4.40	3.10	3.74	1.81	1.24	6.03	4.83	3,191	,032
Diagnostic delay (years)	5.26	3,18	5.20	5.14	3.05	3.38	15.27	12.12	7,573	,001

Significance = $p < 0.05$.

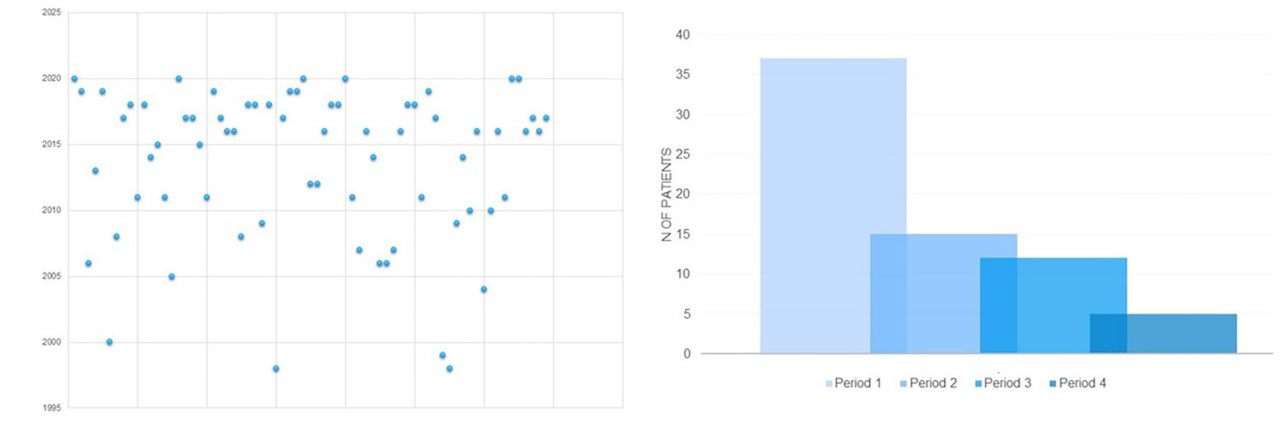

FIGURE 2 | (A) Distribution of molecular genetic diagnosis per year in the whole sample. **(B)** Distribution of molecular genetic diagnosis according to temporal periods in which the molecular genetic diagnosis was made. PERIOD 1: 2016-2020; PERIOD 2: 2011- 2015; PERIOD 3 = 2006-2010; PERIOD 4 = before 2005.

hyperkinetic movement disorders in more than 85% of the cases, with neurometabolic conditions not included in the analysis (2). The proportion of neurometabolic diseases in a Canadian cohort of 197 patients referring to a single pediatric epilepsy center, who underwent targeted gene panels or whole-exome sequencing, accounted for 13% of cases (19). In the same cohort, a co-occurrent movement disorder was reported in almost one-fourth of the cases with a large predominance of dystonia (8,6% of the patients) (19). In a smaller Japanese cohort of 11 patients, 9 different monogenic diseases presenting with early-onset hyperkinetic movement disorders were diagnosed in 7 infants who had a West syndrome and in 2 children with a non-syndromic epileptic encephalopathy (20). Trump et al. (21) identified movement disorders in 4 out of 71 patients with a confirmed genetically determined epilepsy while Cordeiro et al. (22) reported an epileptic syndrome in 13 out of 21 patients with a genetically determined movement disorder.

The herein-reported single center retrospective cohort analysis depicted the heterogeneous clinical panorama and the diagnostic yield that may be observed in a specialistic setting.

The analysis of etiologies suggested a predominant association of chorea with a subset of genes or CNV involved in post-synaptic

signaling (e.g., GNAO1, GNB1, FOXG1, MEF2C, CLN2, MeCP2 duplication) and a high quote of the pathogenic gene or chromosomal variants associated with neurodegenerative and metabolic diseases among patients presenting with myoclonus and parkinsonism.

Patients with hyperkinetic movement disorders, that were diagnosed in our center, presented with an earlier onset of epileptic seizures, a predominant generalized motor semiology of seizures at the onset, and prominence of multifocal EEG abnormalities. The prominent phenotypic features of patients with hypokinetic movement disorders included a later onset of seizures, a higher frequency of focal seizures at the onset, and focal EEG abnormalities.

The onset of seizures was noticed earlier than the onset of movement disorders in patients presenting with hyperkinetic movement disorders while the opposite occurred in patients with hypokinetic manifestations.

A possible explanation might be represented by an underdiagnosis of movement disorders because of their benign nature (e.g., stereotypes) or lack of awareness, vs. the higher social and clinical alarm induced by seizures. A second explanation could rely upon the different etiological spectrum observed in hyperkinetic and hypokinetic groups. In most patients from Group B (60 vs. 20% in Group A), epilepsy is the result or the by-product of a neurodegenerative process, and therefore it can appear later in the disease course, while in most patients from Group A (42/53) epilepsy is one of the first signs of a developmental and epileptic encephalopathy related to disfunction of genes involved in neurotransmission, neuronal excitability, or neural development.

A significant diagnostic delay was experienced in patients in which chorea represented the main movement disorder at the onset. This delay may result from different factors: (a) the highest tendency of physicians, especially if specific expertise in rare diseases lacks, to consider chorea mainly as a symptom of acquired diseases (e.g., Sydenham Chorea or other autoimmune disorders) with a possible underestimation of genetic etiologies; (b) the late diagnosis in patients carrying variants in novel disease-causing genes that were discovered up to several years after the onset of symptoms and the first clinical evaluation of patients in which pathogenic single gene variants or chromosomal aberrations were reported for the first time in the literature) (23).

The significantly longer diagnostic delay in Group B is probably due to the extremely rare occurrence of hypokinetic movement disorders in pediatric ages, especially in patients studied before the era of next generation sequencing. An extensive educational campaign focused on the peculiar phenotypic features and the availability of reliable biochemical (e.g., CSF neurotransmitter measurement in the disorders of monoamine metabolism or urinary copper in Menkes disease) and neuroimaging (e.g., cerebellar atrophy in Neuronal Ceroid Lipofuscinosis or lactate peak at HMRS in Menkes disease) biomarkers might increase the knowledge of genetically determined hypokinetic movement disorders among pediatric neurologists (13).

The limits of the study are strictly correlated with its retrospective design, the rarity of the explored diseases, and the heterogeneous spectrum of genetic etiologies while a bias might be represented by the inclusion of patients with an established molecular genetic diagnosis only (and the subsequent exclusion of patients with comparable spectra but without molecular genetic confirm). Moreover, this study did not systematically explore the longitudinal changes of epilepsy and movement disorder phenotypes from infancy into adulthood with a subsequent possible loss of useful information about prognostic implications even if these details are already available in the literature for some single-gene diseases (24–26). A further limit may be correlated with the current unavailability of an acceptable classification for movement disorders taking into account specific pediatric peculiarities (i.e., the high quote of mixed movement disorders, disorders of tone and postures, weakness, ataxia, and apraxia).

CONCLUSIONS

This paper explored the presenting patterns of genetically determined encephalopathies with epilepsy and movement disorders and highlighted some relevant clinical and diagnostic issues including (a) a more frequent etiological role of abnormalities of genes/chromosomal regions involved in neurotransmission, neuronal excitability, or neural development in patients with hyperkinetic movement disorders; (b) a higher frequency of neurodegenerative courses and a longer diagnostic delay in patients with hypokinetic movement disorders.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MM and SG contributed to the conception and design of the study and wrote the first draft of the manuscript. SC, AR, and LC contributed to data collection and data analysis and wrote some sections of the manuscript. VL revised the manuscript. All authors contributed to the manuscript and read and approved the submitted version.

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REVIEW
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Gene Therapy: Novel Approaches to Targeting Monogenic Epilepsies

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Genetic epilepsies are a spectrum of disorders characterized by spontaneous and recurrent seizures that can arise from an array of inherited or de novo genetic variants and disrupt normal brain development or neuronal connectivity and function. Genetically determined epilepsies, many of which are due to monogenic pathogenic variants, can result in early mortality and may present in isolation or be accompanied by neurodevelopmental disability. Despite the availability of more than 20 antiseizure medications, many patients with epilepsy fail to achieve seizure control with current therapies. Patients with refractory epilepsy—particularly of childhood onset—experience increased risk for severe disability and premature death. Further, available medications inadequately address the comorbid developmental disability. The advent of next-generation gene sequencing has uncovered genetic etiologies and revolutionized diagnostic practices for many epilepsies. Advances in the field of gene therapy also present the opportunity to address the underlying mechanism of monogenic epilepsies, many of which have only recently been described due to advances in precision medicine and biology. To bring precision medicine and genetic therapies closer to clinical applications, experimental animal models are needed that replicate human disease and reflect the complexities of these disorders. Additionally, identifying and characterizing clinical phenotypes, natural disease course, and meaningful outcome measures from epileptic and neurodevelopmental perspectives are necessary to evaluate therapies in clinical studies. Here, we discuss the range of genetically determined epilepsies, the existing challenges to effective clinical management, and the potential role gene therapy may play in transforming treatment options available for these conditions.

Keywords: genetic epilepsy, AAV9, Lafora, SLC13A5, SLC6A1, gene therapy (GT)

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INTRODUCTION

While 20–30% of epilepsies are acquired nongenetically, 70–80% are due to 1 or more genetic factors (1). Developmental and epileptic encephalopathies (DEE) are rare disorders characterized by early-onset, refractory seizures that occur in the context of developmental regression or plateauing. DEE are severe and difficult to treat and may result from a single gene mutation that causes gain-of-function (2) or loss-of-function epilepsy (3, 4). Monogenic epilepsies may

be autosomal recessive (e.g., EPM2A/B or *SLC13A5*), autosomal dominant (e.g., *CHRNA4*), autosomal haploinsufficiency (e.g., *SLC6A1*), or X-linked (e.g., *ARHGEF9*) (5–10). Further, different pathogenic variants in the same gene may result in different epilepsy phenotypes, as seen in the *KCNQ2* gene, where the R213W variant causes benign familial neonatal seizures, and the R213Q variant causes neonatal epileptic encephalopathy with severe pharmacoresistant seizures (11).

Precision medicine describes a rational treatment strategy that is highly specific and aims to address the underlying cause of disease (12). One avenue of precision medicine involves the selection of a therapy that is directed toward modulating or bypassing the dysfunction caused by the underlying genetic defect (12). In the era of gene therapy, avenues that may be applied to epilepsy syndromes include treatments that aim to restore cellular function such as gene replacement therapy (GRT) for disorders due to loss-of-function pathogenic variants (13, 14); genetic substrate reduction therapy (gSRT) [reviewed in Coutinho et al. (15)] to reduce the overproduction of substrates; or transcriptional enhancement, designed to upregulate endogenous expression of a given gene *via* the introduction of regulatory elements (16, 17). Monogenic epilepsies are of particular interest for precision medicine, as simplified GRT, gSRT, and transcriptional enhancement therapies are promising in ameliorating disease. Here, we will focus specifically on Lafora disease, *SLC13A5* deficiency disorder (SDD), and *SLC6A1*-related disorder (SRD).

CLINICAL CARE

Current treatment approaches focus on treating the epilepsy syndrome *via* antiseizure medications, diet, and/or neurostimulation, rather than the underlying genetic basis of disease (9). Combinations of antiseizure medications may be necessary to achieve adequate seizure control. Further, patients may become refractory to antiseizure medications over time (18) and for some patients, specific antiseizure medications are contraindicated, as they may exacerbate neurodevelopmental disability associated with their specific epilepsy syndrome (19). Ketogenic (high fat/low carbohydrate) diets and vagus nerve stimulation approaches also have been attempted in patients with inadequate seizure control, however, with limited success (20–25). Notably, there are no currently approved treatments that address the underlying cause of disease for genetic epilepsies, presenting an urgent need for the community and an opportunity for novel approaches such as GRT and gSRT.

HISTORICAL CONTEXT

Advances in Genetic Diagnosis

Prior to modern genetic approaches, epilepsies were examined for their genetic basis in families using gene mapping and applied linkage analysis. The first discoveries in the 1990s identified ion channels and led to the "channelopathy" hypothesis that suggested that ion channel defects were a common underlying cause of epilepsy (1). Additionally, it is now recognized that other single-gene pathogenic variants contribute to seizure disorders.

Starting in the late 2000s, next-generation sequencing has increasingly led to discovery of pathogenic variants in specific genes and microdeletions resulting in epilepsies (26). Commercially available epilepsy panels are available to test for many genetic epilepsies.

Still, many genetic epilepsies and their natural histories are not well understood. The prognosis for genetic epilepsies is often not promising, and there is a need for innovative solutions to improve patient outcomes. In addition to the development of novel pharmaceuticals, genetic epilepsies may be approached *via* gene therapy.

Advances in Gene Therapy

The first successful human trial of gene therapy occurred in 1990 (27). The field has rapidly expanded in the twenty-first century. One approach is GRT, which utilizes a vector such as adeno-associated virus (AAV) serotype 9 (AAV9), to deliver a functional copy of a gene to correct loss-of-function pathogenic variants, including recessive disorders (e.g., *SLC13A5*) and haploinsufficiencies (e.g., *SLC6A1*) (7, 9, 13). One example is the recent FDA approval of a gene therapy product to treat spinal muscular atrophy—a rare disease that causes infant mortality—which was the first gene therapy approval for children >2 years of age (28). There are also AAV9-based gene therapies in neurodevelopmental disorders in clinical trials (NCT02362438) following promising preclinical results (13).

The gSRT approach may utilize an AAV vector to deliver small interfering RNA that will reduce the overproduction of substrates (15). For example, the *GYS1* gene may be knocked down to prevent the overproduction of the substrate glycogen, which accumulates to cause Lafora disease (16, 29). Transcriptional enhancement approaches may be effective in haploinsufficiencies such as Dravet syndrome, where 1 allele of the *SCN1A* gene possesses loss-of-function pathogenic variants, and the other normal endogenous allele can be modified to increase its expression levels (17). These approaches have the potential to address the underlying cause of disease in inherited epilepsies that are the result of loss-of-function pathogenic variants and provide significant seizure relief to patients.

AAV vectors have been extensively studied for treatment of central nervous system (CNS) diseases (30). AAV9, specifically, is a vector with great potential for treating neurological disorders, as it crosses the blood-brain barrier and targets CNS neurons (31). While other viral vectors transduce neurons, AAV9 is the most studied AAV vector for CNS disorders, and there is more clinical evidence of safety, efficacy, and stability of gene transfer to the CNS with this serotype than with other vectors (32).

Further, to aid in the development of next-generation gene therapy technologies for diagnosis of genetic epilepsies, a better understanding of natural history of disease will be required and is addressed in the next section. These studies inform clinical development and help identify outcome measures for clinical investigation.

TABLE 1 | Potential monogenic epilepsy candidates for gene therapy.

Disorder	Gene	Protein	Protein function	Most common seizure type	Mouse model
Dravet syndrome	*SCN1A*[#]	$Na_v1.1$	Voltage-gated sodium channel (34, 35)	GTCS (36)	*Scn1a* +/– (35)
EIEE (8)	*SLC13A5*	NaCT	Plasma membrane sodium-dependent citrate transporter (37–39)	Clonic or Tonic (40)	*Slc13a5* KO (41)
	*ARHGEF9**	Collybistin	GABA receptor clustering at inhibitory synapses (42)	GTCS (5)	*Arhgef9* KO (5)
	WWOX	WWOX	Development and function of CNS (43)	GTCS (44)	*Wwox* KO (43)
Familial infantile myoclonic epilepsy or EIEE	*TBC1D24*	TBC1D24	Vesicle trafficking for neuronal signal transmission (45)	Myoclonic or clonic seizures (46)	*S324Tfs*3* (45)
Lafora—PME	*EPM2A*	Laforin	Glycogen phosphatase (47)	GTCS (48)	*Epm2a* KO (47)
	EPM2B	Malin	Ubiquitin E3 ligase (47)		*Epm2b* KO (47)
Pyridoxine dependent epilepsy	*ALDH7A1*	ALDH7A1	Lysine catabolism (49)	Focal Seizures (50)	*Aldh7a1* KO (49)
SLC6A1-related disorder	*SLC6A1*[#]	GAT-1	Sodium- and chloride-dependent GABA transporter (51)	Absence seizures (52)	*Slc6a1* KO (51)

**X-linked.*

[#]*Haploinsufficiency.*

All others are autosomal recessive.

CNS, central nervous system; EIEE, early infantile epileptic encephalopathy; GABA, gamma-aminobutyric acid; GAT, GABA transporter; GTCS, generalized tonic-clonic seizures; KO, knockout; PME, progressive myoclonus epilepsy.

CLINICAL TRIAL READINESS

Studies into the natural history of disease are essential to understanding how diseases progress and to inform drug development so that researchers and clinicians can have strong metrics available to evaluate how best to demonstrate efficacy and, ultimately, improve patients' quality of life. Regulatory agencies are increasingly acknowledging the importance of natural history data in the context of rare disease and gene therapy drug development, having released draft guidance on the topics in recent years (33). Natural history studies, although informative, may not accurately represent disease populations due to factors such as study design, variability of supportive care practices, changes in medical care or terminology over time, selection bias, etc. In monogenic epilepsies, due to the relatively recent identification of genetic causes, may particularly be lacking in a detailed and longitudinal understanding of the disease course. Animal models, therefore, also have an important role to play in understanding disease progression. Animal models currently exist for some, but not all, of the recessive and haploinsufficient epilepsies (see **Table 1** for examples of available models) but may not fully replicate the clinical phenotype, which represents a challenge to characterizing the outcomes of potentially disease-modifying investigational drugs. While electroencephalography findings in animal models are comparable to those in humans, neurologic and motor deficits do not always correspond well with the human disease. GRT and gSRT approaches utilizing AAV vector technology may address diseases resulting from pathogenic variants in single genes (13, 15). In particular, AAV9 has shown promise for treating neurological disorders as it crosses into the brain and infects neurons (31). In the following sections, this review will highlight 3 monogenic inherited diseases, areas of active research by our groups: Lafora disease, SDD, and SRD, as well as their clinical picture, mouse models, and approaches to gene therapy for each condition.

Lafora Disease

Lafora disease is a severe, fatal, autosomal recessive progressive myoclonus epilepsy (PME) that results from accumulation of Lafora bodies, abnormal glycogen aggregates (6). Two genes are now known to be involved in Lafora disease: *EPM2A* and *EPM2B* (48, 53–56). Loss-of-function pathogenic variants in *EPM2A* or *EPM2B* lead to an accumulation of Lafora bodies (an abnormal form of glycogen that cannot be metabolized) and subsequent Lafora disease (47).

Presentation and Progression

The mean age of Lafora disease onset is 13.4 years (57). Patients with classical Lafora disease develop normally until adolescence, when they present with action and stimulus-sensitive myoclonus, in addition to tonic-clonic and absence seizures (48). At presentation, it is challenging to distinguish Lafora disease from idiopathic generalized epilepsies (48). Thus genetic testing is critical, as it reveals pathogenic variants in the *EPM2A* and *EPM2B* genes (58).

Patients most often receive antiseizure medications, namely valproic acid, which is typically effective at suppressing seizure activity, however the treatment is palliative (59). Lafora patients quickly develop symptoms of dementia and intractable seizures

(57). Patients tend to lose autonomy by 6 years after disease onset and die from status epilepticus, aspiration pneumonitis, or other complications of neurodegenerative disease within 10 years of disease onset (57).

To date, only one large-scale natural history study for Lafora disease exists, suggesting more studies are needed to describe the heterogenous disease and inform clinical investigation more fully (57).

Gene Therapy Development

It has been shown that *Epm2a* knockout (KO) and *Epm2b* KO mouse models replicate essential features of Lafora disease, such as neuronal degeneration and accumulation of Lafora bodies in muscle, liver, and brain (47, 60, 61). Recently, a proof-of-concept paper demonstrated that a viral vector carrying clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 with a guide RNA could be used to target and cut the *Gys1* gene responsible for producing brain glycogen that leads to Lafora bodies and Lafora disease. In this study, neonatal *Epm2a* KO and *Epm2b* KO mice were injected intracerebroventricularly with an AAV9 vector targeting *Gys1* that led to an editing rate of 17% of *Gys1* alleles. The effect of this editing was a 50% reduction in GYS1 protein, decreased glycogen accumulation, and decreased neuroinflammatory markers (47). This approach addresses the underlying cause of disease using a gene editing strategy, but alternative approaches such as a simpler gene delivery system without CRISPR/Cas9 may have a better safety profile and greater clinical potential.

SLC13A5 Deficiency Disorder

Pathogenic variants in the gene *SLC13A5* impair the sodium/citrate cotransporter, NaCT, with subsequent elevation in plasma and CSF citrate levels (62). These variants result in an autosomal recessive epileptic encephalopathy known as SLC13A5 deficiency disorder as SDD. *SLC13A5* pathogenic variants were first identified in 2014 when whole-exome sequencing was performed in 3 individuals with similar clinical presentation of epileptic encephalopathy from 2 families (7). Whole-exome sequencing is one approach now used to detect SDD (63). Additionally, *SLC13A5* is included in some commercially available epilepsy panels.

Presentation and Progression

Beginning within the first week of life most patients present with seizures and later often have status epilepticus (7, 64). However, there is phenotypic variability, and some patients have onset of seizures later in infancy. Patients with SDD may progress to lifelong drug-resistant epilepsy, with most seizures being convulsive (65). Seizure severity may decrease with age and some patients may even reach seizure freedom (40, 65). Broad-spectrum antiseizure medications often reduce seizure frequency, but targeted treatments are lacking and further innovation is needed.

Affected individuals show global developmental delay with intellectual disability and poor speech and communication (23). Patients often develop significant motor impairments and deficits in cognitive and expressive language (65). Patients typically have persistent neurological symptoms including ataxia, abnormal muscle tone, and abnormal involuntary movements (65). Additionally, patients with SDD may later develop dental enamel hypoplasia (65). It is possible for patients to live well into adulthood (65).

To date, there have been no published natural history studies for SDD. However, one natural history study of SDD is underway (NCT04681781), suggesting more studies may be needed to describe the disease state and inform clinical investigation more fully.

Gene Therapy Development

An *Slc13a5* KO model has been utilized to investigate SLC13A5 disease pathology. It has demonstrated myoclonic and nonconvulsive focal seizures as seen in patients, but with no obvious behavior or pathological abnormalities (66). Recently, a self-complementary AAV9 vector carrying a *SLC13A5* gene was developed (37). Preliminary data showed that delivery of this gene therapy to cerebrospinal fluid in young adult *Slc13a5* KO mice resulted in rescue of epileptic activity. Additionally, treated KO mice had lower plasma citrate levels compared with KO mice that did not receive GRT (37). This approach addresses the underlying cause of the disease, and the clinical potential is under investigation.

SLC6A1-Related Disorder

SLC6A1 pathogenic variants were first identified in 2015 when 2 truncations and 4 missense pathogenic variants were found in patients with epileptic encephalopathies with myoclonic-atonic seizures (67). *SLC6A1* is included in some commercially available epilepsy panels. *SLC6A1* pathogenic variants cause a haploinsufficiency of sodium- and chloride-dependent gamma-aminobutyric acid transporter type-1 (GAT-1), resulting in SRD (65).

Presentation and Progression

The mean age of seizure onset is ∼2.5 years of age in patients with SRD (9). Sixty percent of patients had developmental delay before seizure onset (9). The most prevalent epilepsy syndromes associated with SRD are myoclonic-atonic seizures (24%), genetic generalized epilepsy (23%), and non-acquired focal epilepsy (10%) (9). Further, it was found that absence seizures were the most common type of seizures in SRD (9). Common clinical features are epilepsy, developmental delay or cognitive impairment, and autistic traits (9). In addition patients may develop hypotonia, language disorder, and sleep issues.

Most patients require a care team consisting of neurologists, developmental pediatricians, genetic counselors, and speech and occupational therapists (9). Due to limited clinical data for SRD, treatment is determined based on the presenting clinical epilepsy syndrome and typically includes broad-spectrum antiseizure medications (9).

To date, there have been few natural history studies for SRD (52, 67, 68), indicating more studies are needed to describe the disease state and inform clinical investigation more fully.

Gene Therapy Development

Slc6a1 KO mice have been used to model seizure activity (51). These mice partially recapitulate human SRD as they have tremors, abnormal gait, reduced strength, absence seizures, anxious behavior, and cognitive impairment (9). *SLC6A1* is a potential candidate for gene therapy because it results from pathogenic variants that cause haploinsufficiency, thereby allowing for gene replacement or transcriptional enhancement strategies to potentially alleviate the burden of disease. However, no gene therapy studies have been published on SRD.

Opportunity for Gene Therapy in Monogenic Epilepsies

GRT for CNS disorders has led to promising preliminary safety and efficacy data in clinical trials (31). gSRT has shown promising results preclinically, but additional work is needed in the clinic (16). Single-injection approaches of viral vectors may lead to a safe and effective strategy in the clinic (31). Importantly, these strategies address the underlying cause of disease and have the potential to stabilize the progression of the disease. However, there is still a need for preclinical proof-of-concept research for gene therapy applications for monogenic epilepsies in animal models. Important endpoints to track patient progress and measure success for gene therapy for genetic epilepsies are survival, seizure susceptibility, the number of recurrent seizures, biomarkers such as citrate levels in SDD, and adverse events (37). The development and application of appropriate outcome measures is vital to lead to the next generation of medicines for persons with monogenic epilepsies.

In contrast to targeting the gene underlying the monogenetic epilepsy, an alternative approach may be used, such as gene therapy delivering an AAV vector for an engineered voltage-gated potassium channel to drive down neuronal excitability and thereby reduce seizure (69). Another approach is to virally overexpress neuropeptide Y, which has been shown to suppress seizures in animal models (70). These approaches are not precision medicine addressing the underlying cause of disease, and their clinical applicability must be tested.

REMAINING CHALLENGES IN THE CLINICAL DEVELOPMENT PATH FORWARD FOR GENE THERAPIES

Seizure

By addressing the underlying cause of disease, gene therapy has the potential to impact disease course more than treating seizures alone. Seizure reduction will remain an important clinical goal for patients with epilepsy, yet clinicians rely upon patient and caregiver reports of seizure activity, which are known to have limited reliability (71). Furthermore, nocturnal seizure frequency is inherently difficult to capture through self- or parent-reporting. Reporting and monitoring of seizure activity is therefore often inadequate. Seizures themselves may not be the best target for genetic epilepsies, as they can vary in frequency and severity depending in part on the patient subpopulation. In some genetic epilepsies such as SDD, there may be a reduction in seizures, but continued morbidity due to developmental disabilities, including impairments in motor and cognitive abilities (65). Cognitive dysfunction may result from the underlying disease process itself, which gene therapies may address (72).

Developmental Concerns

In monogenic epilepsies, patients with DEE may miss or have delayed developmental milestones (7) that can negatively impact quality of life and capacity for achieving independent living. These motor and cognitive delays may affect functioning (7) and merit a means of systematic measurement and ongoing monitoring to inform the evaluation of treatment response. Early initiation of gene therapy for genetic epilepsies may mitigate or prevent the development of motor and cognitive manifestations of the diseases. For example, there is a growing body of evidence that patients with a degenerative motor neuron disease, spinal muscular atrophy, treated pre-symptomatically with GRT achieve improved motor outcomes compared to patients treated later in the disease course presumably by preventing or slowing neuronal loss (73).

Motor dysfunction such as hypotonia, stereotypies, and ataxia impair mobility and purposeful use of movement (7, 9). Motor impairment and global developmental delay may be apparent in infancy, such as in EIEE, or may manifest with severe, progressive deterioration following normal development, as experienced by children with Lafora disease (62, 74). It is therefore important to expand our understanding of the spectrum of motor impairments affecting patients with monogenic epilepsy and establish endpoints related to motor ability. Such endpoints would indicate clinical meaningful changes and be applicable across multiple monogenic epilepsy syndromes with early childhood onset.

Cognitive dysfunction, which can result from both recurrent seizure activity and the underlying disease process itself (72), has substantial impact on patient quality of life. It requires that clinicians consider metrics for improving not only seizure frequency and severity but also cognitive function. To this end, more research is needed to understand progressive cognitive decline in epilepsy, especially as the disease course in some genetic epilepsies shows a reduction in seizures, but a continued progression of cognitive decline.

Autism spectrum disorder may also accompany intellectual disability in patients with genetic epilepsies such as Dravet syndrome, and has a substantial impact on a patient's potential to achieve independence (75). There is a need for clearer neurodevelopmental/neurophysiological endpoints to track a patient's developmental abilities both accurately and efficiently over time. It will be important to identify endpoints that can characterize developmental trajectories associated with specific conditions. Such endpoints could subsequently provide an early indication of treatment response when patients' trajectories shift following intervention.

DISCUSSION

Advances in genetic technologies have identified a growing number of monogenic genetic epilepsies potentially amenable

to gene therapies. The state of AAV-based gene therapy has advanced a great deal with extensive study of AAV9 in preclinical models and in the clinic. Loss-of-function pathogenic variants may be highly amenable to gene therapy, namely by GRT and gSRT, which address the underlying cause of disease without the need for gene editing. However, there is still need for translational research to advance new therapeutics to the clinic. Understanding of disease progression through natural history studies may be an important precursor to interventional studies as meaningful clinical endpoints are highly dependent upon the severity and rapidity of clinical decline. Preclinical animal models may also be important to inform optimal timing of dosing relative to disease progression, as rapidly lethal diseases like Lafora disease may have a narrow therapeutic window. While SDD and SRD have different underlying pathology and less severe epilepsy outcomes than Lafora disease, early intervention may be critical in intervention strategies to improve cognitive, behavioral, and functional measures and the chance for good quality of life and greater independence from caregivers. Study design, clinical endpoints, dose selection, inclusion/exclusion criteria, and safety all need to be carefully considered in order to best serve patients.

AUTHOR CONTRIBUTIONS

DB, SP, MH, and BM supported conceptualization of the paper, and reviewed and revised the manuscript. CS, JC, RB, and KG reviewed and revised the manuscript. All authors approved the final draft for submission.

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Motor, epileptic, and developmental phenotypes in genetic disorders affecting G protein coupled receptors-cAMP signaling

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Over the last years, a constantly increasing number of genetic diseases associated with epilepsy and movement disorders have been recognized. An emerging group of conditions in this field is represented by genetic disorders affecting G-protein-coupled receptors (GPCRs)–cAMP signaling. This group of postsynaptic disorders includes genes encoding for proteins highly expressed in the central nervous system and involved in GPCR signal transduction and cAMP production (e.g., *GNAO1, GNB1, ADCY5, GNAL, PDE2A, PDE10A*, and *HPCA* genes). While the clinical phenotype associated with *ADCY5* and *GNAL* is characterized by movement disorder in the absence of epilepsy, *GNAO1, GNB1, PDE2A, PDE10A*, and *HPCA* have a broader clinical phenotype, encompassing movement disorder, epilepsy, and neurodevelopmental disorders. We aimed to provide a comprehensive phenotypical characterization of genetic disorders affecting the cAMP signaling pathway, presenting with both movement disorders and epilepsy. Thus, we reviewed clinical features and genetic data of 203 patients from the literature with GNAO1, GNB1, PDE2A, PDE10A, and HPCA deficiencies. Furthermore, we delineated genotype–phenotype correlation in GNAO1 and GNB1 deficiency. This group of disorders presents with a highly recognizable clinical phenotype combining distinctive motor, epileptic, and neurodevelopmental features. A severe hyperkinetic movement disorder with potential life-threatening exacerbations and high susceptibility to a wide range of triggers is the clinical signature of the whole group of disorders. The existence of a distinctive clinical phenotype prompting diagnostic suspicion and early detection has relevant implications for clinical and therapeutic management. Studies are ongoing to clarify the pathophysiology of these rare postsynaptic disorders and start to design disease-specific treatments.

KEYWORDS

***GNAO1* encephalopathy, *GNB1*, *ADCY5*, *PDE2A*, *PDE10A*, cAMP, GPCR (G protein coupled receptor)**

Introduction

A significant number of genes associated with paroxysmal and non-paroxysmal movement disorders (MDs) and epilepsy have been recognized in recent years, shedding light on the biological substrates and pathways involved in these conditions.

Recently described genes in this field encode for proteins involved in postsynaptic signaling pathways downstream to G-protein-coupled receptors (GPCRs), which are ubiquitously expressed in the central nervous system (CNS) and highly enriched in striatal medium spiny neurons (MSNs) (1, 2). GPCRs control responses to a wide array of sensory stimuli, including light and odorants, and non-sensory stimuli, including neurotransmitters and hormones. Signal transduction *via* GPCRs relies primarily upon the activation of heterotrimeric G-proteins, which consist of an α-subunit that binds and hydrolyzes GTP and a βγ heterodimer (3). GTP binding can induce an allosteric transition leading to βγ release which, in turn, enables Gα and Gβγ signaling to their multiple downstream effectors (4). The variety and expression pattern of individual G-protein subunits define unique GPCR properties in a cell-context-specific manner.

Genes discovered in this pathway and associated with neurological disorders encode transducer molecules or components of the GPCR machinery (i.e., *GNAO1*, *GNB1*, *GNAL*, *GPR88*), or proteins controlling the synthesis and hydrolysis of the second messenger cyclic adenosine monophosphate (cAMP) (*ADCY5*, *PDE10A*, *PDE2A*). Although the precise functions are still largely unknown, *HPCA* (encoding for hippocalcin) is a calcium sensor associated with the plasma membrane that influences the activity of potassium and calcium channels and could be implied in the modulation of dopamine post signaling in striatal neurons (1, 2). Figure 1 represents the organization of this signaling pathway in MSNs (Figure 1). The exact role of these proteins in the pathophysiology of MDs and the functional impact of pathogenic variants have only begun to be explored in preclinical models. For a comprehensive review on this topic, see Golzales-Latapi et al. (2).

GNAL (OMIM 139312) and *ADCY5* (OMIM 600293) genes encode for G_{olf} and adenylyl cyclase type 5 (AC5), the main AC isoform expressed in the striatum, respectively, which are directly involved in the GPCR–cAMP signaling cascade mediated by the activation of dopamine receptor 1 (D1R) and adenosine receptor 2A (A2AR) in MSNs (5).

G_{olf} is responsible for coupling D1R and A2AR stimulation to the activation of ADCY5, causing increased intracellular cAMP levels, which, in turn, enhances neuronal activity (6). Pathogenic variants in *GNAL* and *ADCY5* may manifest through either autosomal dominant or recessive modes of inheritance. Most *GNAL* mutations are heterozygous missense, nonsense, or frameshift variants with a clear loss-of-function (LOF) effect (7, 8) leading to reduced cAMP levels. In contrast, most disease-causing *ADCY5* variants are gain-of-function (GOF) defects causing increased cAMP production (2). These findings suggest a complex scenario in which both increased and decreased intraneuronal cAMP levels may underlie the pathogenesis of MDs.

The critical role of the GPCRs–cAMP signaling pathway in the pathophysiology of MDs has been further highlighted by the identification of disease-causing variants in *PDE10A* (OMIM 610652) and *PDE2A* (OMIM 602658). These genes encode two cyclic nucleotide phosphodiesterases (PDEs) highly expressed in MSNs and critically involved in modulating dopaminergic and adenosinergic responses through degradation of intracellular cAMP and cGMP (9–13). While both homozygous and heterozygous LOF mutations have been reported in *PDE10*, only biallelic changes have been identified in *PDE2A* so far.

GNAO1 (OMIM 139311) and *GNB1* (OMIM 139380) encode proteins that are components of the GPCR machinery, respectively the alpha subunit (Gαo) and the beta-1 (Gß1) subunit of the inhibitory heterotrimeric Go-protein complex (3). *GNAO1* and *GNB1* are co-expressed in the cerebral cortex, hippocampus, and striatum where they are involved in transducing signals downstream to several GPCRs and in the regulation of AC activity.

GNAO1 modulates inhibitory signaling from several GPCRs, including GABA-B, dopamine D2, α2A adrenergic, and adenosine A1, regulating neuronal excitability and neurotransmission (3), and controls neurodevelopment (14). In the brain, Go controls the synthesis of cAMP, directly prevents neurotransmitter release, inhibits N-type and P/Q-type calcium channels, and activates G-protein-coupled inward rectifying potassium (GIRK) channels. Regarding striatal pathways, Gαo activity influences the excitability of neurons of the indirect (inhibitory MSNs, iMSNs) and direct pathways (dMSNs) by tuning inputs from dopamine D2 and adenosine A2A receptors, with crucial effects on movement control (15). In dMSNs, Gao affects both the efficacy (defined as the power to produce an effect) and potency (defined as the capacity to produce strong physiological or chemical effects) of responses to dopamine while only modulating adenosine efficacy. Instead, in iMSNs, Gao affects both efficacy and potency of responses to adenosine while only modulating dopamine efficacy. Taken together, these data indicate that Gao plays a pivotal role in controlling the potency and efficacy of stimulatory neuromodulation while only affecting the efficacy of inhibitory inputs in both populations of striatal neurons (15). A preclinical model of Gao defect revealed a different motor impairment as a result of knocking out Gao in dMSN or iMSN. In the first case, the acquisition and retention of motor skills were mainly affected. In the second one, dystonia and profound coordination deficits were observed (15).

Aberrant cAMP synthesis was originally proposed as the main pathogenic mechanism of the disease in *GNAO1*

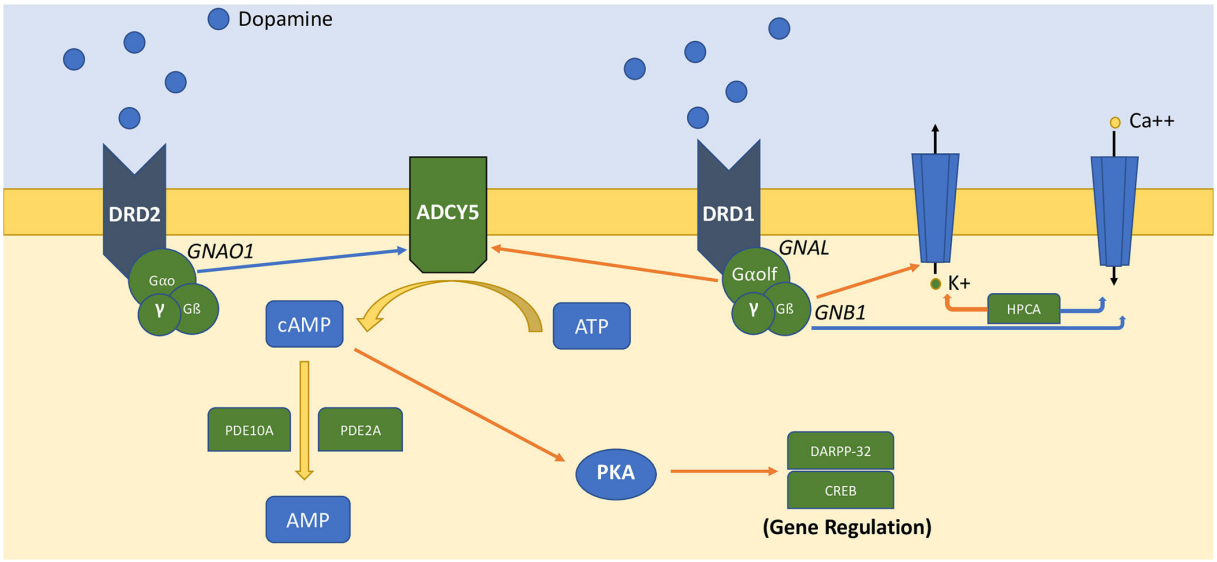

FIGURE 1
Simplified representation of DA-dependent GPCRs–cAMP signaling pathway in medium spiny neurons. Orange arrows represent activation signals, and blue arrows represent inhibitory signals. ATP is converted to AMP by adenyl-cyclase-5 (ADCY5), which is regulated by Gαo (*GNAO1*) and Gαolf (*GNAL*). Gß subunit (*GNB1*) and hippocalcin (HPCA) regulate the activity of potassium and calcium channels. The generated cAMP propagates downstream signaling via cAMP-binding proteins. cAMP is converted to AMP by phosphodiesterase activity (PDE10A, PDE2A).

disorders, with LOF and GOF alleles that appeared to be primarily associated with epilepsy and MD, respectively (16). These data apparently contradict the original findings from Nakamura et al. (17) suggesting a LOF behavior of *GNAO1* variants on $G\alpha_o$-mediated signaling, regardless of the associated clinical presentation. In a recently published and elegant study performed by Muntean and coworkers, *GNAO1* variants were shown to disturb Gαo function in a cell-type-specific manner *via* a combination of LOF and dominant-negative mechanisms that are not mutually exclusive (15). More recently, a strong hypomorphic effect or a complete LOF behavior has been confirmed in genetically modified nematodes harboring *GNAO1* pathogenic variants, leading to excessive neurotransmitter release by different classes of motor neurons (18, 19). Of note, the observed phenotype was shown to be ameliorated by caffeine *via* adenosine receptor antagonism (18).

GNB1 encodes Gβ1, the third component of the heterotrimeric G-protein complex. Upon receptor activation, Gβ1 dissociates from the Gα subunit and, together with the Gγ subunit, activates downstream signaling pathways, leading to inhibition of presynaptic voltage-gated calcium (i.e., Cav2.1 and Cav2.2) and potassium channels, with effects on neurotransmitter release (20, 21). Gβ1 interacts also with $G\alpha_{olf}$ in striatal neurons, and Lohmann et al. showed that pathogenic *GNB1* variants might reduce association with $G\alpha_{olf}$ thus reducing coupling to D1R (22). The functional impact of dominant *GNB1* mutations is still debated.

The clinical spectrum associated to this group of conditions ranges from developmental epileptic encephalopathy with severe early-onset movement disorder to isolated paroxysmal and/or non-paroxysmal movement disorders.

Clinical studies, preclinical models, and systems biology analysis helped to understand the relevance of these genes encoding postsynaptic signaling proteins in different subtypes of striatal cells to the pathogenesis of hyperkinetic MDs (2). The clinical features other than MD and the pathophysiology of epilepsy and developmental issues in these disorders have been less investigated and remain poorly understood.

Here, we reviewed the clinical phenotypes and mutational spectrum associated with GPCRs signaling disorders focusing on the conditions of this group presenting with epilepsy, movement disorders, and neurodevelopmental disorders. We aimed to verify the presence of common clinical features and characterize the core clinical phenotype of this group of severe early-onset genetic neurological disorders. Considering their clinical severity and susceptibility and precipitation with specific triggers, this in-depth clinical characterization has implications for timely diagnosis, management, and therapeutic strategies.

Materials and methods

A comprehensive search of the medical literature (PubMed, Medline, Cochrane CENTRAL, Google Scholar) was conducted to identify papers reporting patients with pathogenic or likely pathogenic variants in *GNAO1, GNB1, ADCY5, GNAL PDE2A, PDE10A*, *HPCA*. "*GNAO1*," "*GNB1*," "*ADCY5*," "*GNAL*," "*PDE2A*," "*PDE10A*," and "*HPCA*" were used as search terms. As

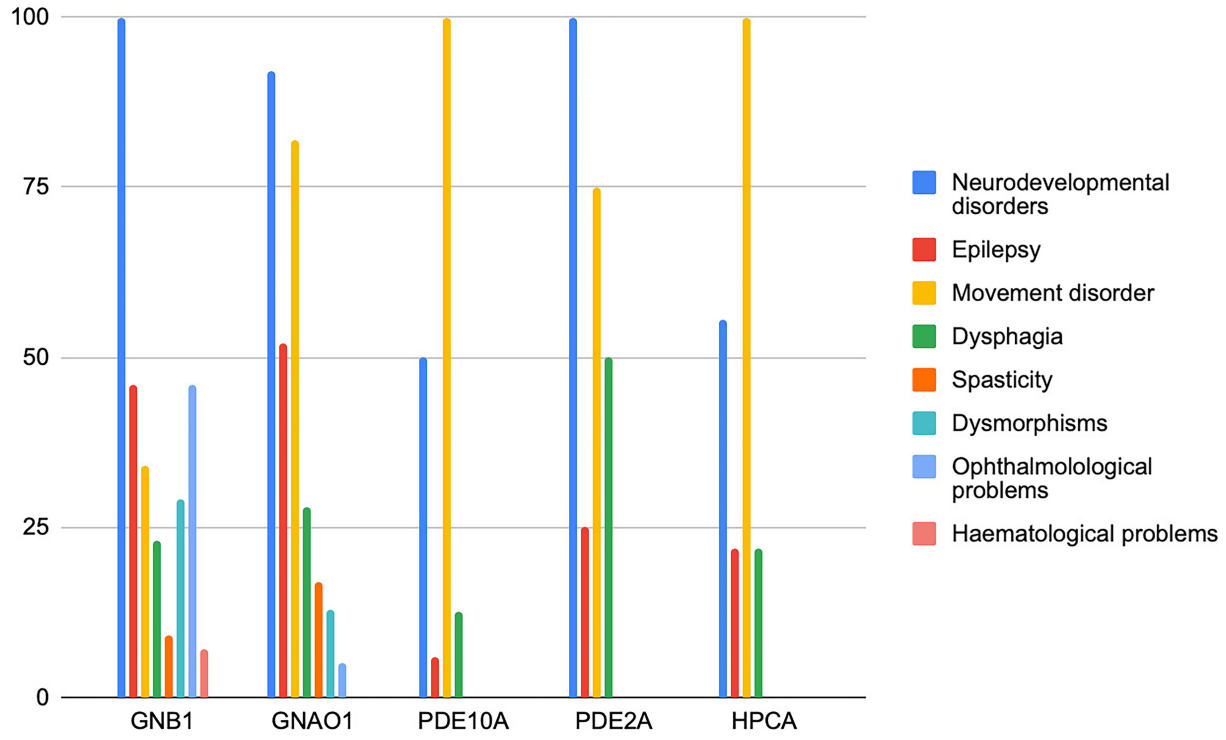

FIGURE 2
Distribution of the main clinical features of genetic disorders affecting the GPCRs–cAMP signaling pathway. The frequency of symptoms is expressed in the percentage of patients.

possible limitations of our search, we declare to have selected only English-written articles. It is possible that by doing so, some information included in non-English-written papers and useful to further delineate the clinical phenotype of these rare disorders could have been missed. Furthermore, variants were not independently re-evaluated as they were already judged as pathogenic or likely pathogenic according to ACMG criteria or reported in multiple patients with similar clinical presentations. We selected and reviewed cases with pathogenic or likely pathogenic variants according to ACMG criteria for which clinical, neuroradiological, neurophysiological, and genetic data were available. We focused on disorders with MDs, epilepsy, and neurodevelopmental disorders in their clinical spectrum.

Results

We collected and reviewed 74 articles (17 for *GNB1*, 46 for *GNAO1*, 6 for *PDE10A*, 2 for *PDE2A*, and 3 for HCPCA) describing motor, epileptic, and developmental phenotype of patients with genetic disorders of GPCRs–cAMP signaling pathway. Six additional papers on *GNAO1* were excluded (four because of insufficient information and two because of the presence of concomitant mutations in other genes). Finally, to the scope of this review 203 cases from 72 papers published up to May 2022 were selected. Figure 2 represents the main clinical features associated with the above-mentioned genes. Characteristics of GNAO1 and GNB1 deficiency were further evaluated to assess for a genotype–phenotype correlation (Figures 3, 4).

GNB1

De novo mutations in *GNB1* cause an autosomal dominant neurodevelopmental disorder (MIM #616973), which may present as isolated or as part of a wide range of both neurological and non-neurological features (22). This gene was first associated with human disease in 2016 (23), and to date, approximately 64 patients carrying 38 different pathogenetic variants have been reported (22–38).

Neurodevelopmental phenotype

Neurodevelopmental delay is described as severe in most individuals and is often the presenting symptom. Motor control is usually limited to the head or trunk, with only a dozen

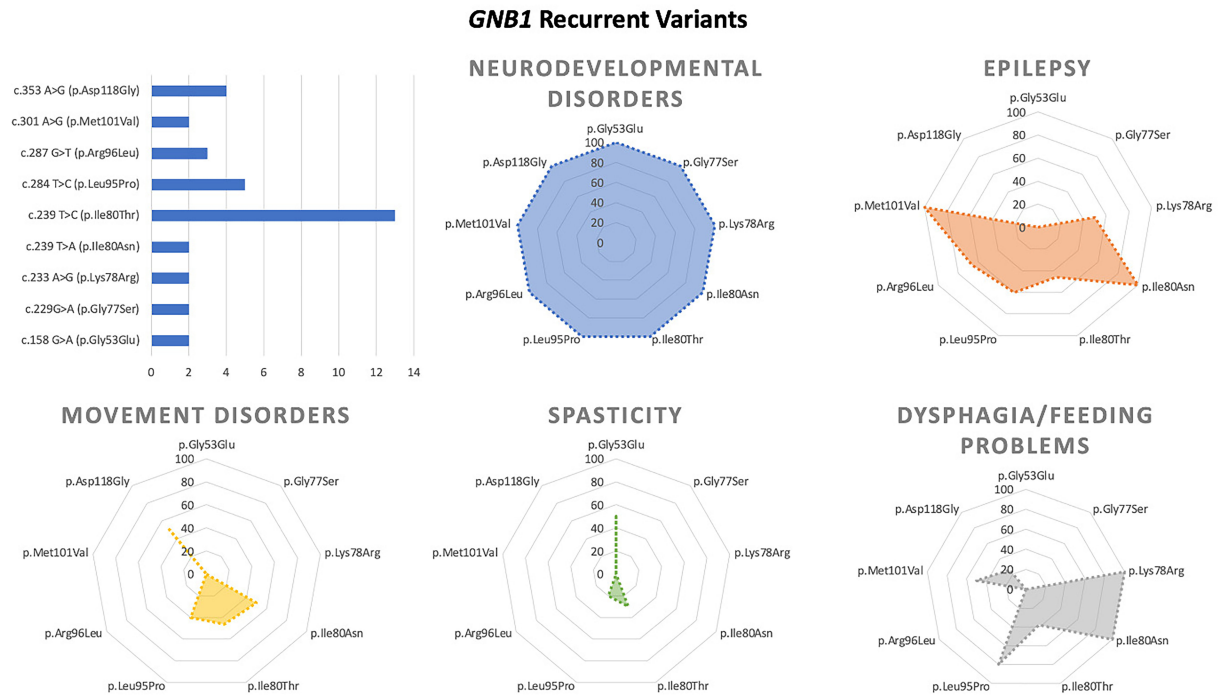

FIGURE 3
Main genotype–phenotype correlations in GNB1 deficiency.

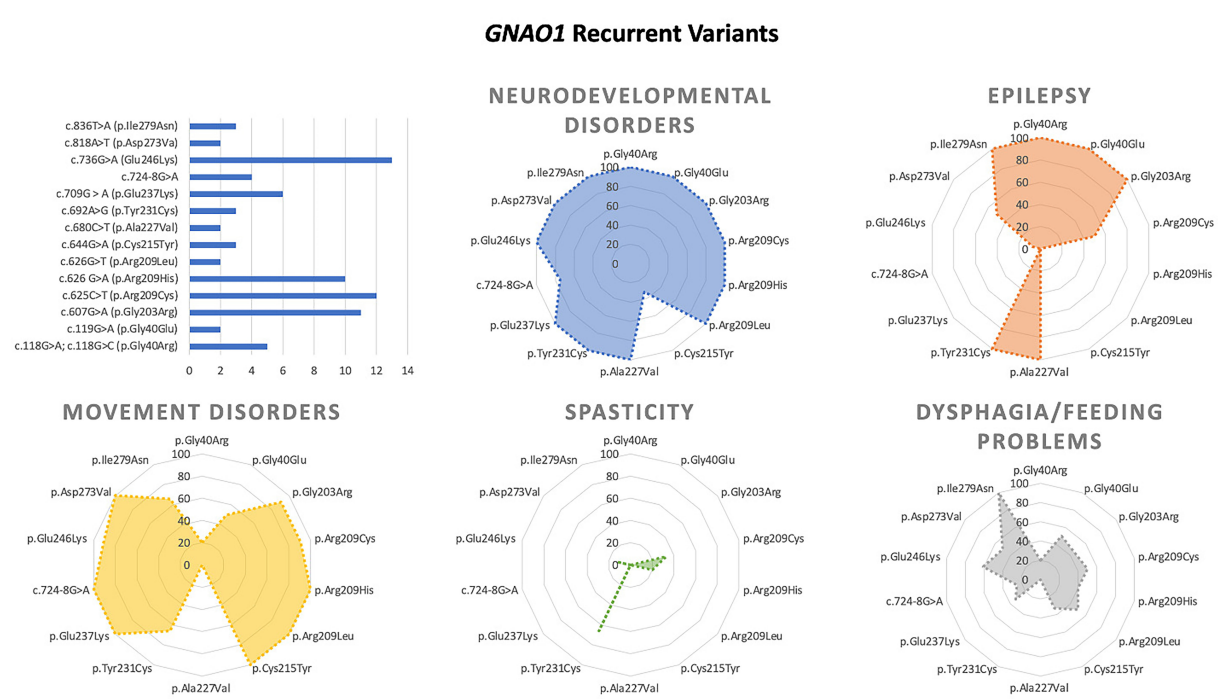

FIGURE 4
Main genotype–phenotype correlations in GNAO1 deficiency.

of patients achieving assisted or independent walking from 3 years of age or later in life (23, 25, 27, 29, 30) and very rare individuals showing normal motor development (22, 27, 29). Language is severely impaired, even compared to motor achievements. Most patients are non-verbal, and a small number of them can only use single words (23–25, 27, 29). A moderate to severe intellectual disability, variably associated with other neurodevelopmental disorders such as ADHD and Autism spectrum disorders, has been reported in most of the patients, whereas only sporadic cases showed mild intellectual disability (29, 30).

Epilepsy

Infancy or childhood-onset epilepsy has been described in 30 individuals and is one of the most challenging issues in GNB1 deficiency. Substitutions located in exons 6 and 7, in particular the ones at residue Ile^{80} (p.Ile80Thr and p.Ile80Asn) and Leu^{95} (p.Leu95Pro), have been associated to infantile-onset seizures. Infantile spasms and hypsarrhythmia represent a common presentation in these patients (23, 28, 31, 32). Other infantile epileptic presentations include sporadic cases of tonic-clonic (one patient), tonic (two patients), and myoclonic seizures (two patients), usually poorly controlled by antiepileptic drugs (23). Febrile status epilepticus anticipating non-febrile seizures has also been reported (23). Patients with childhood-onset epilepsy show a wide range of seizure types, including motor (tonic-clonic and myoclonic) and non-motor seizures with impaired awareness (described as absences or staring spells) (23, 27). Epileptiform discharges are generally multifocal or, less frequently, focal or generalized. In many cases, epilepsy is drug-resistant or controlled by a combination of at least two drugs.

Movement disorder

Movement disorder is frequent in patients with *GNB1* pathogenic variants (23 individuals), usually dystonia and/or ataxia. Dystonia has been reported in 12 patients and was found to be more commonly associated with the p.Ile80Thr, p.Leu95Pro, p.Asp118Tyr, and p.Asp118Gly substitutions (22–25, 28, 30, 34). Myoclonus with dystonia has been reported in two patients (23, 29), suggesting a myoclonus–dystonia phenotype. Non-epileptic "twitches," presumably myoclonus, have been reported in one patient (27). Ataxia has been observed in five patients with different pathogenetic variants (22). A combination of chorea and athetosis was described in three cases (22, 26, 27). Stereotypies such as body rolling and hand stereotypies were reported in four patients (23, 25–27), and tics (vocals and unspecified) in two (23, 27). A single patient with bradykinesia has been described (27). Episodic exacerbations of MD and status dystonicus have been reported in three patients [Galosi et al., 2022 (in press); (27, 28)]. Spasticity (either as quadriplegia or diplegia) was reported in six patients (27, 28).

Only three reports about pharmacological treatment in patients with GNB1 variants are available, and no drugs have been reported to dramatically improve movement disorder. Levodopa administration was not effective in one patient (24, 30). Deep brain stimulation (DBS) improved dystonia in two patients (30, 34).

Other features

Cortical blindness (4 patients) or oculomotor abnormalities including nystagmus (18 patients), strabismus (7 patients), and ophthalmoplegia (4 patients) have been described in nearly half of the GNB1 patients reported in the literature (22, 23, 27, 28).

A normal head circumference was seen in most of the patients (23, 27); macrocephaly and microcephaly were reported, respectively, in five and one cases. Cleft palate (often associated with the p.Leu95Pro variant) (31), growth delay, and other non-specific facial dysmorphisms may be part of the clinical spectrum. Dysphagia and feeding difficulties are frequent in GNB1 (15 pts) (23, 27, 28) leading, in severe cases, to tube feeding (23, 27). Hematological issues, such as cutaneous mastocytosis and acute lymphoblastic leukemia, have been reported in four and one patients, respectively (25–27). Three out of four individuals with cutaneous mastocytosis harbored the p.Ile80Thr substitution (26, 27).

Brain MRI findings range from normal to non-specific findings, such as white matter abnormalities (white matter hyperintensities or abnormal myelination) (10 patients), cerebellar hypoplasia (4 patients), generalized cortical atrophy and/or increased ventricular spaces (4 patients), and abnormalities of cortical gyri (3 patients) (22, 23, 25, 27, 28).

Among recurrent variants, the p.Asp118Gly substitution has been associated with neurodevelopmental disorder and dystonia without epilepsy, whereas the p.Ile80Thr and p.Leu95Pro changes are associated with both epilepsy and movement disorder.

GNAO1

Dominant *GNAO1* mutations were first associated with human disease in 2013 (17) in a small cohort of patients with epileptic encephalopathy and the development of dyskinetic movement disorders in a subset of affected individuals. Three years later, Saitsu and colleagues first recognized the phenotype most frequently associated to this gene, namely, involuntary movement disorder and severe developmental delay with or without seizures (39).

To date, two main GNAO1-related disorders are reported in OMIM: early infantile-onset epileptic encephalopathy (EIEE17) (MIM#615473) and neurodevelopmental disorder with involuntary movements (NEDIM) (MIM#617493). Despite the present classification, the clinical practice is a variety

of overlapping phenotypes with a small number of patients presenting isolated MD or epileptic manifestations. Thirty-nine patients show a mixed phenotype with movement disorder and epilepsy, 52 display isolated movement disorder, 16 experience epilepsy without movement disorder, and 3 have an unspecified neurodevelopmental disorder.

According to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), 60 pathogenic/likely pathogenic variants have been reported in 111 patients. The vast majority of them are missense changes affecting more than 40 highly conserved residues. Approximately, half of the affected subjects harbor mutations affecting residues Gly^{203} (p.Gly203Arg), Arg^{209} (p.Arg209Cys/His/Gly), and Glu^{246} (p.Glu246Lys). Recent structural and functional data indicated that these and other *GNAO1* mutations variably affect Gα- and Gβγ-mediated signaling (15, 16, 19). Seventeen pathogenic variants recur in more than one patient.

Neurodevelopmental phenotype

Most patients presented with hypotonia (84/111 patients) and early developmental delay with significant impairment in both motor and language areas (102/111 patients).

Epilepsy

Epilepsy has been described in 58 individuals (nearly 50% of patients), with onset during the neonatal period (29 patients), infancy (18 patients), or childhood (8 patients) and outcome ranging from severe early-onset cases to milder forms.

Severe forms include developmental and epileptic encephalopathies (DEE) manifesting with infantile spasms or epilepsy of infancy with migrating focal seizures (EIFMS) (17). The p.Gly203Arg substitution appears to be the variant most frequently associated to early-onset epilepsy and movement disorder (40). Epilepsy in these patients is resistant to multiple medications. Other patients may manifest generalized and focal epilepsies at different ages (41). Patients carrying the c.625C>T (p.Arg209Cys) transition have childhood-onset epilepsy (3–12 years), with a high rate of generalized seizures and good response to treatment.

Overall, the highest rate of drug resistance was found in early-onset forms, while later-onset forms are usually better controlled by antiepileptic therapy.

The electroencephalographic abnormalities include hypsarrhythmia and burst suppression in early-onset forms, and focal and multifocal discharges in later presentations. Slow abnormalities have also been reported in several patients, even in the absence of epileptic manifestations.

Movement disorder

Movement disorder with onset during infancy or childhood (1 neonatal onset, 50 infantile onset, 13 childhood onset,) represents the core symptom (92 patients, 83% of cases) in patients with *GNAO1* variants. Chorea, athetosis, ballism, and dystonia, with a high impact on motor functioning, are the most commonly associated MDs (42).

Dystonia is reported in 63 patients, while chorea, athetosis, and ballism (often coexisting) are described in 52 patients. Moreover, almost constant is the finding of dyskinesia, reported in 37 patients, involving the orofacial district in one-third of patients (11 patients). More than one-third of patients with MD (34/87) experienced severe exacerbations. Specific variants appear to be more frequently associated with the occurrence of exacerbations: c.625C>T (p.Arg209Cys), c.736G>A (p.Glu246Lys), and c.709G>A (p.Glu237Lys). In these patients, the hyperkinetic movement disorder seems to be more disabling and potentially life-threatening, leading to a drug-resistant dystonic–dyskinetic state requiring surgical treatment (DBS and/or pallidotomy). Tetrabenazine appears to be the most used and effective pharmacological treatment, although sporadic cases of response to other drugs have been reported (risperidone, levetiracetam). In a minority of patients, myoclonus, ataxia, tremor [resting tremor in two patients (43, 44), not specified tremor in two patients (45, 46), tongue tremor in one patient] and parkinsonian features have been reported.

Other features

Dysphagia, often described, could lead in severe cases to tube feeding. Finally, MRI brain findings range from normal (59 patients) to different non-specific abnormalities, such as cerebral atrophy, abnormalities of myelination, or basal ganglia atrophy. In some cases, repeated MRI shows a lesional progression, suggesting a degenerative course.

ADCY5

The constellation of neurological disorders associated with ADCY5 mutations includes conditions of variable severity, ranging from severe early-onset neurodevelopmental disorder with dyskinesia to familial dyskinesia with facial myokymia (FDFM). Complex combinations of paroxysmal and persistent MDs are possible, including chorea, dystonia, tremor, myoclonus, myokymia, and plegic attacks. Day and nighttime episodes (47) and facial dyskinesia are clue features to the diagnosis. Additional interictal features include axial and oral hypotonia, gaze abnormalities, spasticity, dysarthria, learning issues, and ADHD. Thus, far epilepsy has not been reported as a part of the phenotype. Nearly 119 cases have been described with only three patients reported with confirmed (one patient) (48) or suspected epileptic episodes (two patients) (49).

PDE10A

Different studies described mutations in *PDE10A* causing childhood-onset chorea. Both heterozygous and compound heterozygous/homozygous mutations have been reported, with the latter showing an earlier onset of symptoms (8, 50). Based on OMIM classification, infantile-onset dyskinesia (MIM #616921) and striatal degeneration (MIM #616922) are associated with recessive and dominant modes of inheritance, respectively.

Diggle et al. described two different consanguineous families with biallelic mutations in *PDE10A*, c.320A>G (p.Tyr107Cys) and c.346G>C (p.Ala116Pro), both affecting exon 4. These individuals presented within infancy (mean age of 3 months) with axial hypotonia, dysarthria, and hyperkinetic movement disorder characterized by dyskinesia of the limbs, trunk, and face (50).

The c.320A>G (p.Tyr107Cys) variant was associated with orofacial dyskinesia, and drooling, generalized developmental delay but no cognitive impairment. Symptoms were less severe in older individuals than in younger ones.

No epilepsy was reported, and MRI, when done, was normal.

Two brothers carrying the c.346G>C (p.Ala116Pro) variant presented with developmental delay and at 7 years of age were able to speak single words. The second-born was more severely affected, and he was fed *via* a gastrostomy tube and presented focal epilepsy from 3.5 years of age. EEG revealed arrhythmic delta activity without focal epileptiform discharges, and treatment with carbamazepine had been effective.

Three *de novo PDE10A* mutations [c.898T>C (p.Phe300Leu), c.1000T>C (p.Phe334Leu), and c.1001T>G (p.F334C)] (9, 51–53) have been associated with childhood-onset chorea (5 to 10 years of age) with normal cognitive development and no epilepsy.

Interestingly, patients harboring dominant variant showed the presence of bilateral striatal abnormalities on brain MRI (9, 51, 52, 54).

PDE2A

Biallelic *PDE2A* mutations cause a neurodevelopmental disorder with paroxysmal dyskinesia or seizures (MIM #619150). Four affected individuals have been described so far (9, 10). Interestingly, an intra-familiar phenotypic variability was evident in two siblings, with c.1180C>T predicting the formation of a premature stop codon (p.Gln394*), with one mainly suffering from epilepsy and the other from dystonia (12). All patients present intellectual disability or developmental delay.

MD is characterized by childhood-onset paroxysmal dyskinesia, with different triggers including emotional stress, sudden movements, or sudden sensorial stimuli. Episodes are usually brief (< 1 min) but frequent, until 100 episodes/day. Two patients developed sustained chorea-dystonia. Two cases showed persistent truncal hypotonia (12). Deep brain stimulation reduced the frequency and intensity of dyskinetic attacks in one case (11).

Infantile epilepsy with spasms and tonic seizures was reported in a single patient, resistant to multiple antiepileptic drugs, ketogenic diet, and vagus nerve stimulation (11). Ictal EEG recording showed epileptic spasms and right frontal seizures. The administration of vigabatrin and prednisolone was effective only for the first month, then also associated with ketogenic diet. However, at 5 months he experienced a focal status epilepticus lasting 24 h. MD was not present in this patient at the age of 15 months (12).

No brain MRI abnormalities were detected in these patients.

HPCA

HPCA mutations cause autosomal recessive dystonia (MIM # 224500).

This gene encodes for the neuron-specific calcium-binding protein HPCA. HPCA is widely expressed in the brain, particularly in the hippocampal pyramidal neurons and in medium spiny neurons of the striatum, where it is likely to modulate dopamine signaling, influencing the activity of potassium and calcium channels (2, 53).

HPCA deficiency was initially associated to isolated dystonia (53), but subsequent reports expanded the clinical phenotype, describing patients presenting with variable combinations of seizures, developmental delay, intellectual disability, psychiatric symptoms, and dysphagia.

In two cases, a mild neurodevelopmental delay was noticed. Six individuals with ID are reported. When available, the neuropsychological assessment revealed prominent issues in verbal comprehension and/or verbal fluency.

Psychiatric symptoms such as severe anxiety and mild to severe depression are described.

Dystonia remains the core feature and is reported in seven out of nine described individuals, although other hyperkinetic MDs such as chorea and athetosis have been reported (53). Dystonia usually appears in childhood, mainly affecting the trunk, arms, and face. Orofacial dyskinesia and dysarthria have been reported, more frequently in patients with the c.182C>T p. (Ala61Val) variant. The age of onset of the disease ranges from eight months to eight years (52, 55). Therapeutic management of MD is not systematically reported. In some patients, treatment was not required, while in the two treated patients dopaminergic, anticholinergic, and antiepileptic drugs (valproate and clonazepam) were all equally ineffective.

Infantile seizures were reported in two individuals, associated with the c.225C>A, (p.Asn75Lys) pathogenetic

variant (55). A further patient suffered from two episodes of febrile seizures (55). No epileptiform abnormalities were seen in most patients.

Brain MRI was normal in all patients.

Discussion

Here, we reviewed the clinical phenotypes and mutational spectrum associated with genetic disorders affecting the GPCRs–cAMP signaling pathway and having epilepsy and movement disorder in their clinical spectrum. Common pathophysiology has been proposed but not fully investigated, as well as the degree of clinical overlap, which is the object of this work.

A complex hyperkinetic MD with or without paroxysmal exacerbations seems to be the clinical signature of the whole group. Dominant mutations in *GNAO1*, *GNB1*, and *PDE2A* have been associated to a complex early-onset neurological disorder characterized by a variable association of hyperkinetic MD, epilepsy, and developmental delay generally evolving into intellectual disability (23, 42, 56). *GNAL* and *ADCY5* genes have not been associated with epilepsy so far. A homozygous non-sense variant affecting the *GPR88* gene has been reported in four sisters of a single family with childhood-onset chorea and psychomotor retardation. Since then, no additional patients carrying LOF variants in this gene have been identified, raising concerns on the effective relevance of *GPR88* as a disease-causing gene. Dyskinetic storms or minor paroxysmal choreo-dystonic spells, baseline dystonia and/or chorea, prominent cranial involvement leading to dysarthria and dysphagia, orofacial dyskinesia, axial hypotonia, and severe impairment of postural development characterize the MD phenotype of these conditions. Susceptibility to a wide range of triggers and severe paroxysmal exacerbations evolving into status dystonicus are typical (*ADCY5*, *GNAO1*, *GNB1*, *PDE2A*). Febrile and upon awakening exacerbations of movement disorder have been described for *GNAO1*, *ADCY5*, *PDE2A*, and GNB1 (12, 42, 48, 57). Other typical triggers are emotional stress, sudden movements, and sudden sensorial stimuli. Benzodiazepines (clonazepam, lorazepam, midazolam) are useful for controlling or aborting paroxysmal episodes (*GNAO1*, *GNB1*), while tetrabenazine can partially control the baseline hyperkinetic MD and prevent MD exacerbations (*GNAO1, ADCY5).* Neuromodulation (GPi-DBS or pallidotomy) has been successful in controlling paroxysmal exacerbations and evolution into status dystonicus in GNAO1, GNB1, and PDE2A-related MDs.

Epilepsy can be prominent in *GNB1* and *GNAO1* encephalopathy, while it is anecdotical in individuals with *HPCA, PDE2A,* and *PDE10A* pathogenic variants.

DEE, described in the literature as Ohtahara syndrome, infantile spasms, or EIFMS can be the epileptic presentation of *GNAO1*, *GNB1, and PDE2A*. Onset with tonic seizures or infantile spasms with EEG patterns of burst suppression or hypsarrhythmia has been described for *GNAO1* and *GNB1*.

These infantile forms are usually drug-resistant. Childhood-onset presentations with focal (motor and non-motor) and/or generalized seizures are usually milder. Febrile status epilepticus and febrile seizures are typical of *GNB1* and *GNAO1*.

Global developmental delay and subsequent moderate to severe intellectual disability are observed in almost all patients with *GNB1*, *GNAO1*, *PDE2A*, and *HPCA* variants. Severe motor impairment with limited postural control, absence of independent walking, and absent speech are more frequent in *GNB1* and *GNAO1* encephalopathy. Patients with *PDE10A* and *ADCY5* disorders can be cognitively normal.

Language impairment can be profound, especially in *GNAO1* and *GNB1* encephalopathy, and it is not clear if it is related to the prominent oromandibular distribution of movement disorders, or if it depends on cognitive impairment with or without oral dyspraxia. Unfortunately, this differentiation is not detailed in the actual literature where the language impairment is reported early in life, and in most cases, it is not clear if MD with oromandibular involvement coexisted at that time. MRI findings are usually nonspecific and include delayed or abnormal myelination (*GNB1, GNAO1*), cortical atrophy (*GNAO1, GNB1*), increased ventricular spaces (*GNB1*), and abnormalities of basal ganglia (*GNAO1, PDE10A*).

The presence of a core of very distinctive and shared features suggests that genetic disorders affecting the GPCRs–cAMP pathway can recognize, at least in part, the same pathophysiology.

Conversely, the different distribution and differential expression of clinical manifestations among the different disorders suggest that differentiated biological substrates, neuronal populations, or brain areas can be involved in pathophysiology and phenomenology.

GNB1 mutations have been found in different hematological neoplasm cell lines and are thought to increase the activation of AKT/ERK/mTOR signaling (58). This finding may account for the presence of cutaneous mastocytosis and acute lymphoblastic leukemia in *GNB1* patients and, possibly, even for the presence of polymicrogyria (23).

Interestingly, a recent study in human blood neoplasms has also found *GNAO1* mutations to activate AKT/ERK/mTOR signaling (59). It is thought that the hyperactivation of mTOR pathway, as seen in other disorders such as sclerosis tuberous, *PTEN*, and *GATOR1* complex deficiency (*including DEPDC, NPRL2, and NPRL3* deficiencies), may cause epilepsy through the alteration of normal neural networks (60). The *GNAO1* variant associated to mTOR hyperactivation is the R209C variant (58). Interestingly, we found the same variant to be associated with epilepsy in 50% of the cases. Preclinical studies are needed to understand the pathophysiology of epilepsy in GNAO1 and GNB1 deficiency and to clarify the role of the mTOR signaling pathway activation.

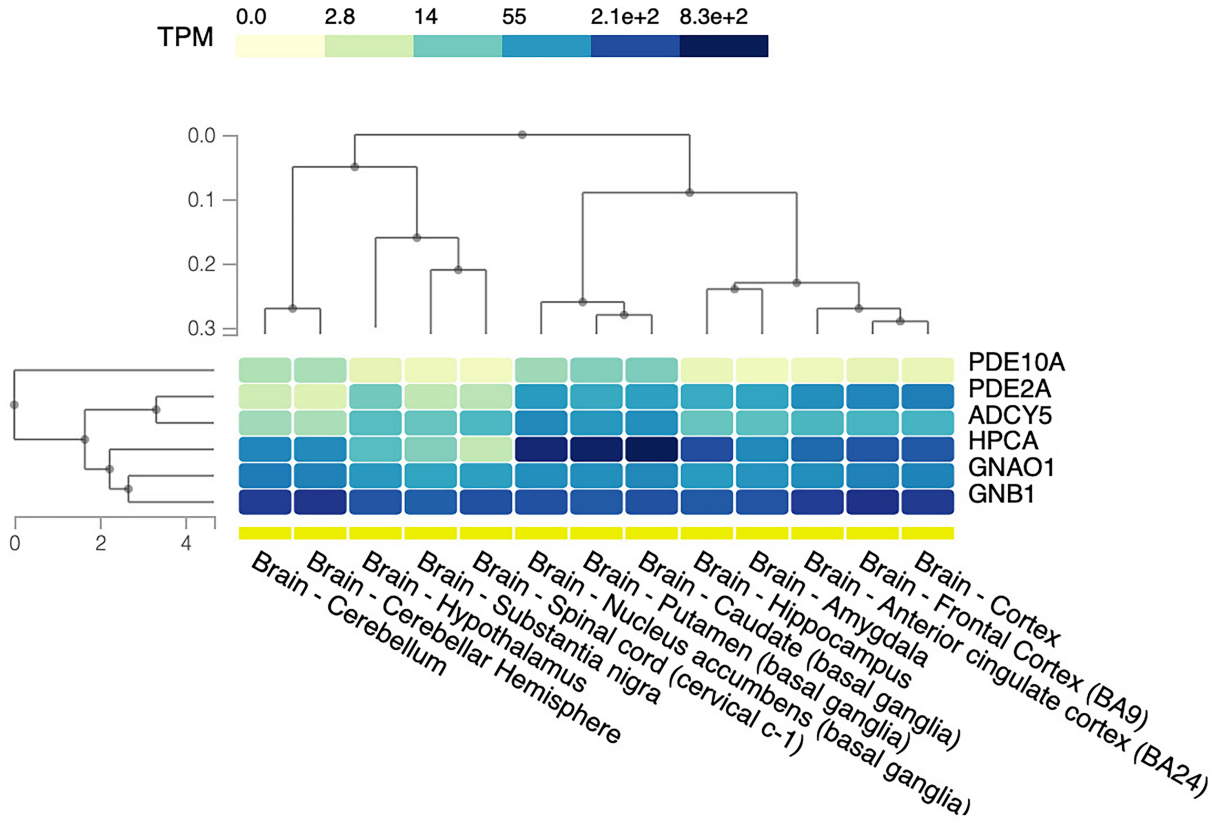

FIGURE 5
Brain tissue expression of protein related to GPCRs–cAMP signaling. The heat map shows relative gene expression indicated as transcripts per kilobase per million mapped reads (TPM) based on the color scale. The data and image were obtained from: https://www.gtexportal.org, the GTEx Portal on 29/04/22. BA, Brodmann area.

Coherently with previous observations, a different brain tissue expression of these proteins has been reported. GNAO1, GNB1, and PDE2A are ubiquitously expressed in the brain, with high levels in cortical areas, while the highest HPCA, ADCY5, and PDE10A expressions are within putamen, caudate, and nucleus accumbens [data from the Genotype-Tissue Expression (GTEx) project (https://gtexportal.org/home/)] (61) (Figure 5).

Transduction of dopaminergic and adenosine inputs from heterotrimeric GPCRs activates a cascade contributing, together with other molecular actors (e.g., PDEs), to cAMP production. The generated cAMP propagates downstream signaling *via* specific cAMP-binding proteins (e.g., cAMP-dependent kinases, transcription factors, or ion transporters). This pathway, ubiquitously expressed in the central nervous system, seems to be particularly relevant for the proper functioning of MSNs of the direct and indirect pathways, and therefore for postural control, initiation of voluntary movements, prevention of unwanted movements, and motor learning (2, 5, 15).

Specifically, cAMP levels finely regulate the activity of protein such as the cAMP-regulated phosphoprotein molecular mass 32 (DARPP-32) and the cAMP-response element-binding protein (CREB). These proteins are thought to play an important role by mediating the dopaminergic neuromodulatory effects on GABAergic transmission and regulating the long-term synaptic plasticity and neuronal growth at MSNs level (62). Thus, through an altered basal ganglia activity, altered cAMP levels may underpin movement disorders such as dystonia, chorea, and parkinsonism.

The contribution of this pathway to neurodevelopment has been less explored. The possible individuation of common mechanisms rather than mechanisms specific to certain disorders deserves further studies.

Here, we reviewed the motor, epileptic, and neurodevelopmental phenotype of genetic neurological disorders affecting GPCRs–cAMP signaling pathway. This group of disorders presents with a highly recognizable clinical phenotype with distinctive movement disorder, epileptic, and neurodevelopmental features. While no biomarker is available for this group of potentially life-threatening disorders, the existence of distinctive clinical features prompting diagnostic

suspicion and early detection has relevant implications for patient management.

Author contributions

SG and LP: data collection, first draft writing. VL and SM: critical review. MD, KB, and MN: data collection. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Case report: A novel *PPP3CA* truncating mutation within the regulatory domain causes severe developmental and epileptic encephalopathy in a Chinese patient

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Introduction: Developmental and epileptic encephalopathy 91 (DEE91; OMIM#617711) is a severe neurodevelopmental disorder caused by heterozygous *PPP3CA* variants. To the best of our knowledge, only a few DEE91 cases have been reported.

Results: This study reports a boy who experienced recurrent afebrile convulsions and spasms at the age of 2 months. After being given multiple antiepileptic treatments with levetiracetam, adrenocorticotropic hormone (ACTH), prednisone, topiramate, and clonazepam, his seizures were not completely relieved. At the age of 4 months, the patient exhibited delayed neuromotor development and difficulty in feeding; at the age of 6 months, he was diagnosed with developmental regression with recurrent spasms and myoclonic seizures that could respond to vigabatrin. At the age of 1 year and 4 months, the patient showed profound global developmental delay (GDD) with intermittent absence seizures. Whole-exome sequencing (WES) identified a novel loss-of-function variant c.1258_1259insAGTG (p. Val420Glufs*32) in *PPP3CA*.

Conclusion: This finding expands the genetic spectrum of the *PPP3CA* gene and reinforces the theory that DEE91-associated truncating variants cluster within a 26-amino acid region in the regulatory domain (RD) of *PPP3CA*.

KEYWORDS

***PPP3CA*, DEE91, epilepsy, regulatory domain, case report**

Introduction

Developmental and epileptic encephalopathy 91 (DEE91; MIM: #617711) is a severe, early-onset neurodevelopmental disease. Patients with DEE91 tend to be clinically diagnosed with West syndrome (WS) or infantile spasms syndrome, developmental regression, and hypsarrhythmia (1, 2). DEE91 is characterized by a delay in infantile neuromotor development, resulting in severely to a profoundly impaired intellectual development, refractory epilepsy, and developmental regression (3). Unlike the diverse genetic causes of WS (2), DEE91 is a well-defined, *PPP3CA*-associated monogenic disease. Thus, the clinical diagnosis of DEE91 is dependent on genetic tests.

Calcineurin is a serine/threonine protein phosphatase that is dependent on calcium and calmodulin. It is a heterodimeric protein consisting of the catalytic subunit calcineurin A and the tightly bound calcium ion-binding subunit calcineurin B. The primary sequences of subunits and heterodimeric quaternary structures are highly conserved from yeast to mammals (4–6). *PPP3CA*, which is also known as calcineurin A, is a catalytic subunit of calcineurin that mediates $Ca2^{+}$-dependent signal transduction (7, 8). Calcineurin defects are associated with various human disorders (9–12). Notably, the heterozygous loss-of-function (LOF) and gain-of-function variants (GOF) in the *PPP3CA* gene are presumed to cause DEE91 and multiple congenital abnormalities (ACCIID; MIM: #618265), respectively (1). Mizuguchi et al. (1) suggested that variants in the catalytic or metal binding domains led to nonsyndromic epileptic encephalopathy, which is related to LOF, while variants in the AI domain cause ACCIID, which belongs to GOF (1).

In this study, we reported a 1 year and 4 month-old male patient who was clinically diagnosed with EE and had refractory epilepsy. During the patient's treatment, seizure progression, developmental delay, and the onset of developmental regression were observed.

Case description

We reported a 1 year and 4 month-old male patient who was the fourth pregnancy and the second birth to a normal non-consanguineous couple. His perinatal period was normal. The second pregnancy was aborted due to non-medical reasons, and the third pregnancy was terminated at 5 months of pregnancy because of cardiac dysplasia. The elder sister presents no abnormal conditions at the age of 20 years, and there is no family history of disease.

At the age of 2 months, the patient was admitted to a hospital after experiencing 3 days of recurrent afebrile convulsions. The seizures lasted approximately 10 s every time, occurred five to six times per day, and involved staring eyes, rigid limbs, and cyanosis face and lips, and the patient was unresponsive without incontinence during the attacks. His physical examination and developmental assessment were normal. A brain magnetic resonance imaging (MRI) showed that the bilateral frontotemporal extracerebral space was broadened, especially on the left side (Figure 1). An electroencephalography (EEG) showed multifocal discharges of irregular sharp-slow waves in the frontopolar, lateral frontal, central, and temporal regions during both consciousness and sleep (Figure 2A). The results of laboratory tests were unavailable. The patient was diagnosed with epilepsy and treated with 10 mg/kg/day levetiracetam, divided by q12H, and the dose was added to 20 mg/kg/day at day 3 of the antiepileptic treatment. His convulsions were relieved, the patient was discharged, and 10 mg/kg/day levetiracetam was prescribed as a maintenance dose.

After levetiracetam was administered for ∼15 days, the patient developed 10–20 spasms/spasticity in a cluster per day. The features of the spasms included instant onset of involuntary nodding, forward flexion of head and torso, eyes staring upwards, and unresponsiveness. He was then sent for medication for a second time. His physical examination and laboratory tests were normal. An EEG showed modified hypsarrhythmia (Figure 2B). The patient was diagnosed with WS (infantile spasms syndrome) and treated with ACTH at 3 units/kg/day through an i.v. for 12 days, an antiepileptic treatment of 3 mg/kg/day topiramate, divided by q12H, and 0.03 mg/kg/day clonazepam. Levetiracetam was sequentially added at 10 mg/kg/day with divided q12H. His spasms were relieved, the frequency of attacks was reduced to 2–3 times per day, milder involuntary nodding and spasticity were observed, and no facial cyanosis occurred. The patient was discharged with a prescription of prednisone at 1 mg/kg/day, p.o., instead of the i.v. ACTH treatment, and topiramate, clonazepam, and levetiracetam were administered as antiepileptic medication.

At the age of 4 months, the patient was found to have significantly delayed neuromotor development. He was unable to look up or turn over, and he lost the ability to pronounce vowels, accompanied by hypotonia in the limbs. At the age of 6 months, the patient lost a previous developmental milestone, which was manifested as unstable neck erection, and this was accompanied by feeding difficulties, including an unwillingness to eat and refusal to drink milk, as well as recurrent spasms and bilateral tonic-clonic. For the third time, he was treated by adding 45 mg/kg/day vigabatrin divided by q12H instead of levetiracetam, and maintenance doses of topiramate, clonazepam, and prednisone were administered. At the age of 8 months, 60 mg/kg/day vigabatrin, divided by q12H, and 0.1 mg/kg/day clonazepam, divided by q12H, were added to the treatment, and the patient's seizures were significantly relieved but he still experienced hypotension in his limbs.

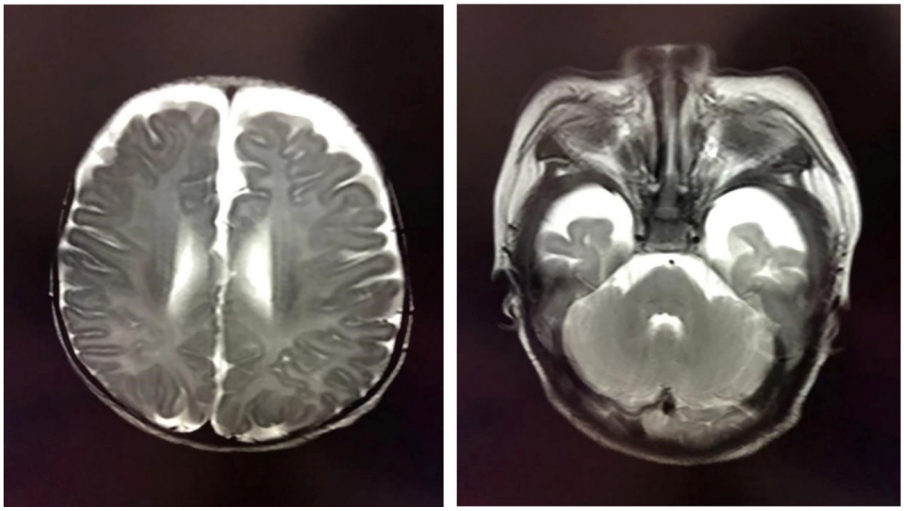

FIGURE 1
Brain MRI showing the broadened bilateral frontotemporal extracerebral space, which is especially apparent on the left side.

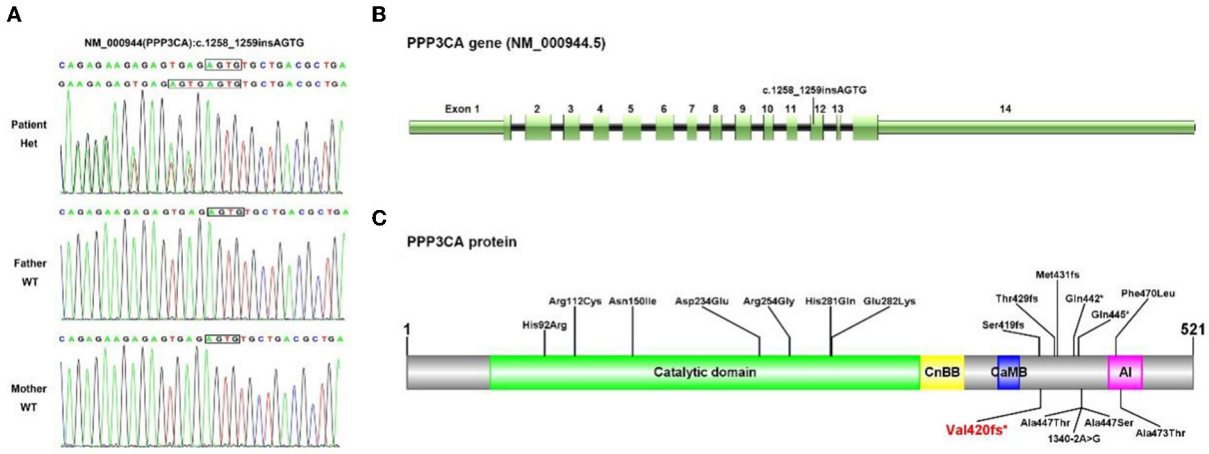

FIGURE 2
EEG of the patient diagnosed with developmental and epileptic encephalopathy 91 at ages of 2 months **(A)**, 2 and a half months **(B)**, and one year and four months **(C)**.

At the last follow-up examination, the patient was 1 year and 4 months old. The patient weighed 9 kg and had no significant deformity. His motor and language developmental milestones equalled those of normal 1- and 3-month-old infants, respectively, indicating that profound global developmental delay (GDD), including an unstable neck erection and inability to raise his head, turn over, and laugh, occurred with limb hypotension. The patient could only pronounce vowels. Under a treatment with 45 mg/kg/day vigabatrin, divided by q12H, 0.1 mg/kg/day clonazepam, divided by q12H, 6 mg/kg/day topiramate, divided by q12H, and 1 mg/kg/day prednisone, involuntary nodding and forward flexion of head and torso in the patient disappeared; however, he still had mild seizures (with staring eyes for approximately 10 s every time). A follow-up EEG showed modified hypsarrhythmia during sleep (Figure 2C).

Methods

Whole-exome sequencing

The whole-exon capture chip used by Trio-WES is the IDT xGen Exome Research Panel version 1.0, which was sequenced by an Illumina NovaSeq 6000 series sequencer (PE150). The

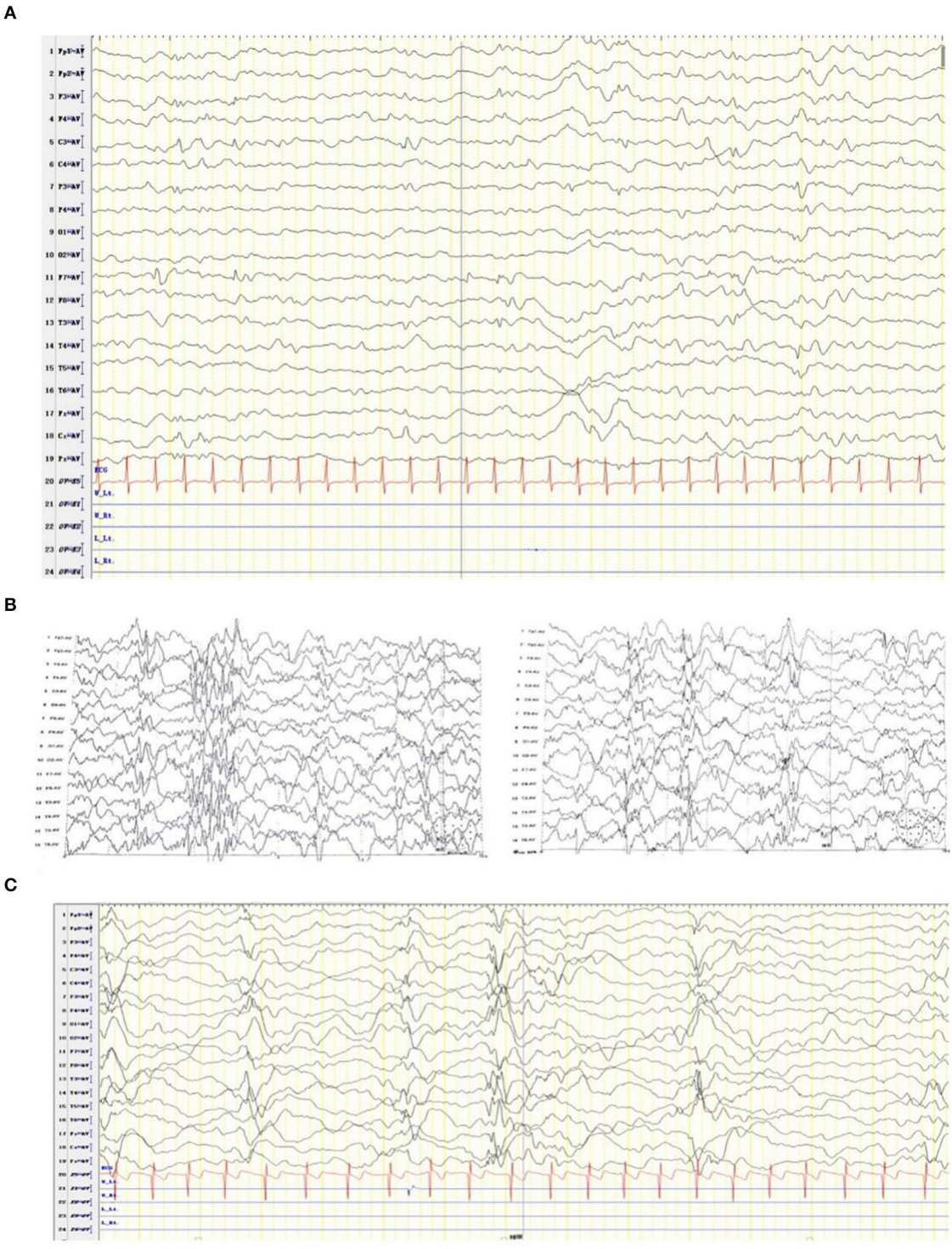

FIGURE 3
Sanger sequencing **(A)** confirmed the mutation NM_000944.5(PPP3CA):c.1258_125 9 insAGTG on exon 12 **(B)** and the pattern diagram of PPP3CA functional domains **(C)**.

sequencing coverage of the target sequence was not <99%. The raw sequencing data were screened, and low-quality reads were filtered through a quality control (QC). Then, the BWA software was used to perform a sequence alignment with the

TABLE 1 Truncating mutations in the *PPP3CA* gene in the historical literature and this report.

Variant number	Nucleic acid change	Protein change	Mutant type	Disease or phenotype	References
1	1255_1256delAG	Ser419fs	Frameshift	Epileptic encephalopathy, early infantile	(13)
2	1283dupC	Thr429fs	Frameshift	Epileptic encephalopathy, early infantile	(14)
3	1290dupC	Met431fs	Frameshift	Epileptic encephalopathy	(1)
4	1324C>T	Gln442*	Non-Sense	Epileptic encephalopathy	(15)
5	1333C>T	Gln445*	Non-Sense	Neurodevelopmental disease, severe with seizures	(3)
6	1258_1259insAGTG	Val440fs	Frameshift	Seizures, generalized developmental delay, epileptic seizures, infantile spasms, feeding difficulties	This study, 2022

*means "stopping translation".

reference human genome. Repeated read operations were excluded, the remaining read operations were statistically analyzed, and the GATK software was used to identify variables. Detected variants were bioinformatically annotated based on public databases such as ClinVar, HGMD, gnomAD, and the 1,000 Genomes Project and were predicted to have pathogenic or deleterious effects based on database data. Variation classification mainly refers to the 2015 ACMG Genetic Variation Classification Standards and Guidelines gene mutation classification system.

Results

Trio-whole-exome sequencing (WES) was performed after signed consent was obtained from the parents, according to the medical ethics statement. We identified a heterozygous variant (NM_000944.5: c.1258_1259insAGTG, p. Val420Glufs*32) of the *PPP3CA* gene exon 12 in the proband. The variant was *de novo*. The result was confirmed by Sanger sequencing (Figure 3A). The variant p. Val420Glufs*32 was pathogenic (PVS1 + PS2 + PM2) according to the American College of Medical Genetics and Genomics (ACMG) practical guidelines (16). The variant has not been documented in public variant databases or reported in historical literature. In addition, pathogenic variants in the list of the OMIM genes (https://www.omim.org/phenotypicSeries/PS308350) associated with WS or developmental and epileptic encephalopathies were excluded by WES.

Discussion and conclusion

We identified a frameshift mutation in *PPP3CA* (p. Val420Glufs*32) that caused DEE91, an early-onset WS-like disorder, in a Chinese male pediatric patient. The specific type of seizure, infantile spasms, and developmental regression were significant in our patient, which prompted the initial diagnosis of WS. In detail, infantile spasms, myoclonus, and multifocal hypsarrhythmia were observed in the EEG in the present case, and these observations share the core phenotype of WS (2). However, the onset of infantile spasms in WS cases usually occurs between 4 and 8 months of age (2), while patients with DEE91 have highly variable epilepsy. Of the 6 cases of DEE91 previously reported by Myers et al. one patient experienced no seizures, two patients had mild seizures that were not detected until 1 year of age, and three patients had seizures and focal seizures at 3 months of age. In another case, seizure onset was observed at the age of 4 years (3). The types of epilepsy also vary and include focal, epileptic spasms, tonic, myoclonic, generalized tonic-clonic, and atonic seizures in previous studies (1, 3, 13, 17). In this study, however, the transformation of seizure types, e.g., spasms to bilateral tonic-clonic seizure, was demonstrated, suggesting that the types of epilepsy in a patient with DEE91 may change, which is similar to patients with WS (2). In addition, the developmental regression observed in our case indicates that regressed development may be relatively common with DEE91. The developmental regression and loss of neck erection in our study could not be identified unless the clinical data before the first 4 months of the patient's life were available.

In our case, the variant c.1258_1259insAGTG (p. Val420Glufs*32) of the *PPP3CA* gene was detected in the proband, which is located on exon 12 (Figure 3B). This mutation is between the metal-binding (calmodulin binding, CaMB) and autoinhibitory (AI) domains (18, 19), resulting in the loss of the latter AI domain (Figure 3C). At present, only the following truncating variants are included in HGMD (Table 1): two non-sense variants and three frameshift variants. These truncating variants are all located between the CaMB and AI domains, which cause the main clinical features of epileptic encephalopathy, neurodevelopmental disease, and severe seizures (Table 1).

Recently, Panneerselvam et al. revealed that all the reported truncating variants are located in a cluster within a 26-amino acid region in the regulatory domain (RD) in relatively more severe DEE91 cases. It concluded that patients with a truncating variant experienced more severe seizures with earlier onsets compared to those of patients with an LOF missense variant, while autism spectrum disorder was relatively common in the latter (20). Rydzanicz et al. described the discovery of a novel *de novo* c.1324C>T (p. (Gln442Ter) *PPP3CA* variant by WES in a boy with severe early-onset epileptic encephalopathy. Western blot experiments in patient cells (EBV-transformed lymphocytes and neural cells obtained by reprogramming) showed that the protein expression levels of both mutant and wild-type proteins were significantly reduced despite normal mRNA abundances (15). Our findings support this theory.

In conclusion, we identified a novel *PPP3CA* frameshift variant through WES, confirming the disease of the proband at the molecular level. The results expand the spectrum of pathogenic variants of DEE91. The clinical features and molecular evidence in the patient support the theory that LOF mutation in *PPP3CA* causes DEE91 and truncating variants within RD lead to severe phenotype. Based on the above results, WES has become an important method for diagnosing rare genetic diseases.

Data availability statement

The datasets presented in this article are not readily available because of ethical and privacy restrictions. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

JL enrolled the patient, collected, and interpreted the clinical information and wrote the manuscript. JC designed the study and corrected the manuscript. Both authors approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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