SUPPLEMENTARY MATERIALS AND METHODS

Subjects and biological samples

A 8 year-old girl was diagnosed with DEE according to clinical manifestations, routine tests and pedigree information at the Pediatric Neurology Unit, Vall d'Hebron University Hospital in Barcelona. DNA from both the proband and her parents was extracted from peripheral blood samples following standard methods. Written informed consent was obtained from the parents for participation in the study.

A 14 year-old girl diagnosed with global cognitive dysfunction and dysmorphism at Parc Taulí University Hospital in Sabadell with a de novo variant at *CHRM1* was detected through URD-Cat project Cohort (https://www.urdcat.cat/home) and then uploaded to GeneMatcher [1] DNA from both the proband and her parents was extracted from peripheral blood samples following standard methods. Written informed consent was obtained from the parents for participation in the study.

Genetic Analysis

Trio Whole Exome Sequencing (WES) of Patient 1 and their parents was performed with NimbleGenSeqCap EZ Exome v3.0 exome enrichment kit (Roche, Madison, WI, USA). Genomic data was aligned to NCBI build 37/ hg19 reference genome sequence. Variants were called with GATK Haplotype Caller[2] and annotated using Variant Effect Predictor[3] and ANNOVAR[4] softwares. Variants were classified according to predicted functional effect and filtered in a two-tiered analysis consisting of inspection of variants affecting genes previously associated with epilepsies followed by scrutiny of variants in all remaining genes. Filtering and further prioritisation was based on quality and coverage parameters, general population frequencies in gnomAD database[5] and phenotype-driven variation databases [ClinVar[6], HGMD[7] and CentoMD[8]] and variant inheritance and *in silico* prediction tools. Further analyses included ontology and protein association data. Putative pathogenic variants were validated using direct Sanger sequencing.

Whole Exome Sequencing (WES) of Patient 2 was performed using Nimblegen SeqCap EZ MedExome + mtDNA 47Mb kit (Roche, Madison, WI, USA). CHRM1 variant identified (NM_000738.2:c.1274T>C;p.(Phe425Ser)) was validated and segregated using direct Sanger sequencing.

Molecular biology

Plasmids were constructed using standard molecular biology techniques employing recombinant PCR and the Multisite Gateway System (Invitrogen, Carlsbad, CA, USA). For localization and Western blot studies, all CHRM1 constructs were flag tagged at their C-terminus (three flag copies) and cloned into the pCDNA3 vector.

Cell culture and transfection, Western blot and Immunofluorescence

HEK293T and HeLa cells were maintained in 5% CO_2, 90% humidity at 37ºC in Dulbecco's Modified Eagle Medium; DMEM (Biological Industries, CT, USA) supplemented with 1% Glutamine, 10% FBS and 1% antibiotic. Transfectin (BioRad, CA, USA) protocol was used to transiently transfect cell cultures.

For Western blotting, HEK293T cells were used. After 48h incubation in a 6-well plate, protein extracts were obtained with lysis buffer (PBS, 1% Triton X-100, 150mM NaCl and protease inhibitors). Immunoblot quantification was done using ImageJ.

For immunofluorescence experiments, HeLa cells were co-transfected with pcDNA3-h*CHRM1*-3Flag, or pcDNA3-h*CHRM1*(P380L)-3Flag together with pcDNA3-PH-GFP expressing the GFP-tagged pleckstrin homology domain of PLCδ1 (PH-GFP). After 24h incubation in 6-well plates, cells were seeded on 10 mm glass coverslips for 24h in 24-well plates and then fixed with 3% PFA. CHRM1 immunostaining was done with α-Flag mouse (Sigma-Aldrich, MO, USA) and Alexa Fluor 568 dye (Abcam, UK). DAPI was also used to stain cell nucleus. An Olympus DSU spinning disk inverted fluorescent microscope was used to visualize the experiments. ImageJ software and Pearson Correlation Analysis plugin were used to analyze plasma membrane colocalization.

Reporter assays

Vectors pGL4.30[*luc2P/*CRE/Hygro] and pGL4.30[*luc2P/*NFAT-RE/Hygro] (Promega, WI, USA) were used to report cAMP and calcium cell signaling respectively. HEK293T were transfected with pcDNA3-*CHRM1*/3Flag or pcDNA3-*CHRM1*(P380L)/3Flag in combination with CRE or NFAT reporter vectors. After one-day incubation, cells were cultured for 24 hours in DMEM without supplementation in a 96-well plate, and then cells underwent a 1µM Carbachol treatment (Cayman Chemical, MI, USA) in DMEM 0.5% FBS during 6 hours. BrightGlo Luciferase Assay System

(Promega, WI, USA) was used to measure cAMP and calcium levels through a luciferase reporter-dependence emission. Bioluminescence signal detection was performed in a CLARIOstar instrument (BMG LABTECH, Germany). Positive controls were stimulated with 1 µM Forskolin ≥ 98% (HPLC) for cAMP signaling and with Calcimicine 5 µM and PMA 10ng/mL (Sigma-Aldrich, MO, USA) for calcium pathways.

Statistics

GraphPad software (La Jolla, Ca, USA) was used to perform the statistic analysis of this study. Kolmogorov-Smirnov test (n>30) and Shapiro-Wilk test (n<30) was used to establish variable normality. For normal variable comparison one-way ANOVA followed by Bonferroni correction was used. Kruskal-Wallis test followed by Dunn multiple comparation test was run when comparing groups with not normal variables.

Supplementary references

1. Sobreira N, Schiettecatte F, Valle D, Hamosh A. GeneMatcher: A Matching Tool for Connecting Investigators with an Interest in the Same Gene. Hum Mutat. 2015;10:928-30.

2. Van der Auwera GA, Carneiro MO, Hartl C, et al. From fastQ data to high-confidence variant calls: The genome analysis toolkit best practices pipeline. Curr Protoc Bioinforma. 2013; (SUPL.43).

3. McLaren W, Gil L, Hunt SE, et al. The Ensembl Variant Effect Predictor. Genome Biol. 2016; 17(1).

4. Wang K, Li M, Hakonarson H. ANNOVAR: Functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010; 38(16).

5. Lek M, Karczewski KJ, Minikel E V., et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature. 2016; 536(7616):285–91.

6. Landrum MJ, Lee JM, Benson M, et al. ClinVar: Improving access to variant interpretations and supporting evidence. Nucleic Acids Res. 2018; 46(D1):D1062–7.

7. Stenson PD, Ball E V., Mort M, et al. The human gene mutation database (HGMD) and its exploitation in the fields of personalized genomics and molecular evolution. Curr Protoc Bioinforma [Internet]. 2012 [cited 2020]; Chapter 1(SUPPL.39):Unit1.13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22948725

8. Trujillano D, Oprea GE, Schmitz Y, et al. A comprehensive global genotype–

phenotype database for rare diseases. Mol Genet Genomic Med [Internet]. 2017 [cited 2020]; 5(1):66–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28116331