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DLG4-related synaptopathy: a new rare brain disorderAgustí Rodríguez-Palmero et al.[#]

PURPOSE: Postsynaptic density protein-95 (PSD-95), encoded by *DLG4*, regulates excitatory synaptic function in the brain. Here we present the clinical and genetic features of 53 patients (42 previously unpublished) with *DLG4* variants.

METHODS: The clinical and genetic information were collected through GeneMatcher collaboration. All the individuals were investigated by local clinicians and the gene variants were identified by clinical exome/genome sequencing.

RESULTS: The clinical picture was predominated by early onset global developmental delay, intellectual disability, autism spectrum disorder, and attention deficit–hyperactivity disorder, all of which point to a brain disorder. Marfanoid habitus, which was previously suggested to be a characteristic feature of *DLG4*-related phenotypes, was found in only nine individuals and despite some overlapping features, a distinct facial dysmorphism could not be established. Of the 45 different *DLG4* variants, 39 were predicted to lead to loss of protein function and the majority occurred de novo (four with unknown origin). The six missense variants identified were suggested to lead to structural or functional changes by protein modeling studies.

CONCLUSION: The present study shows that clinical manifestations associated with *DLG4* overlap with those found in other neurodevelopmental disorders of synaptic dysfunction; thus, we designate this group of disorders as *DLG4*-related synaptopathy.

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INTRODUCTION

The human brain is formed by about 100 billion neurons that are highly interconnected through synapses, which regulate the brain circuit functions. The molecular structure of the synapse is highly complex, and its function is regulated by several proteins at different levels. In excitatory synapses, the postsynaptic submembrane space contains a multiprotein complex called the postsynaptic density (PSD), which has crucial roles in the structural organization and function of the synapses. It contains several scaffold proteins including PSD-95, encoded by *DLG4* (discs large MAGUK scaffold protein 4). PSD-95 belongs to the MAGUK (membrane-associated guanylate kinases) family and has 3 PDZ domains at the N-terminus, an SH3 (Src homology 3) domain and a guanylate kinase-like domain (GKLD). PSD-95 participates in synaptic maturation and dendritic morphology and regulates function of the glutamate receptors NMDA (N-methyl-D-aspartic acid) and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid).¹ PSD-95 is also involved in the structural organization of the PSD through the interaction and stabilization of adhesion molecules, such as NLG1 (neuroligin 1); the voltage-gated, *Shaker*-type K⁺ (K_v1) channels are also key binding partners of PSD-95.¹ Notably, the PSD is a very dynamic structure and its composition (i.e., PSD-95 expression) and morphology are dependent on neuronal activity, which determines synaptic plasticity, essential for learning and memory processes.¹

DLG4 (encoding PSD-95) has three other paralogs (*DLG1* encoding SAP97; *DLG2* encoding PSD-93/Chapsyn-110; and *DLG3* encoding SAP-102), all of which are evolutionarily conserved and have diverse functions.² Homozygous *Dlg1* knockout mice (*Dlg1*^{-/-}) are embryonic lethal while the knockouts of other paralogs are viable. *Dlg3*^{-/-} animals do not show observable cognitive deficits, but in humans, truncating *DLG3* variants have been identified in individuals with X-linked intellectual disability with or without comorbidities (OMIM 300850).^{3,4} On the other hand, *Dlg2*^{-/-} mice show impairments in cognitive flexibility, learning, and attention²

and *DLG2* missense variants are associated with autism,⁵ and gross *DLG2* deletions are described in individuals with schizophrenia, autism, and bipolar disorder.^{6,7} The *Dlg4*^{-/-} mice show increased repetitive behaviors, abnormal communication, impaired motor coordination, increased stress reactivity, anxiety-related responses, and abnormal learning and working memory.^{2,8,9} On the other hand, male *Dlg4*^{+/-} mice present with hypersocial behavior with increased aggression and territoriality levels, while female mice show increased vocalization, and both genders show hypoactivity without motor deficits.¹⁰

In humans, pathogenic *DLG4* variants are rare, and to date only 11 individuals with a *DLG4* variant have been reported. Eight of these published individuals were identified as part of screening cohorts to find new candidate genes for ID ($n = 4$), cerebral visual impairment ($n = 1$), developmental disorders ($n = 1$), and schizophrenia and autism spectrum disorders ($n = 2$).^{11–15} The remaining three individuals were identified by Moutton et al. in a series of 64 individuals with ID and skeletal signs suggestive of Marfan syndrome (OMIM 154700) but do not meet the international criteria, termed Marfanoid habitus (MH).¹⁶

Here we report phenotype and genotype information on 53 individuals (including the 11 previously published cases, clinical features of whom are updated when possible) with heterozygous *DLG4* variants collected through an international collaboration. The effect of the missense variants on protein function was further investigated through structural modeling and molecular dynamics simulation studies. Our results establish *DLG4*-related synaptopathy as a new and rare brain disorder.

MATERIALS AND METHODS

Individuals included in this study

We ascertained the genotype and phenotype information for 53 individuals with a variant in *DLG4* (Fig. 1, Table 1, Figs. S1, S2, Tables S1, S2). Eleven individuals were reported previously.^{11–17} Phenotypes of four of

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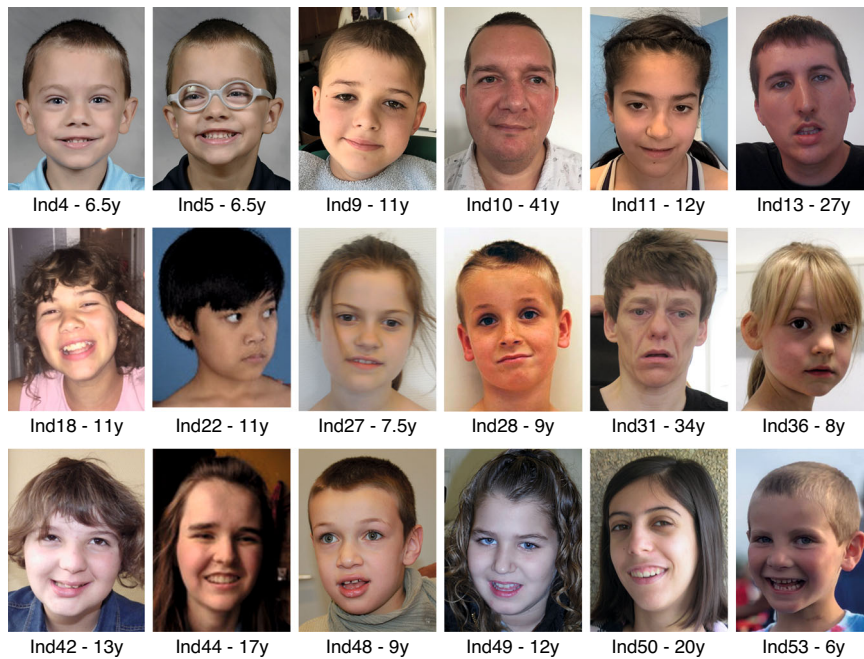


Fig. 1 Facial features of the individuals. Individuals 4 and 5 are twins, and individuals, 49 and 50 have the same *DLG4* variant. Ind individual.

these individuals have been updated, while further clinical information could not be obtained for seven individuals. The previously unreported individuals were identified through GeneMatcher (<http://genematcher.org/statistics/>)¹⁸ or the Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (DECIPHER, <https://decipher.sanger.ac.uk/>).¹⁹ The clinical information of each individual was reviewed including neurodevelopment, growth parameters, neurological manifestations, behavior, dysmorphology, and MH by the local clinicians (Tables 1 and S1). The prevalence of each clinical manifestation related to the total number of cases for whom this information was available is shown in Table S2. The presence of ID was evaluated in individuals over 5 years old and ASD in those over 3 years old. Regression was defined as a loss of previously acquired skills. Statistical calculations were made using Pearson chi-squared test with Yates continuity correction. A heat map for clinical features was built by means of *gplots* package in R and hierarchical clustering was made according to binary distances (Fig. 2S).

Identification and evaluation of the variants

DLG4 variants were identified in the probands using massively parallel sequencing (next-generation sequencing; NGS) based technologies (exome/genome sequencing with or without employing virtual gene panels) in clinical diagnostic or research settings, and parental testing for the identified variant was performed when possible ($n = 48$). Pathogenicity of the identified *DLG4* variants was established according to American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) criteria (Table S3).^{20,21} The Genome Aggregation Database (gnomAD v.2.1.1; <https://gnomad.broadinstitute.org/>) was employed to check the presence of the variants in control populations. NMDescPredictor tool²² (<https://nmdprediction.shinyapps.io/nmdescpredictor/>) was employed to predict whether the truncating variants escaped nonsense-mediated decay (NMD). SpliceAI²³ (<https://github.com/illumina/SpliceAI>), a deep learning-based splice variant prediction tool was used to annotate the variants for their predict effect on splicing (Table S3). All the variants are described using the NM_001365.4 (GRCh37/hg19) transcript of *DLG4* (Fig. 2, Tables 2 and S3).

Structural modeling and molecular dynamics simulation of the missense variants

The 3D structure of the human PSD-95 protein (UniProtKB id: P78352-2, The UniProt Consortium 2019) was modeled using as templates the homologous structures present in the Protein Data Bank: 1KJW²⁴ and 2XKX²⁵ (Fig. 3). Models for the wild type protein and the Gly220Val, Asp229Val, Gly241Ser, Asp375Gly, Arg629Gln and Thr654Ile variants were

built using the SWISS-MODEL server (<https://swissmodel.expasy.org>), and their structural quality was within the range of that accepted for homology-based structure (Anolea/Gromos/QMEAN4).²⁶ Molecular dynamics simulation was carried out as described previously²⁷ and the details can be found in the Supplementary text.

RESULTS

Phenotypic spectrum

The phenotype information is shown in Tables 1 and S1 and the frequencies of clinical features in Table S2. There were no significant gender-specific differences in the clinical severity and the median age at last evaluation was 11 years (18 months–47 years). The mean age for initial clinical presentation was 1.3 years (4 months–5 years). Most individuals presented with the first symptoms in form of developmental delay before age two, except for five individuals with later clinical onset, including four who became symptomatic at age 3 years and one at age 5 years. Of 45 individuals, GDD was reported in 38 (84%) whereas specific motor developmental delay was present in six and predominant language delay in two individuals. Speech was completely absent at the last evaluation in eight individuals older than 3 years. Regression in motor development and/or language was observed in 17/43 individuals with an average age of onset of regression of 4 years (6 months–18 years). ID was present in 44/45 individuals (98%) (all of whom were older than 5 years of age at the last evaluation) and was classified as severe in 29%, moderate in 34%, mild in 29%, mild–moderate in 2% and unspecified in 4%. Of those older than 3 years with clinical information available, ASD manifestations were documented in 24/43 individuals. ASD was reported as a comorbidity in 15/26 (57%) individuals with moderate to severe ID but in only 3/10 (30%) individuals with mild ID ($p = 0.562$). Among the 12 individuals with language regression (with or without motor regression), ten individuals also had ASD, but not all the individuals with ASD had language regression. Attention deficit hyperactivity disorder (ADHD) features were present in 20/35 individuals and tended to occur more frequently in individuals with ASD (11/17, 64%) than in those without ASD (8/17, 47%; $p = 0.49$).

Table 1. Clinical features.

ID of the affected individual	Gender, current age	Age at onset	Developmental delay	Developmental regression	ID	Autism	Anxiety	ADHD	Abnormal movements	Behavior other	Muscle tone	Epilepsy (age onset)	Ophthalmological	Dysmorphic face	Skeletal	MH	MRI
1	M, 5 years	NI	+	(Motor)	NI	+	NI	NI	NI		-	-	Nystagmus	+	Joint laxity	NI	Normal
2	M, 10 years	6 months	+	(Motor)	+	+	+	+	-	OCD Repetitive behaviors	-	+ (9 years) General	Hyperopia	+	-	-	Normal
3	F, 15 years	5 years	-		+	+	+	+	Tremors	Hallucinations Suicidal ideations Thoughts of harming others (sexually abused in childhood)	-	-	Myopia	+	Joint laxity Scoliosis	+	Normal
4	M, 6.5 years	10 months	+	(GDD)	+	+	-	-	-	Overfriendly	Hypotonia	-	Strabismus, hyperopia requiring surgery	NI	Joint laxity Hyperextensible knees requiring AFOs	+	Normal
5	M, 6.5 years	9 months	+	(GDD)	-	+	-	-	-		Hypotonia	-	Strabismus, hyperopia requiring surgery	NI	Joint laxity Hyperextensible AFOs requiring	+	NA
6	F, 16 years	1 years	+	(GDD)	+	-	-	-	Stereotypies Ataxia	Overfriendly	Hypotonia	+ (15 years)	-	-	Joint laxity Scoliosis	-	Normal
7	M, 7 years	3 years	+	(GDD)	+	-	-	-	-	Hyperactivity Dyspraxia	Hypotonia	+ (15 years) Focal ESES	Strabismus (left exotropia)	-	-	-	Normal
8	F, 2 years	NI	NI	NI	NA	NA	NI	NA	NI		NI	NI	NI	NI	NI	NI	Normal
9	M, 11 years	6 months	+	(GDD)	+	+	+	+	Stereotypies Ataxia	Overfriendly High sensory needs	Hypotonia	+ (9 years) Focal	Strabismus Nystagmus	-	Joint laxity	-	Hippocampal asymmetry (left smaller, enlargement of the gray-white matter differentiation) Mild periventricular WMH Mild cerebral and cerebellar atrophy. Ventricular dilatation Thin CC
10	M, 36 years	NI	+	(GDD)	-	+	+	+	-	Obsessed with football and jigsaw puzzles	-	+ General	-	-	-	-	NI
11	F, 11 years	3 years	+	(GDD)	-	-	-	-	-	Withdrawn behavior	-	+ (6 years) FS	Strabismus (alternating exotropia) Astigmatism	-	-	-	Normal
12	M, 18 years	<1 year	+	(Motor)	+	+	+	+	Apraxia Tremor Stereotypies Tics	Catatonia (14 years)	-	-	-	-	-	-	Normal
13	M, 28 years	3 months	+	(GDD)	-	+	+	+	-	Aggressive, shy	Hypotonia	-	NI	+	-	-	NI
14	M, 1.5 years	6 months	+	(GDD)	NA	NA	NI	NA	NI		NI	+ (6 months) IS	-	-	-	-	NI
15	F, 4 years	NI	+	(Language)	NA	+	NI	NI	NI	No aggressive/ repetitive behavior	NI	NI	NI	NI	NI	NI	NI
16	M, 12 years	15 months	+	(GDD)	+	NI	NI	NI	Episodes of whole body shaking		-	+ Focal	-	-	NI	NI	Normal
17	M, 7 years	2 years	+	(GDD)	+	+	+	+	(Mild)	Easily overstrained by external stimuli, played rather on his own in infancy	Hypotonia	-	-	+	Joint laxity	-	Normal

Table 1 continued

ID of the affected individual	Gender, current age	Age at onset	Developmental delay	Developmental regression	ID	Autism	Anxiety	ADHD	Abnormal movements	Behavior other	Muscle tone	Epilepsy (age onset)	Ophthalmological	Dysmorphic face	Skeletal	MH	MRI
18	F, 12 years	NI	NI	NI	+(Mild)	+	NI	NI	NI	Tantrums	NI	-	NI	NI	NI	NI	NI
19	F, 3 years	NI	+(GDD)	NI	NA	NI	NA	NI	Easily overstimulated	-	-	-	Cortical blindness	+	NI	NI	Normal
20	F, 11 years	3 years	+(GDD)	-	+(Moderate)	+	+	-	OCD	Hypotonia	+	+(9 years)	Myopia	NI	Joint laxity Scoliosis	-	Large perivascular space (left frontal)
21	M, 7 years	18 months	+(GDD)	-	+(Moderate)	+	NI	+	Hyperactivity	-	-	-	NI	-	-	-	NI
22	M, 11 years	3 years	+(GDD)	+(3 years)	+(Severe)	+	-	-	-	-	-	-	-	-	Joint laxity Pectus anomaly	+	NA
23	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
24	M, 21 years	NI	NI	NI	+(Moderate)	-	NI	NI	NI	Self-injurious behavior	NI	-	Strabismus	NI	Joint laxity Pectus anomaly	+	Vermis atrophy
25	M, 4 years	6 months	+(GDD)	-	NA	+	-	-	-	Behavioral outburst with frustration (can be physical)	Hypotonia	-	Strabismus	-	Joint laxity	-	Normal
26	M, 7 years	6 months	+(GDD)	-	+(Moderate)	+	-	+	-	-	Hypotonia	+(6 months)	-	+	-	NI	Mild frontotemporal corticosubcortical atrophy with enlarged anterior peripherical liquor spaces Dysmorphic corpus callosum
27	F, 10 years	6 months	+(Motor)	-	+(Moderate)	-	-	+	-	Social	-	-	Strabismus	+	Joint laxity Kyphosis Pes planus	-	Normal
28	M, 9 years	2 years	+(GDD)	-	+(Mild)	-	-	+	-	Tantrums	Toe walking (persist 9 years)	-	-	-	-	-	Frontotemporal corticosubcortical atrophy with enlarged anterior peripherical liquor spaces Thin corpus callosum (anterior predominance)
29	F, 20 years	<1 year	+(GDD)	+(18 years) motor	+(Severe)	+	-	+	Stereotypies	Two episodes of psychosis at 12 and 13 years of age respectively	Hypotonia	+	Amblyopia	-	Scoliosis	-	NI
30	F, 23 years	6 months	+(GDD)	-	+(Moderate)	-	+	-	-	Withdrawn behavior	-	-	-	-	Cubitus valgus	-	Vermis atrophy
31	F, 34 years	1 year	+(GDD)	+(6 months-3 years)	+(Severe)	-	+	-	Dystonia Stereotypies	Inappropriate laughing/ screaming spells	Dystonia	+(1) General	-	-	Scoliosis	-	NI
32	M, 5 years	<1 year	+(GDD)	-	+(Mild)	-	-	+	Stereotypies Ataxia	Easily frustrated, frequent use of dirty words	Hypotonia	+(1.1 years) FS	Strabismus (exotropia)	-	-	-	NI
33	M, 16 years	<1 year	+(GDD)	+(2 years) language	+(Severe)	+	NI	+	-	Impulsive	Hypotonia	+(5 years) General Refractory	-	+	-	-	Periatrial bilateral T2 HI
34	M, 18 years	<1 year	+(GDD)	+(22 months) language	+(Moderate)	+	-	-	-	Behavioral outbursts	Hypotonia	+(7 years) Focal	-	+	Scoliosis	-	Normal
35	M, 11 years	NI	NI	NI	+(Mild)	NI	+	NI	NI	-	NI	NI	Cortical blindness	NI	NI	NI	Normal
36	F, 8 years	<1 year	+(GDD)	-	+(Moderate)	-	+	NI	Stereotypies	Tantrums	-	+(10 years)	-	-	-	-	Normal
37	F, 47 years	NI	+(motor)	NI	+(Moderate)	+	NI	NI	NI	Depression and aggressive outbursts; bipolar disorder	NI	NI	NI	NI	NI	NI	Normal
38	M, 3.5 years	8 months	+(GDD)	+(18 months)	NA	+	NI	NA	-	Hyperactive, sensitive to high-pitched noises, loves playing with	Hypotonia	-	-	-	Joint laxity	-	Incomplete inversion of the left hippocampus

Table 1 continued

ID of the affected individual	Gender, current age	Age at onset	Developmental delay	Developmental regression	ID	Autism	Anxiety	ADHD	Abnormal movements	Behavior other	Muscle tone	Epilepsy (age onset)	Ophthalmological	Skeletal	MH	MRI
39	F, 23 years	2 years	+	(GDD)	-	-	+	-	Ataxia	Social	NI	Yes (7 years) Focal Refractory	-	-	-	Asymmetric dilatation of the lateral ventricles White matter volume loss Thin corpus callosum
40	F, 13 years	6 months	+	(GDD)	+	-	+	+	Tics	water Head banging to fall asleep	Hypotonia	+	(4 months) 15, focal	-	-	Normal
41	M, 35 years	NI	+	(Language)	NI	+	NI	NI	NI	Social	NI	+	(14 years)	Pectus anomaly Limited elbow extension	+	Mild corticospinal atrophy
42	F, 13 years	9 months	+	(GDD)	+	+	+	+	Stereotypies	Aggressive and self-injurious behavior, trichotillomania, pica, bruxism	-	+	(2 years)	-	NI	Normal
43	F, 13 years	NI	+	(GDD)	+	NI	NI	+	Spasticity Dystonia Ataxia	Mild behavioral problems	Dystonia	+	Focal	Scoliosis	NI	Incomplete hippocampal inversion and ipsilateral dysmorphic temporal horn
44	F, 19 years	NI	+	(GDD)	+	+	NI	NI	Tremors Stereotypies	Period of hearing voices	Hypotonia	+	(8 years) Focal	Joint laxity Scoliosis	+	Normal
45	F, 2 years	2 years	+	(GDD)	-	NA	+	NA	Ataxia	Withdrawn behavior	Hypotonia	-	-	-	-	NI
46	M, 23 years	3 years	+	(GDD)	-	-	+	-	Dystonia	Withdrawn behavior	Dystonia	-	Nystagmus	Joint laxity Scoliosis Dolichostenomelia	+	Normal
47	M, 6 years	<1 year	+	(GDD)	-	-	+	-	Stereotypies Ataxia	Inappropriately contact seeking and trustful, happy, smiling, laughing Hyperactive Repetitive behaviors	Hypotonia	-	-	-	-	Normal
48	M, 18 years	9 months	+	(GDD)	-	-	+	+	Dystonia Stereotypies Ataxia	Very happy, distanceless, attention seeking	Hypotonia Dystonia	+	(5 years) General Refractory	Joint laxity	-	Delayed myelination Reduced cholin concentration
49	F, 11 years	NI	NI	NI	NI	+	NI	NI	NI	Cortical blindness	NI	NI	Cortical blindness	NI	NI	Delayed myelination
50	F, 20 years	3 years	+	(GDD)	-	-	-	-	-	Happy, affable, obsessive behavior	-	+	(6.5 years) Focal	+	+	Normal
51	M, 8.5 years	1 year	+	(GDD)	+	+	+	+	-	Phobias; social communication disorder	Hypotonia	-	+	-	-	Normal
52	F, 8.5 years	1 year	+	(Motor)	-	+	+	+	-	-	-	+	(7 years) ESES	-	-	Normal
53	M, 6 years	18 months	+	(GDD)	+	+	+	+	Stereotypies Ataxia	Excessive jumping	Hypotonia	+	(2 years) ESES	-	-	Normal

ADHD attention deficit-hyperactivity disorder, AFOs ankle-foot orthosis, CC corpus callosum, ESE electrical status epilepticus in sleep, F female, FS febrile seizures, GDD global developmental delay, ID intellectual disability, IS infantile spasms, M male, MH marfanoid habitus, MRI magnetic resonance image, NA not applicable, NI not informed, OCD obsessive-compulsive disorder, WMIH white matter hyperintensity.

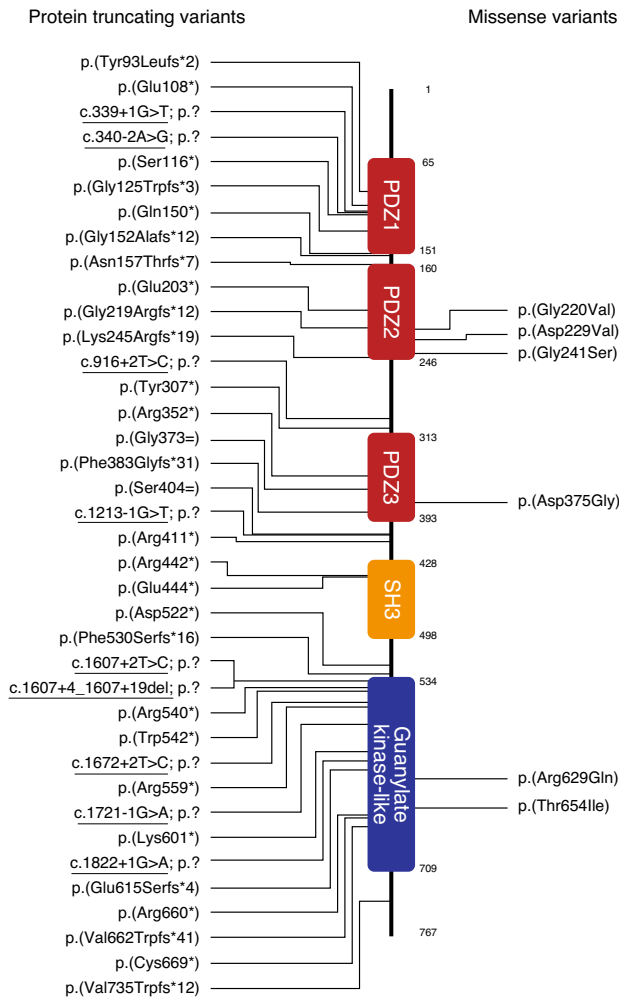


Fig. 2 *DLG4* variants shown on PSD-95. Among the 44 different variants, 6 were missense and two were synonymous while the remaining were predicted to be protein truncating variants. The missense variants were localized to the functional domains of the protein. Splice variants are underlined. PDZ1–3 domains (red) SH3 (light brown), GKLD (blue). All the variants are annotated using the *DLG4* transcript, NM_001365.4.

Epilepsy was reported in 25/47 individuals (53%) and classified as generalized epilepsy in five individuals and focal epilepsy in ten. The type of epilepsy was not specified in the remaining individuals. Two individuals were reported to have febrile seizures, three had infantile spasms and three individuals had an electroclinical presentation compatible with status epilepticus during slow-wave sleep (ESES). The mean age of epilepsy onset was 6 years (6 months–15 years). For six individuals, seizures were reported as refractory to pharmacologic treatment. Electroencephalography (EEG) information was available in 17 of the 25 individuals with epilepsy and showed focal abnormalities in 12 individuals (seven multifocal), generalized abnormalities in two and abnormal background in seven. Epilepsy tended to be more frequent in individuals with moderate/severe ID (18/26, 69%) than in those with mild ID (4/12, 33%; $p = 0.084$) and in those with autistic features (13/22, 59%) versus those without (8/19, 42%; $p = 0.44$). Of the individuals with epilepsy, 12 (50%) had regression in motor development and/or language, whereas it was present in 4/18 (22%) of those without epilepsy ($p = 0.13$).

Other neurological manifestations included hypotonia (23/43, 53%) and abnormal movements (19/41, 46%) such as stereotypies

($n = 12$), ataxia ($n = 9$), dystonia ($n = 4$) and tremor ($n = 3$). Significant facial dysmorphic features were reported in 15/39 individuals (38%), though establishing a characteristic gestalt was not possible as features were variable. Some of the more frequently observed common features were long face, thin eyebrows, thin upper lip and wide nasal bridge with a broad tip of nose (Fig. 1). Skeletal abnormalities were reported in 23/44 (41%) individuals.

Brain magnetic resonance imaging (MRI) was performed in 40 individuals and 13 (32%) were noted to have abnormalities (Fig. 15). Abnormal MRI was found in 20% of the individuals with mild ID (2/10) and in 40% (9/22) of those with moderate/severe ID. The reported abnormalities include variable degrees of brain atrophy ($n = 5$), cerebellar atrophy ($n = 4$), thin corpus callosum, dysmorphic hippocampus ($n = 3$, two with incomplete hippocampal inversion [IH]), delayed myelination ($n = 2$) and periventricular T2 white matter hyperintensity ($n = 2$).

Spectrum of the *DLG4* variants

Among the 53 individuals, 44 different variants were identified (Table 2, Fig. 2). Six variants were found more than once—three unrelated individuals each had the c.1054C>T, c.1324C>T, c.1618C>T and c.1978C>T variants. The c.340-2A>G variant was identified in monozygotic concordant twins, and the c.1587del variant was detected in a set of brothers, and after excluding nonpaternity, germline mosaicism in one of the parents was presumed as these variants were found to be de novo. Among the 48 individuals for whom parental studies were conducted, 43, including a monozygotic twin pair (ID-4 and ID-5), had de novo variants, and one individual inherited the variant (ID-22) from his mother with somatic mosaicism in buccal tissue (blood was not investigated and the percentage of mosaicism could not be obtained). One individual (ID-15) had a maternally inherited *DLG4* variant, but clinical information on the mother was not available.¹⁵

Among the 44 different variants, six were annotated as missense and two were predicted as synonymous while the remaining were predicted to be protein truncating variants: 15 nonsense variants, four single-nucleotide duplications, six single-nucleotide and one eight-nucleotide deletion, one indel and nine splice-site variants. Eight splice-site variants within the canonical splice sequences and the intronic variant (c.1607+4_1607+19del) were all expected to alter splicing (Fig. 2). All the duplications and deletions as well as the indel were frameshift variants. One of these variants, c.2203_2207delinsT (ID-53) was predicted to escape nonsense-mediated decay (NMD), while all the other protein truncating variants were predicted to be subject to degradation by NMD.

Two individuals had de novo variants annotated as synonymous. Individual 25 had the p.(Ser404=) (c.1212G>A) variant, which was at the last nucleotide of exon 11 and using SpliceAI it was predicted to cause a canonical splice donor loss with a probability of 63% with a new donor gain 4 bp downstream, which would result in a frameshift (p.[Val405Thrfs*17]). The other synonymous variant, p.(Gly373=) (c.1119C>T), identified in individual 22 was in the middle of exon 11 and SpliceAI predicted a donor gain with a probability of 44%. Further analyses with reverse transcriptase polymerase chain reaction (RT-PCR) revealed a deletion in the RNA transcript (r.1118_1212del) predicted to result in a frameshift, p.(Glu374Glnfs*11).

The missense variants were localized to the functional domains of the protein (three in the PDZ2 domain, one in the PDZ3 domain, and two in the Guanylate kinase-like domain). None of these variants were detected among the 125,748 exomes and 15,708 genomes from unrelated individuals in the gnomAD database, and they were all classified as pathogenic or likely pathogenic, except for two missense variants that were classified as variants of uncertain significance (VUS) according to

Table 2. *DLG4* variants and their predicted effects.

gDNA Chr17(GRCh37)	cDNA (NM_001365.4)	Exon/ intron	Predicted effect on PSD-95	PSD- 95 domain	Predicted coding effect	CADD score	Inheritance	ID of the affected individual
g.7107520dup	c.277dup	5	p.(Tyr93Leufs*2)	PDZ1	Frameshift	32	<i>dn</i>	1
g.7107344C>A	c.322G>T	6	p.(Glu108*)	PDZ1	Nonsense	40	<i>dn</i>	2
g.7107326C>A	c.339+1G>T	IVS6	p.?			35	<i>uk</i>	3
g.7107137T>C	c.340-2A>G	IVS6	p.?			34	<i>dn</i>	4 ^a
							<i>dn</i>	5 ^a
g.7107128G>C	c.347C>G	7	p.(Ser116*)	PDZ1	Nonsense	36	<i>dn</i>	6
g.7107103dup	c.372dup	7	p.(Gly125Trpfs*3)	PDZ1	Frameshift	32	<i>dn</i>	7
g.7107027G>A	c.448C>T	7	p.(Gln150*)	PDZ1	Nonsense	37	<i>dn</i>	8
g.7107020del	c.455del	7	p.(Gly152Alafs*12)		Frameshift	32	<i>dn</i>	9
g.7106909del	c.468del	8	p.(Asn157Thrfs*7)		Frameshift	31	<i>dn</i>	10
g.7106770C>A	c.607G>T	8	p.(Glu203*)	PDZ2	Nonsense	37	<i>dn</i>	11
g.7106629dup	c.654dup	9	p.(Gly219Argfs*12)	PDZ2	Frameshift	32	<i>dn</i>	12
g.7106624C>A	c.659G>T	9	p.(Gly220Val)	PDZ2	Missense	26.6	<i>dn</i>	13
g.7106597T>A	c.686A>T	9	p.(Asp229Val)	PDZ2	Missense	27.7	<i>dn</i>	14
g.7106562C>T	c.721G>A	9	p.(Gly241Ser) ^f	PDZ2	Missense	27.7	<i>mat</i>	15
g.7106549del	c.734delA	9	p.(Lys245Argfs*19)		Frameshift	32	<i>dn</i>	16
g.7106220A>G	c.916+2T>C	IVS10	p.?			33	<i>dn</i>	17
g.7100367A>T	c.921T>A	11	p.(Tyr307*)		Nonsense	36	<i>dn</i>	18
g.7100234G>A	c.1054C>T	11	p.(Arg352*)	PDZ3	Nonsense	37	<i>dn</i>	19
							<i>dn</i>	20
							Not <i>mat</i> ^b	21
g.7100169G>A	c.1119C>T	11	p.(Gly373=) ^c	PDZ3	Synonymous	13.7	<i>dn</i>	22
g.7100164T>C	c.1124A>G	11	p.(Asp375Gly) ^f	PDZ3	Missense	25.7	<i>uk</i>	23
g.7100134_7100141del	c.1147_1154del	11	p.(Phe383Glyfs*31)	PDZ3	Frameshift	33	<i>dn</i>	24
g.7100076C>T	c.1212G>A	11	p.(Ser404=) ^c		Synonymous	25.4	<i>dn</i>	25
g.7099895C>A	c.1213-1G>T	IVS11	p.?			35	<i>Mat/ mosaic</i>	26
g.7099876G>A	c.1231C>T	12	p.(Arg411*)		Nonsense	41	<i>dn</i>	27
g.7099645G>A	c.1324C>T	13	p.(Arg442*)	SH3	Nonsense	38	<i>dn</i>	28
							<i>dn</i>	29
							<i>dn</i>	30
g.7099639C>A	c.1330G>T	13	p.(Glu444*)	SH3	Nonsense	40	<i>dn</i>	31
g.7097682dup	c.1563dup	14	p.(Asp522*)		Frameshift	33	<i>dn</i>	32
g.7097658del	c.1587del		p.(Phe530Serfs*16)		Frameshift	28.2	Germline mosaicism ^d	33
							Germline mosaicism ^d	34
g.7097636A>G	c.1607+2T>C	IVS14	p.?			34	<i>dn</i>	35
g.7097619_7097634del	c.1607+4_1607 +19del	IVS14	p.?			16.38	<i>dn</i>	36
g.7097309G>A	c.1618C>T	15	p.(Arg540*)		Nonsense	44	<i>uk</i>	37
							<i>dn</i>	38
g.7097301C>T	c.1626G>A	15	p.(Trp542*)	GKLD	Nonsense	51	<i>dn</i>	39
g.7097302C>T	c.1625G>A						<i>dn</i>	40
g.7097161A>G	c.1672+2T>C	IVS16	p.?			34	<i>uk</i>	41
g.7097031G>A	c.1675C>T	17	p.(Arg559*)	GKLD	Nonsense	45	<i>uk</i>	42
g.7096904C>T	c.1721-1G>A	IVS17	p.?			35	<i>dn</i>	43
g.7096823T>A	c.1801A>T	18	p.(Lys601*)	GKLD	Nonsense	44	<i>dn</i>	44
g.7096801C>T	c.1822+1G>A	IVS18	p.?			35	<i>dn</i>	45
g.7096416del	c.1843del	19	p.(Glu615Serfs*4)	GKLD	Frameshift	33	<i>dn</i>	46

Table 2 continued

gDNA Chr17(GRCh37)	cDNA (NM_001365.4)	Exon/ intron	Predicted effect on PSD-95	PSD- 95 domain	Predicted coding effect	CADD score	Inheritance	ID of the affected individual
g.7096373C>T	c.1886G>A	19	p.(Arg629Gln)/ p. (His608Argfs*14) ^e	GKLD	Missense/ frameshift	32	<i>dn</i>	47
g.7096298G>A	c.1961C>T	19	p.(Thr654Ile)	GKLD	Missense	26.7	<i>dn</i>	48
g.7096281G>A	c.1978C>T	19	p.(Arg660*)	GKLD	Nonsense	41	<i>dn</i>	49
						41	<i>dn</i>	50
g.7096275del	c.1984del	19	p.(Val662Trpfs*41)	GKLD	Frameshift	34	<i>dn</i>	51
g.7095310G>T	c.2007C>A	20	p.(Cys669*)	GKLD	Nonsense	40	<i>dn</i>	52
g.7094124_7094128delinsA	c.2203_2207delinsT	22	p.(Val735Trpfs*12)		Frameshift	34	<i>dn</i>	53

Combined Annotation Dependent Depletion (CADD) tool was used to score the deleteriousness of the variants (<https://cadd.gs.washington.edu/>) and Mutalyzer (<https://mutalyzer.nl>) was used to check sequence variant nomenclature according to the guidelines of the Human Genome Variation Society (Table S4).

cDNA complementary DNA, *del* deletion, *dn* de novo, *dup* duplication, *gDNA* genomic DNA, *GKLD* guanylate kinase-like domain, *IVS* intervening sequence (intron), *mat* maternal, *NA* not applicable, *uk* unknown.

^aIndividuals 4 and 5 are monozygotic twins.

^bThe variant was not maternal, father not available.

^cFurther analyses revealed a deletion in the RNA transcript (r.1118_1212del) predicted to result in frameshift, p.(Glu373Glnfs*11) in individual 22, and in individual 25 the variant was in the canonical splice sequence and predicted to affect splicing.

^dIndividuals 33 and 34 are brothers (the parents do not have the variant and germline mosaicism is suspected).

^ePrediction tools suggests that this substitution is a splice variant predicted to result in frameshift, p.(His608Argfs*14).

^fThese missense variants are classified as variants of unknown significance (VUS) according to American College of Medical Genetics and Genomics/ Association for Molecular Pathology (ACMG/AMP) criteria, and even though protein studies suggest that they affect protein function, further functional studies are warranted to determine their pathogenicity.

ACMG/AMP criteria.^{20,21} Both of the latter variants were identified as part of a large screening study:¹⁵ p.(Gly241Ser) was maternally inherited (no phenotypic information on the mother is available), and the inheritance of p.(Asp375Gly) was not reported. All the missense variants were scored using SpliceAI tool and no splicing change was predicted for five of these. However, a new splice acceptor was predicted with 99% probability for the c.1886G>A, p.(Arg629Gln) in ID-47), which would result in a frameshift, p.(His608Argfs*14). The family is contacted for verification of this finding.

Prediction of the structural and functional effect of missense *DLG4* variants using homology modeling

To study the functional implications of the six missense *DLG4* variants p.(Gly220Val), p.(Asp229Val), p.(Gly241Ser), p.(Asp375Gly), p.(Arg629Gln), and p.(Thr654Ile), a 3D model of the wild type PSD-95 was generated using standard homology modeling-based techniques (Fig. 3). Subsequently, models for the six PSD-95 mutants were generated using the wild type model as a template and subjected to 100 ns of unrestricted molecular dynamics (MD) simulation. The details of the results of the protein modeling and MD simulation can be found in the Supplementary material.

Three of the modeled missense substitutions [p.(Gly220Val), p.(Asp229Val), p.(Gly241Ser)] occur in the PDZ2 domain of PSD-95 (Fig. 3). The simulation studies suggest that all these substitutions alter the structure of the PDZ2 domain (Fig. 3b, c). Furthermore, the p.(Asp229Val) substitution is predicted to modify the kinase/phosphatase recognition motif, and the p.(Gly241Ser) substitution is predicted to modify the ubiquitylation recognition motif, both of which are likely to affect the protein function. Notably, modeling of the p.(Gly241Arg) variant, which is reported in a single individual in the gnomAD database and alters the same amino acid as the p.(Gly241Ser) variant (ID-15), did not suggest a structural effect on the protein structure supporting that this variant was likely benign (Fig. 3d; Supplementary material).

The Asp375 residue is located in a loop between two beta-sheets in the PDZ3 domain, which is enriched in negatively

charged amino acids (Asp374, Asp375, and Asp377), and it is probably involved in the interaction with other adjacent structures such as potassium channels²⁵ (Fig. 3e). The p.(Asp374Gly) substitution generated a decrease in the negative charge of the surface, which is likely to modify the nature of this interaction and affect its functionality.

The residues Arg629 and Thr654 are both located in the guanylate kinase-like domain (GKLD, Fig. 3f–g). The p.(Arg629Gln) substitution is likely to modify the surface charge and thereby may affect the interaction of PSD-95 with other proteins such as the potassium channels. The p.(Thr654Ile) substitution is likely to modify the guanosine monophosphate (GMP) binding site, as well as modify the structure of the putative active site. In addition, this substitution could modify a kinase/phosphatase recognition motif predicted in the rat.²⁸

Genotype–phenotype correlation

All the individuals share core clinical manifestations that mainly affect the central nervous system, although there is some variability regarding the severity of ID, epilepsy, and the presence of other associated features, such as movement disorders. Most of the individuals ($n = 47$) had predicted loss of function (LoF) variants distributed throughout the protein, while only six had missense variants (two of which were classified as VUS), localized in the highly conserved regions of the functional protein domains (PDZ2, PDZ3, and GKLD). However, given the small sample size and some missing clinical information, it is difficult to make genotype–phenotype comparison. Among the individuals with truncating variants, one had a c.2203_2207delinsT (ID-53) variant, which was predicted to escape NMD. However, the clinical features of this individual were not specifically milder than those of the other individuals with truncating variants. Of note, in individuals with the same *DLG4* variant including two brothers (ID-33 and ID-34) we observed some variability in terms of the presence and severity of clinical manifestations and the MRI findings. The exception was the monozygotic twin brothers (ID-4 and ID-5) with almost identical symptoms.

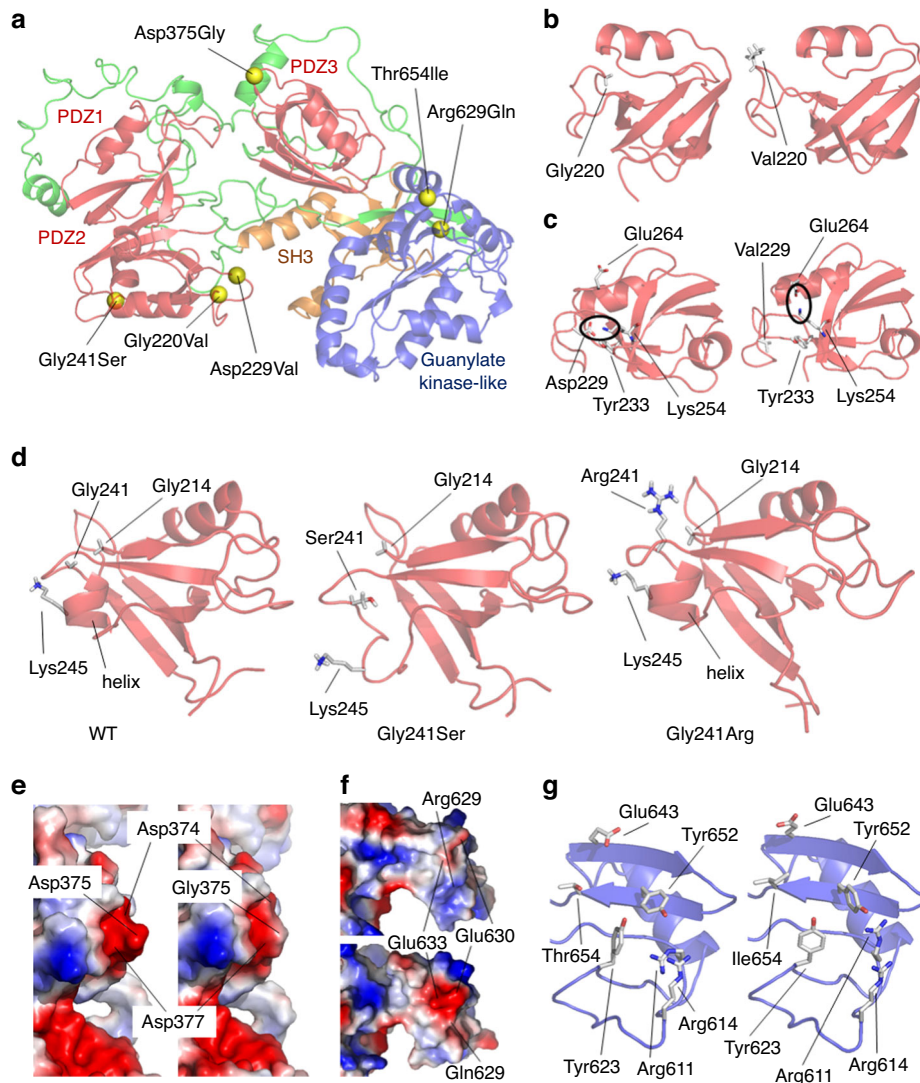


Fig. 3 Structural modeling of *DLG4* missense variants. (a) Structure model of human *DLG4* protein. Locations of domains PDZ 1, 2, and 3; SH3; and guanylate kinase-like (GK-LIKE) are labeled. Positions of variants p.(Gly220Val), p.(Asp229Val), p.(Gly241Ser), p.(Asp375Gly), p.(Arg629Gln), and p.(Thr654Ile) are shown as yellow spheres. (b) Structure of the wild type (left) and p.(Gly220Val) (right) at PDZ2 domain after molecular dynamics (MD) simulation. The positions of residues Gly220 and Val220 are indicated. (c) Structure of the wild type (left) and Asp229Val variant (right) PDZ2 domain after MD simulation. The positions of residues Asp229 (or Val229), Lys254, and Glu264 are indicated. Salt bridges are shown with black circles. (d) Structure of the PDZ2 domain of the wild type, Gly241Ser, and Gly241Arg variants after MD simulation. Locations of amino acids Gly241/Ser241/Arg241, Gly214, and Lys245 are labeled. The Gly241Ser substitution identified in individual 15 alters the structure of the PDZ2 domain, while the Gly241Arg substitution, which is reported in a single individual in the gnomAD database, does not suggest a structural effect supporting that this variant was likely benign. (e) Left: position of negatively charged residues Asp374, Asp375, and Asp377 in the surface of PDZ3 domain. Right: variant of Asp375 to Gly promotes a decrease in the local negative charge of the surface. (f) Surface of the wild type (upper panel) and Arg629Gln variant (lower panel) guanylate kinase-like domain after 100 ns of MD simulation. The positions of residues Arg629 (or Gln629) are indicated. Note the contribution of the Glu630 and Glu633 residues in the negatively charged patch in the surface of the variant protein. (g) Residues conserved in the guanylate kinase-like domain located in the homologous positions to those coordinating GMP binding in the yeast guanylate kinase enzyme²⁴ both in wild type (left) and Thr654Ile variant (right) proteins. PDZ1–3 domains (red) SH3 (light brown), GKLD (blue). Figures were generated using the PyMol Molecular Graphics System (<https://pymol.org/>; Schrödinger, LLC, Portland, OR).

DISCUSSION

This study includes the largest series of individuals harboring variants in *DLG4* published to date and demonstrates that the clinical phenotype is largely neurodevelopmental, with an early onset of symptoms and a clinical picture predominated by GDD/ID, ASD, ADHD, hypotonia, and epilepsy. Given that the synaptopathies are defined as brain disorders associated with synaptic dysfunction²⁹ and that the individuals presented in this study have clinical features overlapping those observed in

individuals with synaptopathies (cognitive disorders such as intellectual disability, motor dysfunction such as ataxia and dystonia, epilepsy and psychiatric diseases such as ASD and ADHD),^{29,30} we coin the phenotypes associated with *DLG4* variants as *DLG4*-related synaptopathy.

Postsynaptic disorders are relatively unknown. Among individuals with *DLG4*-related disorders, cognitive impairment and ASD predominate, as already described in the context of PSD-complex dysfunction in the hippocampus excitatory synapses in mice.^{31,32}

The present work also suggests a certain correlation between the degree of ID and the presence of ASD and epilepsy, these being more frequent in the context of moderate to severe ID. The physiopathology of epilepsy observed in *DLG4*-related synaptopathy is currently unknown, but it is plausible that variants of the functional domains of PSD-95 will affect the function of glutamate receptors (such as NMDA or AMPA) or K_v1 channels, which are known to be dysfunctional in epileptic disorders, and hereby lead to altered excitatory synaptic transmission.

Variants in genes encoding postsynaptic proteins of striatal medium spiny neurons (MSNs) have previously been associated with movement disorders.³³ Taking into account that PSD-95 has been identified in glutamatergic synapses of midbrain dopaminergic neurons³⁴ and the MSNs of the human neostriatum,³⁵ it is not surprising that some individuals with *DLG4* variants had an associated movement disorder. Most of the symptoms observed in *DLG4*-related disorder, may thus be explained by impaired synaptic plasticity due the changes in the structural organization of the PSD. However, further research is warranted to understand the synaptic physiopathology in this disorder.

Brain abnormalities as detected by MRI are diverse and nonspecific. They mainly include cerebral and/or cerebellar atrophy, thinning of the corpus callosum, hippocampal dysmorphia (two individuals with incomplete hippocampal inversion [IHI]) and mild delayed myelination. Although IHI has been reported in the general population, it has been described more frequently in individuals with epilepsy and febrile status epilepticus.^{36,37} Therefore, taking into account that PSD-95 is highly expressed in the hippocampus and diminished activity alters the correct development of excitatory synapses (producing an excitatory/inhibitory imbalance) and modifies dendritogenesis during embryological development, IHI could represent a neuroimaging manifestation of the abnormal neurodevelopment provoked by *DLG4* variants. This possibility should be considered in future studies. In this series, careful review of the neuroimaging studies of the individuals has not been possible, so in some cases, this anomaly could have gone unnoticed. On the other hand, in five individuals (ID-9, 26, 28, 38, 41), there is some component of white matter atrophy, and it is associated with thinning of the corpus callosum in three of them. This is in agreement with an association between genetic variability in *DLG4* and white matter structure in the preterm neonatal brain as described previously.³⁸ Furthermore, corticosubcortical atrophy with anterior predominance is seen in two of the individuals (ID-26 and ID-28; Fig. 15) and could be associated with the high expression of PSD-95 in the prefrontal cortex.³⁹

DLG4 variants have recently been associated with ID through identification of three individuals with de novo LoF variants in a cohort of 820 individuals with ID (0.37%)¹² and, subsequently, three LoF *DLG4* variants were identified in a cohort of 64 individuals with ID and MH (corresponding to 4.7% of the cohort). Considering the higher prevalence in this series compared with the larger series of ID individuals, the authors suggested that *DLG4* was a strong candidate gene in ID individuals with comorbid MH.¹⁶ In the present study, we could re-examine 38 individuals specifically for MH features, which were present only in 9, suggesting that MH is not a major clinical feature of *DLG4*-related synaptopathy. In a very recent study comprising 31,058 individuals, LoF variants of *DLG4* were identified in 15 individuals,⁴⁰ suggesting that incidence of this synaptopathy is about 0.05% among the individuals with ID.

DLG4 is likely to be intolerant to both missense and LoF variants ($Z = 4.93$ for missense and observed/expected (o/e) value = 0.06–0.24 for LoF variants, gnomAD database). All the variants described in this study meet criteria for classification as pathogenic or likely pathogenic, except for two of the missense variants, p.(Gly241Ser) and p.(Asp375Gly), which are classified as VUS. We included these variants as they were associated with ASD

in a previous study,¹⁵ and the modeling studies show an effect on the protein, acting in highly conserved regions of its functional domains. LoF variants reported in this study are distributed throughout the protein, whereas the missense variants are localized to the functional protein domains (PDZ1, PDZ3, GKLD). Structure modeling suggests that the missense variants affect structural conformation and/or protein function and are therefore likely to act as LoF variants. These modifications in highly conserved positions of the protein presumably alter its function, thereby affecting its interaction with other proteins, which is fundamental for synaptogenesis, functional dynamics, and plasticity. Modeling studies were helpful to predict the functional consequences of the missense variants and thus supported the pathogenicity classification. Furthermore, modeling of the p.(Gly241Arg) variant, which is reported in a single presumably unaffected individual in the gnomAD database and affects the same amino acid as the p.(Gly241Ser) variant we had identified in an affected individual, did not suggest a structural effect on the protein and was likely to be benign. However, further functional studies are warranted, especially for the VUS, to understand the effect of the missense variants on protein function. Apart from protein modeling we annotated the variants using an NMD and a splice variant prediction tool. Both synonymous variants p.(Gly373=) and p.(Ser404=) were predicted to affect splicing leading to frameshift, and this was verified with RT-PCR. Notably, the c.1886G>A substitution, which was initially annotated as a missense variant, p.(Arg629Gln), was predicted to result in a frameshift, p.(His608Argfs*14). Furthermore, one of the truncating variants, c.2203_2207delinsT, 100 bp upstream to the TGA stop codon, was suggested to escape NMD, but the prediction was not verified. These findings underline the importance of RNA based studies in clinical diagnosis to understand the consequences of the DNA variants.

This study has certain limitations. The individuals come from different centers and therefore have been clinically evaluated by professionals using different criteria. Employment of Human Phenotype Ontology (HPO, <https://hpo.jax.org>) terms to describe the phenotypic abnormalities as part of the clinical practice and research may help to overcome this limitation. Furthermore, the available clinical information is limited in several individuals, making it more difficult to extract detailed information (and percentages) on certain manifestations such as epilepsy. Finally, information on neuroimaging studies in all the individuals could have enabled a more comprehensive assessment of the presence of abnormalities in the development of the hippocampus and other brain structures. Studies of the cerebrospinal fluid might have enabled us to determine whether a characteristic neurotransmitter profile could serve as biomarker.

In conclusion, haploinsufficiency of *DLG4* is likely to impair PSD-95 activity, interfere with synaptic function during critical developmental windows and alter the synaptic plasticity necessary for the functional adaptation and modulation of learning and behavioral processes, leading to the neurodevelopmental disorder described in this group of individuals. We provide evidence that missense variants affecting the functional domains of PSD-95 can also cause a *DLG4*-related synaptopathy. A better understanding of the pathophysiology of synaptopathies could contribute to the development of new specific therapies in the future.

DATA AVAILABILITY

All data that are not already included in the supplementary material are available upon request.

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AUTHOR CONTRIBUTIONS

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ETHICS DECLARATION

Informed written consent for genetic testing and publication of the clinical information including clinical pictures was obtained from the parents or the legal guardians of each individual according to the Declaration of Helsinki. The work carried out at the corresponding author's (Z.T.) institution has been approved by the Regional Ethical Committee, Capital Region of Denmark (H15007871).

COMPETING INTERESTS

The authors declare no competing interests.


ADDITIONAL INFORMATION

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