BRIEF REPORT

Muscarinic acetylcholine receptor M1 mutations causing neurodevelopmental disorder and epilepsy

Anna Marcé-Grau[1] | Xabier Elorza-Vidal[2,3] | Carla Pérez-Rius[2] | Anna Ruiz-Nel·lo[4] | Júlia Sala-Coromina[1] | Elisabet Gabau[5] | Raúl Estévez[2,3] | Alfons Macaya[1,6]

[1]Pediatric Neurology Research Group, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain

[2]Physiology Unit, Department of Physiological Sciences, Genes Disease and Therapy Program, IDIBELL-Institute of Neurosciences, Universitat de Barcelona, L'Hospitalet de Llobregat, Spain

[3]Rare Disease Network Research Center (CIBERER), ISCIII, Spain

[4]Genetics Laboratory, Institut d'Investigació i Innovació Parc Taulí I3PT, UDIAT-Centre Diagnòstic, Parc Taulí Hospital Universitari, Universitat Autònoma de Barcelona, Sabadell, Spain

[5]Paediatric Unit, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc taulí I3PT, Universitat Autònoma de Barcelona, Sabadell, Spain

[6]Institute of Neuroscience, Universitat Autònoma de Barcelona, Bellaterra, Spain

Correspondence
Raúl Estévez, Department of Physiological Sciences II, Faculty of Medicine, IDIBELL-Universitat de Barcelona, Campus de Bellvitge, Pavelló de Govern, C/Feixa Llarga s/n, 08907 L'Hospitalet de Llobregat, Barcelona, Spain.
Email: restevez@ub.edu

Alfons Macaya, Pediatric Neurology Laboratory, Vall d'Hebron Research Institute, Pg. Vall d'Hebron, 119-129, 08035 Barcelona, Spain.
Email: amacaya@vhebron.net

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Abstract

De novo rare damaging variants in genes involved in critical developmental pathways, notably regulation of synaptic transmission, have emerged as a frequent cause of neurodevelopmental disorders (NDD). NDD show great locus heterogeneity and for many of the associated genes, there is substantial phenotypic diversity, including epilepsy, intellectual disability, autism spectrum disorder, movement disorders, and combinations thereof. We report two unrelated patients, a young girl with early-onset refractory epilepsy, severe disability, and progressive cerebral and cerebellar atrophy, and a second girl with mild dysmorphism, global developmental delay, and moderate intellectual disability in whom trio-based whole-exome sequencing analysis uncovered de novo missense variants in *CHRM1*. Biochemical analyses of one of the NDD-associated variants proved that it caused a reduction in protein levels and impaired cellular trafficking. In addition, the mutated receptor showed defective activation of intracellular signaling pathways. Our data strengthen the concept that brain-reduced muscarinic signaling lowers the seizure threshold and severely impairs neurodevelopment.

KEYWORDS
epileptic encephalopathy, muscarinic receptor, whole-exome sequencing

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Raúl Estévez and Alfons Macaya are last-shared authors.

Developmental and epileptic encephalopathies (DEE), are a group of diseases with onset within the first year of life where developmental impairment is recognized to occur as a direct consequence of the genetic mutation, in addition to the potential deleterious effect of epileptic activity on brain development (Scheffer et al., 2017). With the transition of next-generation sequencing to genetic diagnostics, the pace of DEE gene discovery has accelerated with over 100 currently known genetic etiologies (Brunklaus et al., 2020). Importantly, the genes underlying DEE are known to often cause a range of neurodevelopmental disorders (NDD) with or without epilepsy. Most of these genes encode proteins related to one of six fundamental processes: Ion transport, cell growth and differentiation, regulation of synaptic processes, transport and metabolism of small molecules within and between cells, and regulation of gene transcription and translation (Symonds & McTague, 2020).

Cholinergic signaling has been associated with the maintenance of cortical network excitability balance (Drever et al., 2011). To date, only mutations in the nicotinic acetylcholine receptor have been reported as disease-causing in genetic epilepsies (Steinlein et al., 1995). However, mutations affecting PLCB1, encoding phospholipase C isoform β1, which is placed downstream on the muscarinic cholinergic signaling pathway, have been associated with a DEE phenotype (Kurian et al., 2010) and so are mutations in the subunits of the CHRM1-regulated Kv7 channels (Jentsch, 2000).

In the present study, we identify two patients with point mutations in CHRM1, providing novel insight into the molecular mechanisms underlying DEE and neurodevelopmental impairment and further implicate defects in the cholinergic pathway in severe infantile epilepsies. Methods are described in the Supplementary section. The study was approved by the local Ethics Committee (PR(AG) 223/2017) and informed consent was obtained from the patient's parents according to the Helsinki declaration.

Case 1.   The proband is a 10-year-old girl with epilepsy and severe encephalopathy. She was born at term to healthy, unrelated parents after uneventful pregnancy and delivery. After birth, she was noted to be hypotonic, irritable, and difficult to feed. At age 1.5 months, she developed tonic seizures featuring right arm extension and head version. The reported physical exam showed no dysmorphic features or organomegaly, absent visual fixation, and global hypotonia. Electroencephalography (EEG) revealed multifocal epileptiform discharges and diffuse background slowing. Brain MRI (magnetic resonance imaging) (Figure 1a), array CGH, serum lactate, amino acids, sialotransferrin pattern, very-long-chain fatty acids, and biotinidase, urine organic acids, CSF folate, purines, neurotransmitter metabolites, and CSF/plasma glucose ratio were normal. Seizures persisted daily despite treatment with pyridoxine, phenobarbital, sodium valproate, or carbamazepine, and by age 4 months, manifested as a combination of tonic spasms, myoclonic jerks, and dysautonomic signs. At age 2 years 11 months, she displayed microcephaly with HC: 44.5 cm (−3 SD), right occipital plagiocephaly, and poor contact despite brief visual fixation and smiling in reaction to voice; other findings were horizonto-rotatory nystagmus, spastic quadriparesis, hyperreflexia, extensor plantar responses, and occasional axial or segmental myoclonia. The patient

suffered on average two to three seizures per day, mostly generalized tonic, heralded by a flexor spasm and accompanied by oral automatisms and eyelid myoclonia, each lasting 1–5 min. EEG revealed a high-voltage background with multifocal spikes of occipitotemporal predominance. Seizures proved refractory to treatment with different combinations of topiramate, lamotrigine, gabapentin, clobazam, levetiracetam, lacosamide, and eslicarbacepine acetate. A repeat MRI at age 4 years showed mild enlargement of subarachnoidal spaces, a relatively thick corpus callosum, and marked cerebellar atrophy with vermian predominance (Figure 1a). Magnetic resonance spectroscopy showed the presence of a creatine peak. She currently remains wheelchair-bound, with little awareness of her surroundings (Figure 1c). Seizure frequency has been reduced to a few per week while on perampanel monotherapy.

Whole-exome sequencing (WES) detected the NM_0 00738.2:c.1139C>T; p.(Pro380Leu) de novo variant in the CHRM1 gene, encoding the muscarinic acetylcholine receptor 1 (Figure 1d). This variant modifies a highly conserved residue in the orthologous and paralogous receptors. The p.Pro380Leu variant is considered as damaging or potentially disease-causing by various in silico predictors and is not present in general population databases. Therefore, the p.Pro380Leu variant is considered likely pathogenic. In the recently solved three-dimensional structure of CHRM1 (Maeda et al., 2019), residue Pro380 determines a kink in the C terminal part of helix 6 (Figure 1e).

No additional CHRM1 variants were found by WES reanalysis in a cohort of over 102 patients with DEE. However, a match was detected upon sharing the novel variant in the Matchmaker Exchange platform (Case 2).

Case 2.   This is a 14-year-old girl born at 28 weeks gestation to healthy, unrelated parents. Birth weight was 860 g, length 34 cm, and head circumference 24 cm. Despite prematurity, the neonatal period was relatively uneventful, did not require ventilation and serial cerebral ultrasounds were all normal. She showed some dysmorphic features including right hemifacial microsomia, congenital torticollis with plagiocephaly, high-arched palate, hypertelorism, and bilateral fifth finger clinodactyly. She was hypotonic early on and displayed feeding difficulties and axial titubation. Psychomotor development was globally delayed. She sat unassisted at age 2 years and walked after age 3 years. On follow-up, she displayed general motor clumsiness, crouch gait, right esotropia, very limited language with right-sided sensorineural hypoacusia, and poor social skills. Chromosomal analysis excluded 22q.11 deletion syndrome and the metabolic screen was normal. Brain MRI at age 5 (Figure 1b) showed mild ventriculomegaly, small pons, and cerebellar peduncles, and a small and detached cerebellar vermis. She has developed severe scoliosis, which required surgical intervention. No seizures have been recorded. Current examination reveals global cognitive dysfunction, with reduced and abnormal speech and additional oromotor difficulties. Eye movements are full and no nystagmus is appreciated. Fine motor skills are deficient with no pareses or pyramidal signs. She currently attends a special school and is able to recognize letters but unable to read; she appears withdrawn with frequent bouts of obsessive or aggressive behaviors. Her head circumference is 49 cm (−4 SD).
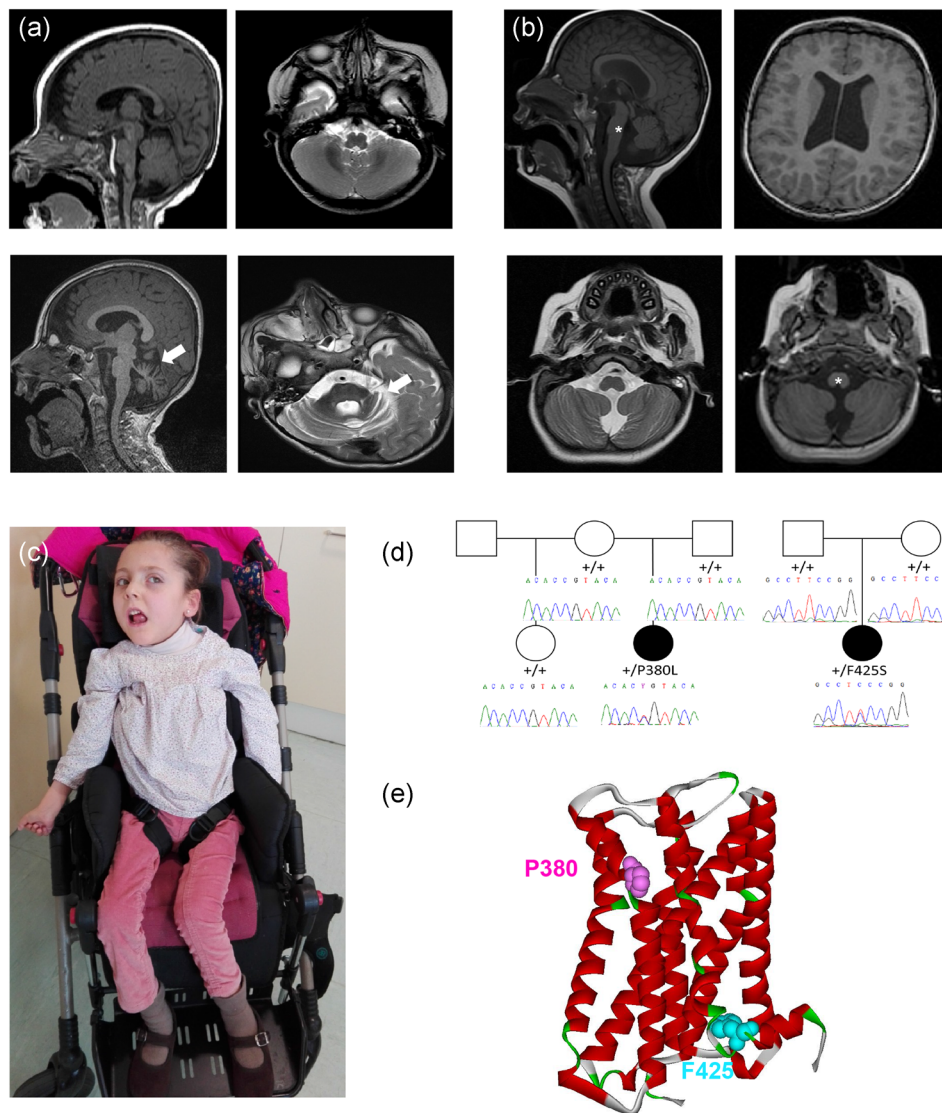
FIGURE 1 Identification of de novo mutations in *CHRM1* in patients with a developmental disorder or epileptic and developmental encephalopathy. (a) Case 1 brain MRI at age 1 year (top) and 4 years (bottom) showed right occipital plagiocephaly, mild enlargement of subarachnoidal spaces, a relatively thick corpus callosum, and marked cerebellar atrophy with vermian predominance (arrows). (b) In Case 2, MRI at age 5 years revealed prominent pontine (asterisks) and mild vermian hypoplasia. Note mild enlargement of the lateral ventricles. (c) Case 1 at age 8 years, exhibiting poor eye contact, hypotonia, and quadriparesis. (d) Pedigrees of both probands, showing the p.(Pro380Leu) heterozygous carrier (filled symbol), and her unaffected parents and half-sister and the p.(Phe425Ser) heterozygous carrier (filled symbol) and her unaffected parents. (e) 3D CHRM1 protein structure model shows the seven transmembrane helixes (in red) and the position of Proline 380 (in pink), which determines a kink in the distal part of helix 6 and Phe425 (in blue), located close to the G11 binding site. 3D, three-dimensional; MRI, magnetic resonance imaging

WES detected the NM_000738.2:c.1274T>C;p.(Phe425Ser) de novo variant in *CHRM1* (Figure 1d). Phe425 is located at the segment H8 (Maeda et al., 2019) located close to the cytoplasmic side and it is also very conserved in all CHRM homologs. See Table S1 for a comparative summary of clinical and genetic features of Cases 1 and 2.

We focused on the mutation p.(Pro380Leu) as the patient with this mutation is more severely affected. Thus, wild-type (WT) CHRM1 and mutant were expressed into HEK293T cells and protein expression levels were analyzed (Figure 2a). The mutant showed a different protein pattern band compared to WT protein, with a reduction of protein band 1 (WT: 71% ± 14%, P380L: 14% ± 2% of total

protein, $n = 5$), corresponding to the mature glycosylated form, and an increase of bands 2 and 3 (WT: 19% ± 10%, P380L: 64% ± 26% of total protein, $n = 5$), corresponding to immature forms, as revealed by glycosidase treatment (not shown). Then, we reasoned that mutation P380L impairs the correct maturation of CHRM1 protein.

To verify this biochemical data, we cotransfected HeLa cells with WT CHRM1 or mutant plus PH-GFP (green fluorescent protein), a fluorescent probe that labels the plasma membrane (Figure 2b). Although WT CHRM1 was detected mainly at the plasma membrane, predominantly colocalizing with PH-GFP (yellow staining) (Pearson's correlation coefficient: Rr = .8 ± .02, $n = 3/65$ cells), the mutant was

almost exclusively detected in intracellular compartments (Figure 2b), showing less degree of colocalization with PH-GFP (Rr = .3 ± .02, n = 3/45 cells). Thus, immunofluorescence studies confirmed that the mutation reduced the surface expression of the CHRM1 protein.

Muscarinic acetylcholine receptors are involved in signaling pathways related to adenosine 3′,5′-cyclic monophosphate (cAMP) and calcium intracellular release. CHRM1 is mainly known to signal through Gq/11-activating phospholipase C that increases IP_3 calcium-related pathways. However, cAMP signaling has also been

associated with CHRM1. Reporter assays were performed in HEK293T cells to detect cAMP- (Figure 2c) or IP_3/Ca^{2+}-associated transcription (Figure 2d). Both reporters displayed a reduced signaling activation for the mutant compared with WT.

In our first patient, seizure onset was in early infancy, yet she displayed neurological and behavioral abnormalities since birth and hence the label DEE. Severely impaired cognitive and motor development was already notable within the first year of life and acquired microcephaly reflected atrophy and/or cerebral underdevelopment in the context of an
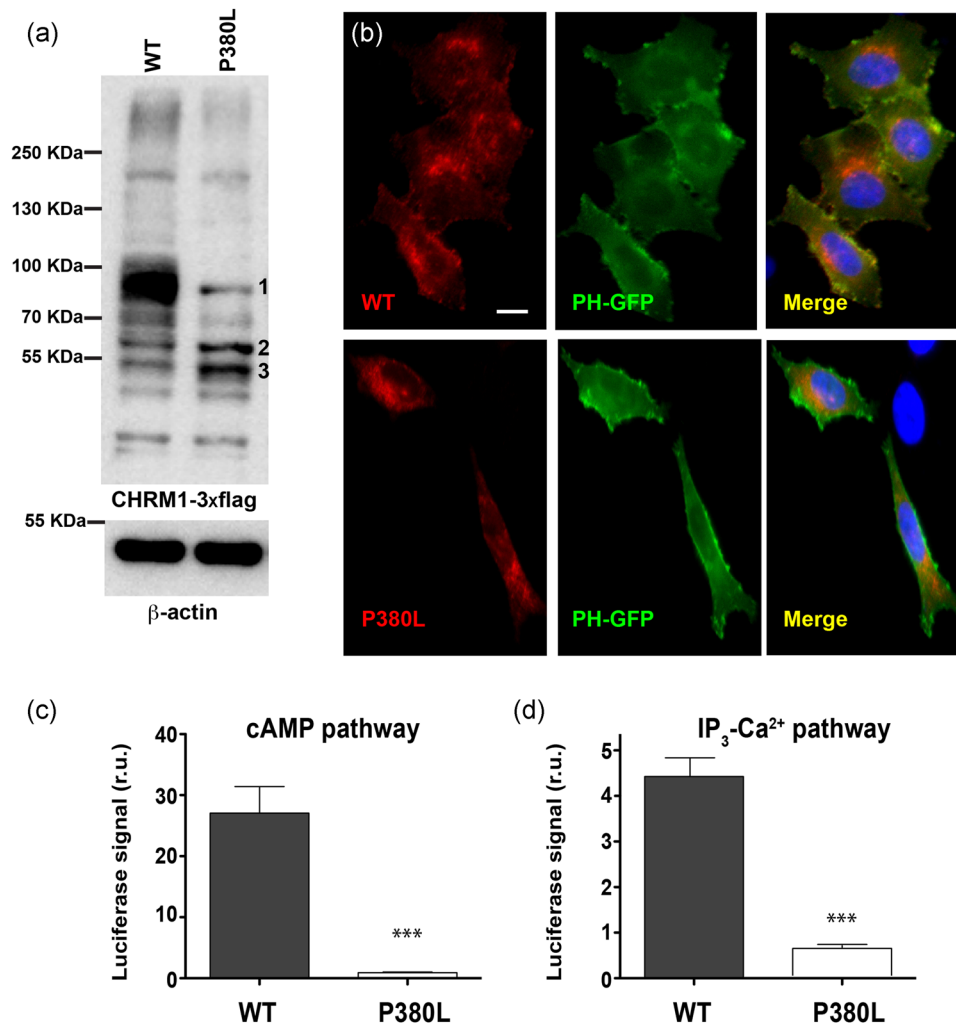
FIGURE 2 The mutation p.P380L causes a trafficking and a functional defect to the CHRM1 protein. (a) Western blot analysis of transfected cells with Flag-tagged CHRM1 WT or carrying p.(Pro380Leu) mutation was performed with an antibody detecting the flag epitope. CHRM1 is a protein whose molecular weight is 52 kDa, but the mature protein increases its molecular weight due to glycosylation. Different protein band pattern was obtained corresponding to (1) mature glycosylated protein form, (2) and (3) immature forms. Cells transfected with the mutant showed an increase in forms 2 and 3 and a decrease in mature glycosylated CHRM1 compared to cells transfected with WT. β-Actin was used to normalize protein levels. Statistics were performed from five independent experiments. (b) HeLa cells cotransfected with WT or mutant CHRM1 and PH-GFP, were studied by immunofluorescence. Co-localization between CHRM1 (red label) and PH-GFP (green label) was analyzed (Merge) by Pearson´s correlation analysis. Statistics were performed from 3 independent experiments. (c, d) Signaling pathways associated to CHRM1 were studied by a luciferase signal assay in HEK293T cells transfected with WT or mutant CHRM1, after treatment with Carbachol, a CHRM1 agonist. (c) cAMP signaling pathway activation was studied through a CRE gene reporter. Negative controls and forskolin-treated cells (positive control) were used to normalize results. (d) IP_3-Ca^{2+} signaling pathway activation was studied through an NFAT gene reporter. Negative controls and Ca+ PMA (phorbol myristate acetate)-treated cells (as a positive control) were used to normalize results. Statistics were performed from three independent experiments. cAMP, adenosine 3′,5′-cyclic monophosphate; GFP, green fluorescent protein; WT, wild-type

epileptic and developmental disorder. Our second patient had early-onset cognitive and motor impairment as well, but her phenotype is better covered under the more unspecific label of NDD. De novo mutations in variance-intolerant genes are a well-known cause of NDD, including DEE. In fact, patients with developmental disorders resulting from mutations in novel genes, as opposed to many classical conditions, are often phenotypically dissimilar and phenotype-driven recognition of these genetic defects is revealing rather unlikely (Kaplanis et al., 2020). In keeping, we have previously reported wide phenotype variability, from epileptic encephalopathy to NDD and autism spectrum disorder, in association with variants in other synaptic genes such as *VAMP2* or *GRIA2* (Salpietro, Dixon, et al., 2019; Salpietro, Malintan, et al., 2019).

Our findings hint at CHRM1 dysfunction as a possible novel cause of NDD. Additional evidence may come from the recent finding of a missense *CHRM1* variant in a young boy included in an autism spectrum disorder cohort sequencing study (Satterstrom et al., 2020). Thus, a total of three putative pathogenic *CHRM1* variants have been associated with neurodevelopmental phenotypes and all of them are assessed as disease-causing by main in silico predictors (Table S1). It is also the first instance where the metabotropic muscarinic receptors are linked to an epileptic disorder. Admittedly, this is based only on the fact that we have identified one de novo mutation in a single patient. However, we believe that this mutation is pathogenic as it affects a conserved residue in all CHRM proteins that is present in an important structural element of the transmembrane segment 6 (TM6). The TM6 suffers a small rotation and an outward displacement during receptor activation that allows the G protein to engage the receptor core (Maeda et al., 2019). Mutation p.(Phe425Ser) may also partially destabilize the binding of the G11 protein, as it is very close to the residues cysteine 421 and asparagine 422, which have been involved in the binding of the G protein. Thus, it could be that both mutations impair receptor activation. In addition, expression of the Pro380Leu mutant protein in transfected cells at the membrane is reduced possibly due to a folding defect. We reasoned that, based on the fact that this mutant is expressed at low levels, it is very difficult to consider that the mutant protein may exert a dominant-negative effect. Rather, we support the hypothesis that the patient may suffer a reduction of cholinergic activity due to haploinsufficiency. This hypothesis is in agreement with the fact that a minor reduction of KCNQ activity, a known target of muscarinic regulation, is enough to cause an epileptic phenotype (Jentsch, 2000).

How a reduction in cholinergic activity in humans may lead to an early epileptic or developmental phenotype? One of the best-known targets of muscarinic regulation is the M current formed by KCNQ channels through $G_{q/11}$ mediated protein signals that increase phospholipase C-beta activity, which results in consumption of phosphatidylinositol 4,5-bisphosphate (PIP2) resulting in KCNQ inhibition. In this pathway, loss of function mutations in KCNQ channels or PLCB1 cause epileptic encephalopathy (Schoonjans et al., 2016). Thus, considering this well-known pathway, one simple hypothesis is that muscarinic inhibition might result in increased KCNQ activity, which may affect neuronal excitability properties. However, gain-of-function variants in KCNQ2 do not show epileptic seizures

(Miceli et al., 2015). Thus, modulation of M current by CHRM1-PLCB1 might not be the only acting pathogenic mechanism.

CHRM1 might regulate many different targets including other ion channels depending on the developmental stage and in a cell-specific manner. It is noteworthy that, although CHRM1 is nearly not expressed in the cerebellum (Bakker et al., 2015), our patient 1 developed prominent cerebellar atrophy suggesting that a defective CHRM1-mediated cholinergic activity may have resulted particularly damaging for Purkinje cells. Patient 2 had also cerebellar vermian and pontine atrophy, while her moderate ventricular enlargement could conceivably relate to white matter injury in association with encephalopathy of prematurity.

In summary, our work further suggests that muscarinic activity in the brain might affect multiple processes regulating seizure susceptibility and neuronal development and therefore, *CHRM1* can be proposed as a novel gene associated with DEE and NDD.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that supports the finding of this study are available in the Supporting Information of this article. Variants are submitted at LOVD database (https://databases.lovd.nl/): https://databases.lovd.nl/shared/variants/0000666233#00005134 and https://databases.lovd.nl/shared/variants/0000708802#00005134.

ORCID

Anna Marcé-Grau 🄳 http://orcid.org/0000-0001-5762-4023
Xabier Elorza-Vidal 🄳 http://orcid.org/0000-0001-9163-6079
Carla Pérez-Rius 🄳 http://orcid.org/0000-0002-6058-7400
Anna Ruiz-Nel·lo 🄳 http://orcid.org/0000-0001-7314-5962
Júlia Sala-Coromina 🄳 http://orcid.org/0000-0001-5116-6316
Raúl Estévez 🄳 http://orcid.org/0000-0003-1579-650X
Alfons Macaya 🄳 http://orcid.org/0000-0001-7998-4185

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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