Muscarinic Acetylcholine Receptor M1 mutation causing developmental and epileptic encephalopathy

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Abstract

Developmental and epileptic encephalopathies are a group of devastating disorders where an underlying, usually genetic, cause leads to brain developmental impairment which can be further aggravated by superimposed, abundant epileptiform activity. Compared to other epilepsies, DEE show much greater locus heterogeneity and de novo rare damaging variants in genes involved in critical developmental pathways, notably regulation of synaptic transmission, have emerged as a frequent cause. Here, in a young girl with early-onset refractory epilepsy, severe disability and progressive cerebral and cerebellar atrophy, trio-based whole exome sequencing analysis uncovered a de novo missense variant in CHRM1. No additional CHRM1 variants were found by WES reanalysis in a cohort of 102 patients with EIEE/DEE nor any matches were produced upon sharing the novel variants in the Matchmaker Exchange platform. Biochemical analyses proved that this variant caused a reduction in protein levels and an impaired cellular trafficking. In addition, the mutated receptor showed defective activation of intracellular signalling pathways. Our data strengthen the concept that brain reduced muscarinic signalling lowers seizure threshold and severely impairs neurodevelopment.

INTRODUCTION

Early Infantile Epileptic Encephalopathies (EIEE) are a group of diseases with onset appearing within the first year of life, where epileptiform abnormalities interfere with brain development and thus are believed to contribute to the progressive disturbance in cerebral function (Berg and Cross, 2010). In contrast, in the recently defined Developmental and Epileptic Encephalopathies (DEE), 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 (Hebbar and Mefford, 2020) with over 100 currently known genetic etiologies (Brunklaus et al., 2020). Most of the genes involved in these conditions encode proteins related with 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 and McTague, 2020).

Cholinergic signalling 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 signalling pathway, have been associated with an EIEE phenotype (Kurian et al., 2010) and so are mutations in the subunits of the CHRM1-regulated Kv7 channels (Jentsch, 2000).

In the present work we identify a patient with a point mutation in *CHRM1* , providing novel insight into the molecular mechanisms underlying DEE and further implicate defects in the cholinergic pathway in severe infantile epilepsies.

METHODS AND ETHICAL COMPLIANCE

An 8 year-old girl diagnosed with DEE underwent trio whole-exome sequencing. HEK293T and HeLa cells were transfected with CHRM1 wild-type and mutated plasmids tagged at C-terminal. Western blot and immunoblotting assays were done to study protein expression and cell trafficking. Luciferase reporter assays were performed to measure cAMP and calcium cell signaling activity. GraphPad software was used to perform the statistic analysis (See Supplementary Information). 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.

RESULTS

Clinical features and genetic studies

The proband is an 8 year-old girl with epilepsy and severe encephalopathy. She was born at term to healthy, unrelated parents after uneventful pregnancy and delivery. A 17 year-old half-sister is healthy. Birth weight was 2950g (27^{th} pctl), height 47,5 cm (13^{th} pctl) and head circumference 32,5 cm (11^{th} pctl). 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 organomegalies, absent visual fixation and global hypotonia. An EEG revealed multifocal epileptiform discharges and diffuse background slowing. Initial tests performed between ages 2 and 4 months were normal and included brain MRI (**Figure 1A**), serum amino acids, urine organic acids, CSF folate, purines and neurotransmitter metabolites and CSF/plasma glucose ratio. 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. The patient did not attain any developmental milestone during the first year of life. Further trials with topiramate, lamotrigine, gabapentin, clobazam, levetiracetam, lacosamide and eslicarbacepine acetate in various combinations did not improve her condition. She was referred to our center at age 2y 11mo. She displayed microcephaly with HC: 44.5 cm (-3SD), 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 2-3 seizures per day, mostly generalized tonic, heralded by a flexor spasm and accompanied by oral automatisms and eyelid myoclonia, each lasting 1-5 minutes and was on valproate and lacosamide. EEG revealed a high-voltage background with multifocal spikes of occipito-temporal predominance. Ictal recording during a tonic seizure showed initial theta-delta generalized waves with irregular temporo-occipital spikes and a posterior global attenuation. New investigations included array CGH, serum lactate, sialotransferrin pattern, very long chain fatty acids, biotinidase, biochemical screen for purine and pyrimidine defects and whole exome sequencing (WES, see below). 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**). MRS showed the presence of creatine peak. She received therapies for increasing spasticity, including oral baclofen, botulinum toxin injections and multilevel lower limb tenotomies. She currently remains wheel chair bound, with little awareness of her surroundings **(Figure 1B)** . Seizures persist unchanged but their frequency has been reduced to a few per week after perampanel was substituted for the previous treatment. Melatonin has been added for prominent insomnia.

Whole exome sequencing detected the NM_000738.2:c.1139C>T; NP_000729.2:p.(Pro380Leu)*de novo* variant in the *CHRM1* gene, encoding the muscarinic acetylcholine receptor 1, which was later validated by Sanger sequencing (**Figure 1C**). 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 (see Supplementary Materials

and Methods). Therefore, the p.Pro380Leu variant is considered likely pathogenic. In the recently solved 3D structure of CHRM1 (Maeda et al., 2019), residue Pro380 determines a kink in the C terminal part of helix 6 (**Figure 1D**)

No additional *CHRM1* variants were found by WES reanalysis in a cohort of over 102 patients with EIEE/DEE nor any matches were produced upon sharing the novel variants in the Matchmaker Exchange platform (Philippakis et al., 2015).

CHRM1 mutant P380L presents an altered traffic to the plasma membrane

CHRM1 is a protein whose molecular weight as a monomer is 52 kDa, but the mature protein increases its molecular weight due to glycosylation. To study the effect of the mutation p.Pro380Leu, wild-type CHRM1 or mutant were expressed into HEK293T cells and protein expression levels were analysed (**Figure 2A**). The mutant showed a different protein pattern band compared to wild-type protein, with a reduction of protein band 1 (wild-type: 71 ± 14 %, P380L: 14 ± 2 % of total protein, n= 5), corresponding to the mature glycosylated form, and an increase of bands 2 and 3 (wild-type: 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 co-transfected HeLa cells with CHRM1 wild-type or mutant plus PH-GFP, a fluorescent probe that labels the plasma membrane (**Figure 2B**). Whereas CHRM1 was detected mainly at the plasma membrane, predominantly colocalizing with PH-GFP (yellow staining) (Pearson's correlation coefficient: Rr = 0.8 ± 0.02, n= 3 / 65 cells), the mutant was almost exclusively detected in intracellular compartments (Figure 2B), showing less degree of colocalization with PH-GFP (Rr = 0.3 ± 0.02, n = 3 / 45 cells). Thus, immunofluorescence studies confirmed that the mutation reduced surface expression of the CHRM1 protein.

Reduced signal transduction of the mutant CHRM1

Muscarinic acetylcholine receptors are involved in signalling pathways related to cAMP and calcium intracellular release. CHRM1 is mainly known to signal through Gq/11 activating phospholipase C that increase IP_3-calcium related pathways. However, cAMP signalling has also been associated with CHRM1, although its role is still unknown(Peralta et al., 1988). To detect the function levels of the mutant vs. wild-type, reporter assays were performed in HEK293T cells to detect cAMP- **(Figure 2C**) or to detect IP_3/Ca^{2+}-associated transcription**(Figure 2D**). Both reporters displayed a reduced signalling activation for the mutant compared with wild-type.

DISCUSSION

In our patient, seizure onset was in early infancy, yet she displayed neurological and behavioural 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 epileptic and developmental disorder.

We here provided evidence that dysfunction of CHRM1 might cause DEE, or CHRM1 encephalopathy. While mutations in the various genes encoding ionotropic nicotinic acetylcholine receptor subunits *CHRNA4* ,*CHRNB2* and *CHRNA2* are typically associated with autosomal dominant nocturnal frontal lobe epilepsy (Steinlein et al., 1995; De Fusco et al., 2000; Aridon et al., 2006), to our knowledge this is the first instance where the metabotropic muscarinic receptors are linked to an epileptic disorder. Admittedly, this is based only in the fact that we have identified one *de novo* mutation in a single patient. However, we believe that this mutation is pathogenic since 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). Thus, it could be that the p.P380L mutation impairs receptor activation as our data suggest. In addition, expression of this mutant protein in transfected cells at the membrane is reduced possibly due to a folding defect. We reasoned that, based on the fact that the mutant protein 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 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 result in consumption of phosphatidylinositol 4,5-bisphosphate (PIP2) resulting in KCNQ inhibition. In this pathway, loss of function mutations in KCNQ channels or PLCB causes epileptic encephalopathy. 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 reason of pathogenesis.

CHRM1 might regulate many different targets including ion channels depending on the developmental stage and in cell-specific manner. For example, in the hippocampus, activation of CHRM1 might stimulate M current, G-protein coupled inwardly rectifying potassium channels and TRPC channels in dentate-gyrus granule cells while it may inhibit M current in CA1 neurons (Carver and Shapiro, 2019). On the other hand, activation of CHRM1 might inhibit calcium-dependent small conductance potassium (SK) channels leading to NMDA receptor disinhibition (Tigaret et al., 2018).

It is noteworthy that, although CHRM1 is nearly not expressed in the cerebellum (Bakker et al., 2015), our patient developed a prominent cerebellar atrophy suggesting that a defective CHRM1-mediated cholinergic activity may have resulted particularly damaging for Purkinje cells.

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 to DEE phenotype.

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Competing interests

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

Data availability statement

The data that supports the finding of this study are available in the supplementary material of this article.

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Figure Legends

Figure 1. Identification of a *de novo* mutation in CHRM1 in a patient with developmental and epileptic encephalopathy. A . Brain MRI at age 1y (top) and 4y showed right occipital plagiocephaly, mild enlargement of subarachnoidal spaces, a relatively thick corpus callosum and marked cerebellar atrophy with vermian predominance (arrows).**B** . The patient at age 8y, exhibiting poor eye contact, hypotonia and quadriparesis. **C.** Left panel: Pedigree of the affected patient, showing the p.Pro380Leu heterozygous carrier (filled symbol), and her unaffected parents and half-sister. Right panel: Sanger sequencing validation. Chromatograms of patient (bottom) and the wild-type sequence as found in parents and half-sister sequencing (top), confirmed the *de novo* occurrence of the heterozygous *CHRM1*c.1139C>T (NM_000738.2) variant. **D**

. 3D CHRM1 protein structure model shows the 7 transmembrane helixes (in red) and the position of Proline 380 (in pink), which determines a kink in the distal part of helix 6.

Figure 2. The mutation p.P380L causes a trafficking and a functional defect to the CHRM1 protein. A . Western-Blot of transfected cells with flag-tagged CHRM1 WT or carrying p.P380L mutation was performed with an antibody detecting the flag epitope. Different protein band pattern was obtained corresponding to (1) mature glycosylated protein form, (2) and (3) immature forms. Cells transfected with the mutated vector showed an increase in forms 2 and 3 and a decrease in mature glycosylated CHRM1 compared to cells transfected with wild-type vector. β-actin was used to normalize CHRM1 protein levels. Statistics were performed as described in Materials and Methods from 5 independent experiments. **B.** HeLa cells co-transfected with pcDNA3-h*CHRM1* -3Flag or pcDNA3-h*CHRM1* (P380L)-3Flag (P380L) and PH-GFP, were studied by immunofluorescence and colocalization 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 pcDNA3-hCHRM1(wt)-3Flag] or pcDNA3-hCHRM1(P380L)-3Flag- when treated with Carbachol, an agonist.**C.** cAMP signaling pathway activation was studied through 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 analyzed through NFAT transcription factor. Negative controls and CA+PMA treated cells (positive control) were used to normalize results. Statistics were performed from 3 independent experiments.

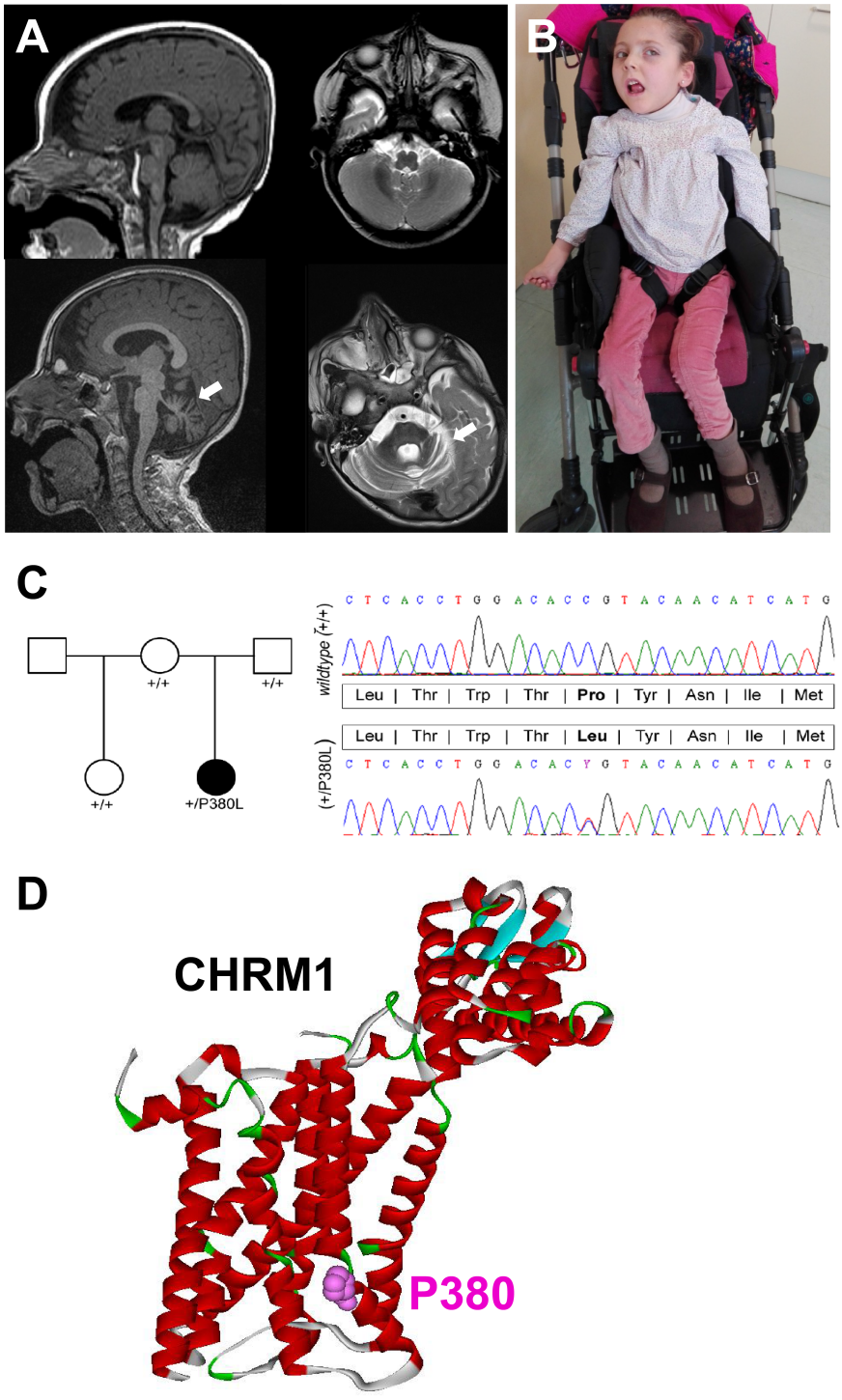

Figure 1

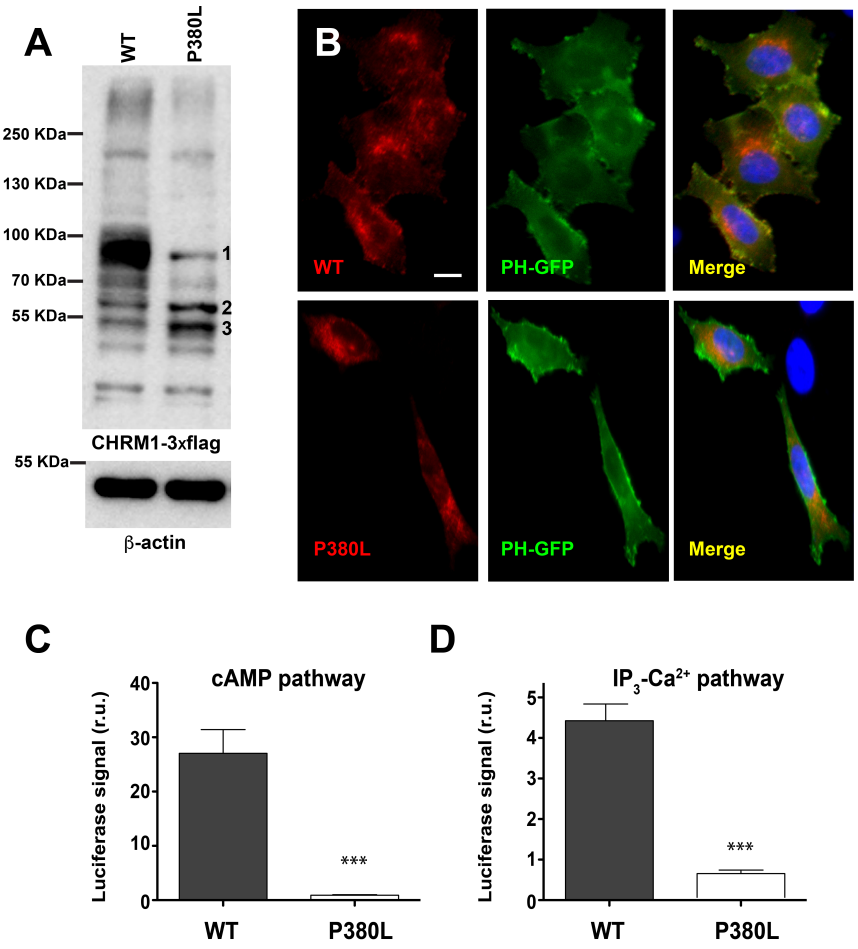

Figure 2