BRAIN REPORT

Endocannabinoid dysfunction in neurological disease: neuro-ocular DAGLA-related syndrome

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The endocannabinoid system is a highly conserved and ubiquitous signalling pathway with broad-ranging effects. Despite critical pathway functions, gene variants have not previously been conclusively linked to human disease. We identified nine children from eight families with heterozygous, *de novo* truncating variants in the last exon of *DAGLA* with a neuro-ocular phenotype characterized by developmental delay, ataxia and complex oculomotor abnormality. All children displayed paroxysms of nystagmus or eye deviation accompanied by compensatory head posture and worsened incoordination most frequently after waking. RNA sequencing showed clear expression of the truncated transcript and no differences were found between mutant and wild-type DAGLA activity. Immunofluorescence staining of patient-derived fibroblasts and HEK cells expressing the mutant protein showed distinct perinuclear aggregation not detected in control samples.

This report establishes truncating variants in the last *DAGLA* exon as the cause of a unique paediatric syndrome. Because enzymatic activity was preserved, the observed mislocalization of the truncated protein may account for the observed phenotype. Potential mechanisms include DAGLA haploinsufficiency at the plasma membrane or dominant negative effect. To our knowledge, this is the first report directly linking an endocannabinoid system component with human genetic disease and sets the stage for potential future therapeutic avenues.

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Abbreviations: eCB = endocannabinoid; NODRS = neuro-ocular DAGLA-related syndrome; PTU = paroxysmal tonic upgaze

Introduction

The endocannabinoid (eCB) system is a modulatory system involved in cell and tissue homeostasis that is highly conserved among species.[1] The pathway consists of G-protein coupled receptors (primarily CB_1 and CB_2), their endogenous ligands (eCBs) and the proteins involved in the synthesis and degradation of eCBs.[2] The two main eCBs are *N*-arachidonoyl-ethanolamine (AEA; anandamide) and 2-arachidonoylglycerol (2-AG). AEA is synthesized by a complex system involving multiple enzymatic pathways.[3] In contrast, 2-AG is synthesized primarily by a pair of enzymes in the brain, diacylglycerol lipase alpha and beta (DAGLA and DAGLB, respectively) encoded by the *DAGLA* and *DAGLB* genes.[3]

DAGLA is expressed in neurons and astrocytes throughout the brain, most prominently in cerebellum, hippocampus, ventral tegmental area and striatum.[4] It is found in the plasma membrane of neuronal cells[5] and contributes to intracellular 2-AG signalling.[6] DAGLA may also be found in the nucleus or cytoplasm of a variety of cell types.[7–9] DAGLB, on the other hand, is mostly expressed in the nervous system by microglia.[10]

In the developing brain, the eCB signalling pathway has important influences on neural development, signalling and brain repair.[1,3] DAGLA activity is required within the developing nervous system for axonal growth and guidance.[6] Once the nervous system is mature, eCBs modulate neuronal activity and network function[2] where eCBs are released from postsynaptic neurons and act retrogradely on presynaptic CB_1 and CB_2, resulting in short- and long-term suppression of neurotransmitter release.[6] This intricate system influences a wide array of pathophysiological functions, including: emotion, cognition, energy balance, pain sensation and neuroinflammation.[11]

DAGLA animal models display a range of neurological phenotypes. *DAGLA* knockdown zebrafish show morphologic abnormalities in mid- and hind-brain regions associated with abnormal locomotor and optokinetic behaviours.[12] *Dagla* knockout mice are viable (mousephenotype.org), display increased seizure susceptibility and severity,[13,14] anxiety and depression-like behaviours,[14]

abnormalities in regions involved in learning and memory including altered cholinergic innervation of CA1 pyramidal cells of the hippocampus[15] and compromised adult neurogenesis in the hippocampus and subventricular zone.[14]

We report nine children from eight families with heterozygous apparently *de novo* premature termination variants in *DAGLA*. Each displays a unique neuro-ocular phenotype characterized by global developmental delay and ataxia with superimposed paroxysms of worsening ataxia and complex oculomotor abnormality of nystagmus or eye deviation accompanied by compensatory head posture.

Subjects and methods

Ethical approval

Consent was obtained according to the respective institutional ethics guidelines and with approval of the local ethics committees in the participating study centres.

Patient ascertainment and review of clinical features

The index case was identified by trio genome sequencing. Genematcher[16] was utilized to identify additional individuals with *DAGLA* variants and initiate an international collaboration of eight centres. For each case, medical records were reviewed. Anonymized data were provided via a standardized study questionnaire.

Subjects

Case 1 is an 11-year-old Hispanic male with global developmental delay, hypotonia, ataxia, dysarthria, congenital nystagmus and intellectual disability. From birth, the child displayed daily paroxysmal spells characterized by eye deviation and/or nystagmus (vertical>>horizontal) with abnormal head posture either alone or in combination with generalized ataxia. Spells are most prominent within 5–10 min of waking in the morning or after a nap. Symptoms typically persist until midday and are less prominent or entirely absent in the afternoon and evening. Abnormal eye movements are mostly upward eye deviation with chin down posture with less common episodes of downward eye deviation with upward head tilt and convergence movements. Simultaneously, he may display whole body titubation, dysarthria and/or wide based markedly unsteady gait. Over time, the child made forward developmental motor progress and demonstrated improved baseline ataxia. He also showed reduced frequency, duration and severity of daily spells, albeit without complete cessation. Parents note improvement in eye movements, balance and speech with acetazolamide and after eating foods high in carbohydrates. They also report reduced severity of morning episodes when cornstarch is consumed at bedtime. Brain MRIs were normal. EEG showed mild epileptiform discharges at age 23 months and mild slowing posterior dominant rhythm at nine years. Muscle biopsy supported a possible mitochondrial dysfunction (Supplementary Table 1). Blood and muscle mitochondrial sequencing revealed no pathogenic variants and mitochondrial depletion studies were normal. Genomic sequencing of proband and parents revealed a *de novo* variant in the *DAGLA* gene NM_006133.3:c.2440G>T, p.(Glu814X) (Supplementary Video 1).

Cases 2–9 are described in the Supplementary material and Supplementary Videos 2–4. All cases are summarized in Table 1 and Supplementary Table 1.

Laboratory methods

Genomic sequencing

Exome or genome sequencing was performed on proband and informative family members using standard methods (Supplementary material).

RNA sequencing

Blood was obtained from the index patient (Case 1) and parents. RNA was isolated using Qiagen Blood RNA kit (Cat. No. 762164). Total RNA [Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus (20040529)] and mRNA libraries (TruSeq Stranded mRNA Library Prep 20020595) were generated and sequenced on NovaSeq to produce ~100 M reads per subject. RNA sequence reads were aligned and variants called using the DRAGEN v3.84 pipeline.

DAGLA activity evaluation

Cloning of full-length and mutant DAGLA (1–813)

Full-length *Homo sapiens DAGLA* cDNA was cloned into expression vector pcDNA3.1 with an N-terminal FLAG-tag as previously described.[17] For mutant *DAGLA*, a stop codon was introduced at E814 using QuikChange site-directed mutagenesis.[17]

Recombinant expression of enzymes by transient transfection of HEK293T cells

Transient transfection of HEK293T cells with DAGLA constructs, either 2 µg of wild-type or mutant DAGLA DNA constructs, were conducted using (JetOPTIMUS-11701) as per the manufacturer's instructions. Transfected cells were plated 24 h after transfection for experiments.

Assessment of DAGLA activity by gel-based activity-based protein profiling

Lysates from HEK293T cells expressing wild-type DAGLA or mutant DAGLA (1–813) (50 µl, 1 mg/ml) were treated with either FP-Rh (1 µM final concentration) or HT-01 (2 µM final concentration) for 30 min at 37°C. The reactions were quenched by adding 20 µl of 4× sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. After separation by SDS–PAGE, samples were visualized by in-gel fluorescence scanning using the ChemiDoc MP system (Bio-Rad). Band intensities were quantified with Image Lab (5.2.1) software (Bio-Rad).

Immunofluorescence staining

For immunofluorescence, 200 000 cells were plated on poly-D-lysine–coated coverslips (neuVitro GG-18-PDL) and incubated for 24 h. Cells were then washed with chilled 1× PBS and fixed in 4% paraformaldehyde (PFA) before staining with primary antibody against DAGLA (Invitrogen PA5-23765) in 1:200 dilution and incubated overnight. Cells were washed in 1× Tris-buffered saline with 0.1% Tween (TBST), incubated with secondary antibody (Invitrogen A32732) in 1:1000 dilution, and incubated for 40 min. ProLong-Gold antifade reagent with DAPI (Invitrogen P36935) was used as nuclear stain. Pictures were taken using Echo microscope.

Data availability

Supporting data for this study are available from the corresponding author upon request.

Table 1 Genotype and phenotype of case cohort

Case #	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9
Genetics									
DAGLA variant	c.2440G>T p.E814X	c.2484delC p.E829RfsX6	c.2551C>T p.Q851X	c.2456_2457delAC p.H819PfsX5	c.2484delC p.E829RfsX6	c.2484delC p.E829RfsX6	c.2370C>G p.Y790X	c.2485G>T p.E829X	c.2613dup p.S872QfsX6
Inheritance	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*[a]	*De novo*[a]	*De novo*	*De novo*	*De novo*
Phenotype									
Gender	Male	Male	Male	Male	Male	Female	Male	Male	Male
Age (year of birth)	11 years (2010)	9 years (2012)	5 years (2016)	7 years (2014)	15 years (2006)	12 years (2009)	12 years (2009)	4 years (2017)	5 years (2016)
Motor development	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed
Language development	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed	Normal	Normal
Age at symptom onset	Birth	6 months	Birth	Birth	9 months	First weeks	3 months	First month	7 months
First symptom	Nystagmus (v,h)	Nystagmus (v,h)	Apnoeic spells; feeding difficulty	Clubfoot	Nystagmus (v)	Nystagmus	Poor head control	Poor head control; pendular nystagmus	Paroxysmal upward eye deviation
Nystagmus (v/h)	Y (v,h)	Y (v,h)	N	Y (v)	Y (v)	Y (v)	Y (v,h)	Y (v)	N
Nystagmus onset	Birth	6 months	None	7 months	9 months	First weeks	13 months	First weeks	None
Other eye movements	Amblyopia; Sac Pur	N	Sac Pur	N	Sac Pur	Sac Pur	Sac Pur; Abnl Sac	Sac Pur	Sac Pur
Dysarthria	Y	Y	Y	Y	Y	Y	Y	N	Y
Hypotonia	Y	Y	Y	Y	Y	Y	Y	Y	Y
Ataxia	Y	Y	Y	Y	Y	Y	Y	Y	Y
Intellectual disability	Y	Y	Too young to assess	N: low average IQ	Y	Y	Y	N	N
MRI brain[b]	Normal	Normal	Abnormal	Normal	Normal	Normal	Possibly anormal	Abnormal	Possibly anormal
Paroxysmal events									
Paroxysmal nystagmus	Y	Y	N	Y	Y	Y	Y	Y	N
Paroxysmal chin down posture	Y	Y	Y	Y	Y	Y	Y	Y	Y
Paroxysmal chin up posture	Y	N	N	N	Y	Y	N	Y	N
Paroxysmal upward eye deviation	Y	Y	Y	N	N	N	N	Y	Y
Paroxysmal ataxia	Y	Y	N	N	Y	Y	N	Y	N
Paroxysmal event triggers	Morning; post sleep; post nap	Morning; post nap; fever	n/a	n/a	Morning; post nap; fever	Morning; post nap; fever	Morning; post sleep; fatigue	Morning; post sleep; post nap	Morning; post sleep
Relieving factors	Acetazol; sweets	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Reverse diurnal fluctuation	Y	Y	N	N: possible diurnal	Y	Y	Y	Y	N
Spell trajectory over time	Improving	Slightly improved	Improving	Improving	Improving	Improving	Improving	Improving	Improving

Abnl Sac = abnormal saccades; Acetazol = acetazolamide; h = horizontal; N = no; n/a = not available; Sac Pur = saccadic pursuits; v = vertical; Y = yes.

[a]Probable parental mosaicism.

[b]Details in Supplementary Table 1.

Results

Sequencing reveals distal truncating variants in DAGLA

Seven unique apparently *de novo DAGLA* variants were identified in nine children from eight families. All variants lead to premature protein truncation within an 82 amino acid window. These truncating variants disrupt multiple predicted phosphorylation sites as well as a protein–protein interaction domain previously shown to interact with HOMER scaffolding proteins[5] (Fig. 1). RNA was isolated from peripheral blood from the index patient and parents. Two libraries of total RNA and mRNA were generated for each subject. The truncating variant was identified in each library from the proband with 10/25 reads (40%), indicating that the transcript does not undergo nonsense-mediated decay (NMD). The library reads of the proband's mother and father (26× and 30× coverage, respectively) showed only the wild-type allele.

Detailed phenotyping identifies a distinct DAGLA associated phenotype

Clinical features of individuals with pathogenic *DAGLA* variants are summarized in Table 1 and Supplementary Table 1. Eight of nine children are male. All displayed hypotonia and gross motor delay; seven also had language delay, but without autistic features. All children had baseline ataxia and oculomotor abnormalities including nystagmus or saccadic pursuits. In addition, all had prominent paroxysmal events during which there was worsened incoordination and eye movement abnormalities including worsened nystagmus, forced vertical versions or both, often accompanied by compensatory head postures. For seven of nine children, episodic events were most prominent in the morning after awakening or after a nap. For most, paroxysmal episodes reduced in frequency and severity as the child aged.

Functional studies

To characterize the effects of the truncating variant on DAGLA protein levels, activity and subcellular localization, fibroblasts from the index case were obtained and immortalized. RNA levels of DAGLA were comparable between patient-derived fibroblasts and cells from an unaffected control. Immunofluorescent staining showed no difference in the proportion of DAGLA-positive cells between patient-derived and control. However, immunofluorescent staining identified distinct perinuclear aggregates of DAGLA in the patient's cells (Fig. 2A). To confirm this cellular characteristic, HEK293T cells were transfected with either wild-type *DAGLA* or *DAGLA* containing the p.Glu814X variant. Lysates from the HEK293T cells were assessed for DAGLA activity by Activity-Based Protein Profiling (ABPP),[17] which showed no difference in DAGLA activity (Supplementary Fig. 1). The perinuclear aggregation of DAGLA observed in patient-derived fibroblasts was validated in HEK293T cells expressing the mutant truncated DAGLA, which had an ~4-fold increase in the cell numbers with perinuclear aggregates relative to isogenic control cells transfected with wild-type DAGLA ($P < 0.005$, Fig. 2B). The total number of DAGLA-positive cells was comparable between mutant and wild-type-expressing HEK293T cells.

Discussion

We define neuro-ocular DAGLA-related syndrome (NODRS), a new clinical syndrome in nine children from eight families with apparently *de novo* premature termination variants in *DAGLA*. The neuro-ocular features of this cohort are unique and consistent across all individuals, characterized by ataxia and developmental delays with daily superimposed paroxysmal exacerbations. The episodic events cause worsened ataxia and/or increased oculomotor abnormalities (nystagmus and/or forced vertical versions) with compensatory head postures. The NODRS acronym reflects the distinctive chin-down posture common across the cohort. The majority report a striking pattern of exacerbation in the morning shortly after waking and after naps. The skewed gender ratio (8/9 male) is statistically unlikely, although whether this is a real phenomenon or a chance finding is unclear.

The NODRS phenotype overlaps, but does not conform with several previously defined conditions, including: non-progressive congenital ataxias, infantile nystagmus syndromes, episodic ataxias, and paroxysmal tonic upgaze (PTU). Of these conditions, NODRS most closely resembles complicated forms of PTU. PTU is characterized by episodic, sustained, conjugate upward eye deviation in association with downbeat nystagmus on attempted downgaze, sometimes with mild ataxia, and symptom resolution in a few months to years.[18] It is a heterologous condition that, in its complicated form, may manifest additional persistent neurological symptoms including developmental delays, significant ataxia and oculomotor abnormalities.[19,20] Episodic ataxia exacerbation, as seen in our cohort, is not generally considered to be part of the PTU phenotype, but several cases displaying this aspect have been reported. For example, one previously described complicated PTU case that was strikingly similar to our cohort demonstrated compensatory head postures and ataxia that were most severe after sleep.[21] The molecular aetiology in this case is unknown. Other children with PTU and episodic ataxia resembling our cohort were found to carry pathogenic variants in the *CACNA1A* gene,[20,22,23] although, in contrast to our cohort, PTU attacks in these individuals were relieved by sleep.

Several clinical aspects of NODRS may provide insight into disease pathophysiology. Anatomically, the prominent features of ataxia and eye movement abnormalities localize to the cerebellum and/or brain stem. Brain imaging identified mild inferior vermian hypoplasia for two children in our cohort. For the remainder, these regions were unremarkable, although subtle or non-anatomic changes in these regions cannot be excluded (Table 1 and Supplementary Table 1). Abnormalities in this region are consistent with high expression of DAGLA in cerebellar Purkinje cells both in human and rodent (www.proteinatlas.org).[24,25] Most individuals report the unusual phenomenon of symptom onset after waking. Early morning symptoms may suggest impaired energy metabolism exacerbated by the overnight fast. Potential secondary mitochondrial impacts are supported by muscle biopsy abnormalities in Case 1 and myopathic changes on electromyogram in Case 7 (Supplementary Table 1). Worsening symptoms after a nap suggest links to circadian rhythm as opposed to fasting; considering this, the circadian regulation of eCB signalling and its light entrainment role in the rat is of potential relevance.[26]

The clustering of pathogenic *DAGLA* variants in our NODRS cohort may provide insight into the mechanism of disease. All variants are located proximally in the final exon of *DAGLA*, permitting the transcript to escape NMD. The termination variants may disrupt a variable number of known phosphorylation sites and all eliminate a HOMER binding domain, which is critical for the recruitment of DAGLA to the plasma membrane but not for 2-AG production[5] (Fig. 1). In concordance with this, transfected cells expressing mutant DAGLA and mutant patient-derived fibroblasts revealed mislocalization of mutant DAGLA with no effect on DAGLA enzymatic activity (Fig. 2 and Supplementary Fig. 1).

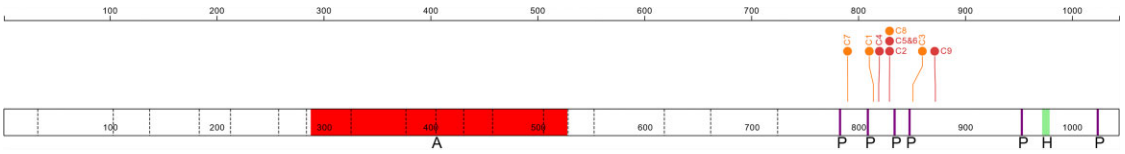

Figure 1 *DAGLA* variants and domains. Schematic of DAGLA (*bottom* box) with exon–exon boundaries (dotted lines) and codon position (*top*). Frameshift and nonsense variants [orange (C1,3,7,8) and red (C2, 4, 5, 6, 9) lollipops, respectively] disrupt HOMER motif (green, 'H') and phosphorylation sites (purple 'P') but leave the active domain (red 'A') intact.

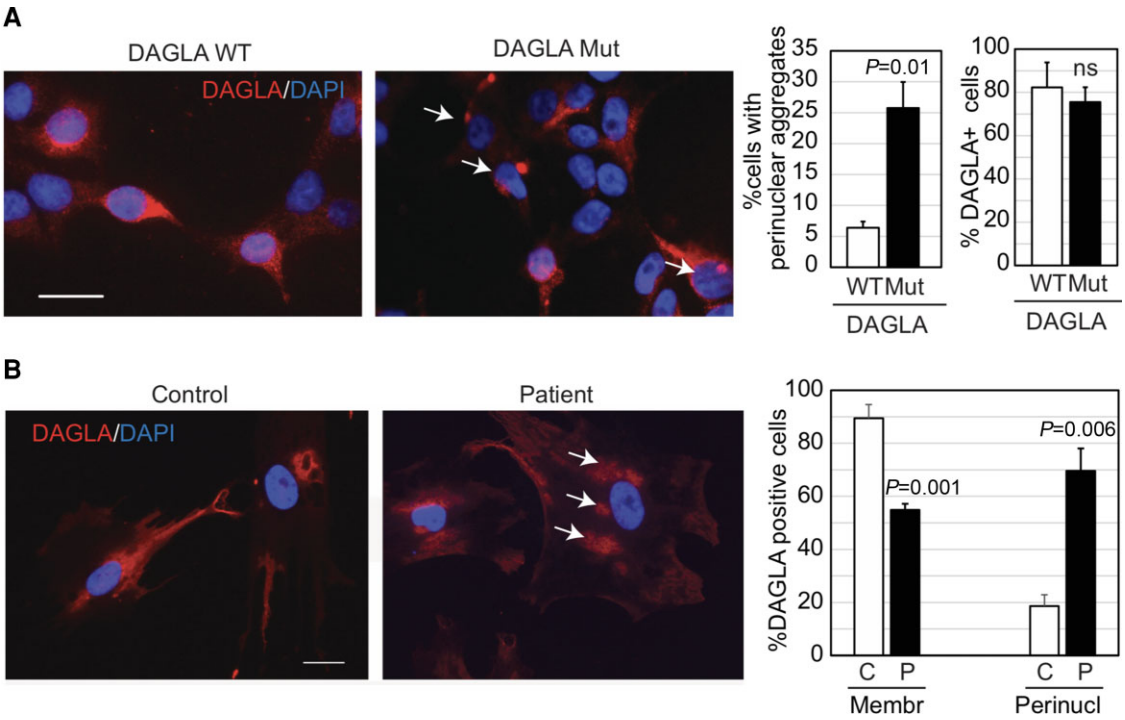

Figure 2 Immunofluorescence studies. Representative photomicrographs of immunofluorescence for DAGLA in HEK293T cells transfected with either wild-type (WT) or mutant (Mut) *DAGLA* cDNA (**A**) and in patient-derived fibroblasts (P) and control (C) cells from an unaffected individual (**B**). DAPI was used for nuclear counterstain. Scale bars = 50 µm. Accompanying bar graphs represent quantification of the number of cells with perinuclear (perinucl) aggregation (arrows) or membrane-bound (membr) DAGLA. Graphs: bars indicate the mean and error bars the standard deviation. Two-tailed Student's *t*-test determined *P*-values.

Variants in *DAGLA* that both disrupt the HOMER motif and escape NMD must fall within a length of 262 amino acids. The probability of identifying eight independent *de novo* variants within this constrained location is small ($P < 1.78 \times 10^{-5}$). Although the precise pathophysiological mechanisms underlying NODRS are uncertain, our findings suggest either a dominant negative effect of truncated and mislocalized DAGLA or effects of reduced DAGLA concentration at the plasma membrane. Given the broad and diverse role of eCBs, either or both mechanisms may be at play in distinct brain regions at different times in development, resulting in static and paroxysmal symptoms that may potentially evolve over time. Truncating variants in more proximal regions of *DAGLA*, predicted to lead to NMD, are vanishingly rare in public databases, implying that globally reduced levels of DAGLA are lethal.[27] In contrast, our findings imply that reduced levels at the plasma membrane are compatible with life, although may be associated with disease.

Prior reports suggested that disruption of DAGLA were associated with various neurological conditions but were not conclusive. Autoantibodies to DAGLA were detected in the sera of patients with CNS syndromes who presented with cerebellitis, epilepsy and hippocampal sclerosis or brainstem encephalitis.[28] One study implicated a duplication of 20 genes, including *DAGLA*, with adult-onset spinocerebellar ataxia, while another study reported rare missense variant enrichment in eCB system genes *DAGLA* and *CNR1* within a cohort of individuals with a wide range of neurological phenotypes.[14,29] Similarly, DAGLA animal models implicate DAGLA in development and function of the nervous system. These models show some similarity but do not fully recapitulate the NODRS phenotype. This may be due to the changing evoluationary importance of DAGLA in humans or that these models do not reproduce the mislocaltization described here.

In summary, we define NODRS, a novel childhood-onset neuro-ocular phenotype associated with premature termination variants in the last exon of *DAGLA*. We hypothesize that complete haploinsufficiency of *DAGLA* is lethal in humans, and that the syndrome described here is caused by reduced concentration of DAGLA at the plasma membrane or potentially a dominant negative effect of mislocalized mutant DAGLA. Development of animal models with truncating variants in the last *DAGLA* exon will be needed to understand the mechanism by which mislocalized DAGLA leads to disease. Additional studies will also be required to explore whether truncating *DAGLA* variants may be identified in other patients with a PTU-like phenotype as well as to develop targeted therapeutic options for these children. As the first report conclusively linking cannabinoid dysfunction to human disease,

we are hopeful this work will play a pivotal role in better understanding the role of eCBs in health and disease and will also contribute to optimization of the use of cannabis-derived medications for a broad range of disorders.

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Competing interests

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Supplementary material

Supplementary material is available at *Brain* online.

Appendix 1

Undiagnosed Disease Network authors

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