Loss of DPP6 in neurodegenerative dementia: a genetic player in the dysfunction of neuronal excitability

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Abstract

Emerging evidence suggested a converging mechanism in neurodegenerative brain diseases (NBD) involving early neuronal network dysfunctions and alterations in the homeostasis of neuronal firing as culprits of neurodegeneration. In this study, we used paired-end short-read and direct long-read whole genome sequencing to investigate an unresolved autosomal dominant dementia family significantly linked to 7q36. We identified and validated a chromosomal inversion of ca. 4 Mb, segregating on the disease haplotype and disrupting the coding sequence of dipeptidyl-peptidase 6 gene (DPP6). DPP6 resequencing identified significantly more rare variants—nonsense, frameshift, and missense—in early-onset Alzheimer’s disease (EOAD, p value = 0.03, OR = 2.21 95% CI 1.05–4.82) and frontotemporal dementia (FTD, p = 0.006, OR = 2.59, 95% CI 1.28–5.49) patient cohorts. DPP6 is a type II transmembrane protein with a highly structured extracellular domain and is mainly expressed in brain, where it binds to the potassium channel Kv4.2 enhancing its expression, regulating its gating properties and controlling the dendritic excitability of hippocampal neurons. Using in vitro modeling, we showed that the missense variants found in patients destabilize DPP6 and reduce its membrane expression (p < 0.001 and p < 0.0001) leading to a loss of protein. Reduced DPP6 and/or Kv4.2 expression was also detected in brain tissue of missense variant carriers. Loss of DPP6 is known to cause neuronal hyperexcitability and behavioral alterations in Dpp6-KO mice. Taken together, the results of our genomic, genetic, expression and modeling analyses, provided direct evidence supporting the involvement of DPP6 loss in dementia. We propose that loss of function variants have a higher penetrance and disease impact, whereas the missense variants have a variable risk contribution to disease that can vary from high to low penetrance. Our findings of DPP6, as novel gene in dementia, strengthen the involvement of neuronal hyperexcitability and alteration in the homeostasis of neuronal firing as a disease mechanism to further investigate.

Keywords Neurodegenerative brain diseases · Dementia · DPP6 · Hyperexcitability · Whole genome sequencing · Oxford nanopore technologies (ONT) PromethION

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Background

Considerable progress has been made towards understanding the genetic origin, the neuropathology and the epidemiology of neurodegenerative brain diseases (NBD) such as Alzheimer’s disease (AD) and frontotemporal dementia (FTD). Linkage analyses and large-scale genomics studies, have shown that a wide genetic heterogeneity is responsible for the neuronal pathologies and dysfunctions in NBD [6,
with yet additional causes to identify and mechanisms to discover. Many disorders of the central nervous system (CNS) show a broad array of clinical features, e.g., impaired behavior, language, etc., but some of them, such as alterations in memory, are shared across disorders [7, 28, 55]. Several emerging concepts suggest a converging mechanism in NBD involving early neuronal network dysfunctions [58, 84] and alterations in the homeostasis of neuronal firing [24] as culprits of neurodegeneration [24, 58, 84]. This is supported by studies in mouse models of AD, in which was shown that higher firing rates can promote amyloid-β (Aβ) production [84] and that the neuronal hyperactivity precedes the deposition of plaques [5]. Studies using functional magnetic resonance (fMRI) demonstrated hippocampal activation in both patients with amnestic mild cognitive impairment (aMCI) [16] and preclinical carriers of inherited causal mutations in familial AD [64] as well as FTD patients [83]. These are only some examples of an emerging field, which spurs the investigation of these new mechanisms to better understand neurodegenerative dementia and to design more effective treatments [3, 58]. Despite the diverse mechanistic insights [24, 58, 84], additional data are necessary to better understand the pathophysiology of the early dysfunctions of circuits and neurons. Our discoveries, started with an in depth examination of an unexplained linked locus on chromosome 7q36 (size 5.44 Mb and LOD score = 3.39 at θ = 0) [65] and resulted in the finding of dipeptidyl-peptidase 6 (DPP6) as novel gene in NBD. DPP6 is a single pass type II transmembrane protein expressed in brain, where it forms a multimeric complex with the potassium channel Kv4.2, regulating the voltage-dependent gating properties and the surface expression of Kv4.2 in the brain [60] playing a crucial role in neuronal excitability [73]. With this study we provided direct genetic evidence to support the involvement of neuronal hyperexcitability and alteration in the homeostasis of neuronal firing as disease mechanisms in dementia.

Materials and methods

Family 1270

The proband of family 1270, aged 47 years, was ascertained in the frame of the Dutch population-based epidemiological study of early-onset AD in 1980–1987 [30]. The 1270 family, with a history of autosomal dominant inheritance [29, 76], was sampled for genetic studies in the 80–90’s. Diagnoses of AD were made according to National Institute of Neurological and Communicative Diseases and Stroke–Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria published in 1984 [49], and of mild cognitive impairment (MCI) according to Honig and Mayeux [31]. Linkage analyses for loci on chromosomes 14, 19, and 21 [76], and mutation screening of the AD genes, APP (21.q21.1), PSEN1 (14q24.3) and PSEN2 (1q42.1), were negative [11, 75].

Follow-up clinical studies of family 1270, including neurological examination of incident patients, interviews of first-degree relatives and review of medical records and CT scans, identified four additional patients [65]. The onset age in the updated family was highly variable with a mean age at onset of 66.8 ± 7.4 years and range of 47–77 years. In the proband, and most other patients, the disease initially presented with memory impairment, except for one patient, in whom a change of personality was the initial complaint, which later in the disease course was followed by memory loss. In all patients the disease progressed into other areas of cognition, such as praxis and speech [65]. Neuroimaging was available for two patients, III-48 at age 74 years and III-21 at age 82 years and showed cortical atrophy in both [65]. For the only living patient, III-21, who received the diagnosis of possible AD, a CT scan (at age 82 years) showed that cortical and subcortical atrophy was most notable in the temporal and frontal regions (supplementary material) [65]. A cohort of 82 Dutch EOAD patients [76], mean age at onset (AAO) ± standard deviation (SD) of 57.0 ± 5.6 years (82.3% women) was included for candidate gene resequencing.

Belgian patient and control cohorts

The Belgian AD cohort consisted of 558 patients (mean onset age 61.6 ± 6.8 years, range 33–70 years), of whom 221 Belgian AD patients were referred to our molecular diagnostic unit for PSEN1, PSEN2, and/or APP mutation screening. Post-mortem brain analysis was performed in 17 patients confirming AD pathology. The majority of the patients were recruited at the memory clinic of the hospitals Middelheim and Hoge Beuken of the Hospital Network Antwerp (ZNA), Belgium (P.P.D.D. and S.E.) [19, 20]. Another subset was collected at the Department of Neurology and the Memory Clinic of the University Hospitals of Leuven (UHL), Belgium (R.V.) as well as through the neurology centers of the clinical partners of the Belgian Neurology (BELNEU) consortium. Diagnosis of possible, probable or definite AD was obtained by consensus of at least two neurologists based on the NINCDS-ADRDA diagnostic criteria [49] and the National Institute on Aging-Alzheimer’s Association (NIA-AA) diagnostic criteria [32, 50]. Each AD patient underwent a neuropsychological examination, including mini-mental state examination (MMSE) [23] and structural neuroimaging, while functional neuroimaging and cerebrospinal fluid analysis was done in a subset of patients [4]. The Belgian FTD cohort consisted of 614 patients (mean AAO 66.1 ± 9.9 years; age range 20–89 years), which included 35 patients with a concomitant amyotrophic lateral sclerosis (FTD-ALS), recruited in the framework of...
Genomics Inc. (Mountain View, CA USA) [18]. Raw short-read paired-ends WGS was performed at Complete Whole genome sequencing (WGS) of the BELNEU consortium, Belgium. uninformed consent forms were approved by the Ethics Committee of the University Hospital Antwerp and the respective hospitals of the members of the University of Antwerp, and the Memory Clinic of the University Hospitals Leuven, Gasthuisberg, Leuven, Belgium.

Ethical assurances

Ascertainment of the family 1270 relatives and the Dutch patients was performed in the Netherlands using a study protocol approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam. All Belgian participants and/or their legal guardian signed a written informed consent form for their participation in the clinical and genetic studies. The clinical and genetic study protocols and the informed consent forms were approved by the Ethics Committee of the University Hospital Antwerp and the University of Antwerp, and the respective hospitals of the members of the BELNEU consortium, Belgium.

Whole genome sequencing (WGS)

Short-read paired-ends WGS was performed at Complete Genomics Inc. (Mountain View, CA USA) [18]. Raw sequencing reads were aligned to the reference genome (National Center for Biotechnology Information (NCBI) build 36 (hg18). Sequence alignment and variant calling were performed by Complete Genomics Inc. while data annotation and analysis were performed with the GenomeComb package [68]. Good quality variants were selected as previously described [68]. Additionally, novel or rare (minor allele frequency (MAF) < 1% in the 1000 Genome Project [2] and/or in our in-house database of WGS data of unrelated individuals (n = 82)), heterozygous variants shared among the four WGS patients were investigated. Sequenom MassARRAY® (Agena Bioscience, CA, USA) and Sanger sequencing (BigDye Terminator Cycle Sequencing kit v3.1; analysis on an ABI 3730 DNA Analyzer, both Thermo Fisher Scientific, MA, USA) were used for variants validation. Structural variations (SV) were called by Complete Genomics and/or using a SV detection tool integrated in GenomeComb [68] version 0.90.0 (available at http://genomecomb.sourceforge.net). This tool scans the genome sequencing data for groups of read pairs that map at a distance that is markedly different from the expected insert size. Under normal conditions, the distance between the two sequence reads of a mate pair is expected to be approximately 350 bp corresponding to the size of selected fragments during WGS library construction. For the detection of inversions, groups of discordant mate pairs must be present for which the reads map at a different distance than expected and in opposite orientation. The linkage region between markers D7S636 and D7S559 [65] was analyzed for both single nucleotide variants (SNVs) as well as SV. Additionally, the public genome data repository of Complete Genomics Inc. containing freely accessible WGS data of 69 individuals, 52 unrelated individuals (software version 1.10.0; http://www.complegetomics.com/public-data/) [18], as well as WGS data from 427 individuals (157 unrelated) sequenced by Complete Genomics Inc. (software version 2.2.0) and distributed by the 1000 genome project [2] were used for comparative purposes.

Long-read direct WGS was performed in-house on the PromethION sequencing platform (Oxford Nanopore Technologies (ONT), UK). Prior to library preparation, the DNA was sheared to 35 kb using the Megaruptor (Diagenode, BE) and size selected to a minimal length of 6 kb on the BluePippin (Sage Science, MA, USA) using a High-pass protocol and the S1 external marker on a 0.75% agarose gel (Sage Science, USA). The recommended SQK-LSK109 protocol for library preparation for PromethION (ONT) sequencing was followed with slight increases in all enzymatic incubation times and during elution. In short, DNA template damage and ends were repaired in a combined step using NEBNext FFPE DNA Repair Mix and NEBNext Ultra II ER/dAT Module (New England Biolabs, USA) followed by AMPureXP (Beckman Coulter, CA, USA) bead purification and ligation of platform-specific adapter sequences. The final library (100 fmol) was loaded on a PromethION flow cell with 8021 active pores at the start, following the default protocol for PromethION DNA sequencing. Base calling of the raw reads was performed using the ONT basecaller Guppy (v1.4.0) on the PromethION compute device. Run metrics were calculated and
visualized using NanoPack [12]. Reads were aligned to hg19 using ngmlr (v0.2.6) [70] using default parameters. Inversions were detected using npInv inversion caller [71]. The coverage was assessed using mosdepth [59]. Long-read WGS of ten unrelated individuals (eight dementia patients and two controls) generated in-house following the same pipeline was used for comparison purposes.

DNA local alignment analysis of the NCBI hg19 reference sequence of chromosome 7: 149,169,800–154,794,690 bp was performed using YASS [57].

Directional genomic hybridization (dGH™) analysis

Directional genomic hybridization (dGH™) [67] analysis of chromosome 7 was performed by KromaTiD, Inc. (Fort Collins, Colorado, http://www.kromatid.com/) using the dGH™ C7 Paint assay (D3P-HC710) on Epstein–Barr virus (EBV) immortalized lymphoblast cell lines fixed in metaphase after one replication cycle in the presence of Brd-U and Brd-C. Single-stranded sister chromatids were hybridized with high density directional probes. Inverted fragments inherently possess an opposite 5' → 3' orientation, resulting in a switch of fluorescent hybridization signal from one sister chromatid to the other.

Whole gene resequencing

Resequencing of the complete coding region (CDS) of DPP6 (NM_130797.3), including two alternatively spliced exon 1 (NM_001936.4 and NM_001039350.2) and intron/exon boundaries, was achieved using a custom-designed gene panel (Agilent Technologies, CA, USA) [26] combined with massive parallel sequencing (MPS) on a MiSeq® sequencer (Illumina®, San Diego, CA, USA). Read processing, alignment and variant calling were performed using GenomeComb [68]. The pipeline used fastq-mcf [1] for adapter clipping. Reads were then aligned using bwa [41]. Realignment in the neighborhood of indels was performed with GATK [15]. All positions with a coverage ≥5 were variant called using GATK [15]. At this initial stage positions with a coverage <5 or a score <30 were considered unsequenced. The resulting variant sets of different individuals were combined and annotated using GenomeComb [68]. Downstream data analysis was further performed with the same software [68]. Exon 1 of DPP6 isoform 1 (NM_130797.3), due to high GC content and genomic complexity, was sequenced upon PCR amplification with specific primers. PCR products were processed by direct Sanger sequencing as described earlier in the text. Sanger sequencing was also used for validation of the identified variants after gene panel sequencing assay, using exon-specific primer pairs (sequences available upon request).

Variants modeling

Prediction of deleteriousness of the nucleotide changes for the DPP6 variants was performed using Combined Annotation Dependent Depletion (CADD) version 1.3. The rescaled (PHRED) score is reported, which correlates with allelic diversity and variants pathogenicity [37]. The investigation of the effect of the amino acid changes on protein stability (difference in free Gibbs energy) and the interaction with functional residues (i.e., glycosylation sites) were performed with FoldX (http://foldx.crg.es/) and YASARA [39, 77]. This analysis was limited to the extracellular domain, because only the crystallographic structure of this protein domain is available. DPP6 protein data bank accession number: 1XFD.

DPP6 transmembrane protein stability assay

Gateway and In-Fusion cloning (both Invitrogen, Thermo Fisher Scientific, Waltham MA, USA) were used to generate the wild-type DPP6 pCR3 expression construct C-terminally fused with the HiBit sequence as well as a control construct including a PEST sequence in between DPP6 and the HiBit sequence (constructs available upon request). Mutations of interest were introduced in the wild-type DPP6 construct by site directed in vitro mutagenesis using KAPA HiFi Hot-Start DNA polymerase (Kapa Biosystems, MA, USA). A construct containing the sequence of the secreted Gaussia luciferase (GLuc) was used for normalization purposes. HEK293T cells were co-transfected with DPP6 and GLuc constructs (4:1 ratio) using XtremeGene9 (Sigma-Aldrich, MO, USA). Non-transfected cells were included as a control. Gaussia luciferase and Nano-Glo® HiBit luciferase signals were detected 48 h after transfection by the use of BioLux Gaussia Luciferase Assay Kit (New England Biolabs, MA, USA) and a Nano-Glo® HiBit Extracellular detection System (Promega Corporation, WI, USA), respectively. Both the GLuc and the Nano-Glo® HiBit luminescence signal (LUC) were measured following the manufacturer guidelines using a GloMax®96 microplate luminometer (Promega Corporation, WI, USA). The injector option was used for detection of GLuc activity. All construct concentrations and LUC signals were initially optimized to be within the linear range of detection. For data analysis, relative LUC activity was calculated as Nano-Glo® HiBit luciferase signals normalized to GLuc signals. Six independent experiments were performed and the resulting data per construct were pooled together for statistical analysis.
DPP6 mRNA and protein analyses

Semi-quantitative real-time PCR (qRT-PCR) was used to quantify brain expression levels of total DPP6. Expression levels were measured in the frontal cortex (BA10) of patient-specific variant carriers ($n=3$) and control individuals ($n=4$). Total RNA was isolated from fresh frozen brain tissue using the RiboPure™ kit followed by DNase treatment (both Ambion, Thermo Fisher Scientific, MA, USA). First-strand cDNA was synthetized utilizing the SuperScript® III First-Strand Synthesis System (Thermo Fisher Scientific, MA, USA) with random hexamer primers. qRT-PCR reactions were performed using the Fast SYBR® Green chemistry (Thermo Fisher Scientific, MA, USA) and run on the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, MA, USA). Quantification of mRNA levels was achieved with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA box binding protein (TBP) as internal control genes, all with moderate to high expression in neurons (https://www.proteinatlas.org/). Normalization to the reference genes was achieved through geometric averaging of the expression levels, as described by Vandesompele and colleagues [80]. Each sample was measured in triplicate and three independent experiments were performed.

Protein lysates from fresh frozen human brain tissue were made in modified radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 50 mM Tris–HCl; pH 8.0) supplemented with 1% sodium dodecyl sulfate (SDS), as described previously [38]. Protein preparations from mutation carriers and control individuals were separated on 4–12% NuPAGE® Bis-Tris gel (Thermo Fisher Scientific, MA, USA) and electroblotted onto a polyvinylidene difluoride membrane (PVDF, Hybond P; Amersham Biosciences, GE Healthcare Life Sciences, Buckinghamshire, UK). Membranes were probed with primary antibodies to detect DPP6 (1:10,000 AVIVA System Biology, ARP44867_P050, San Diego, CA, USA), Kv4.2 (1:1000 Abcam, (EP982Y) ab46797, Cambridge, UK) and GAPDH (1:20,000 GeneTex, GTX100118, Irvine, CA, USA). Immunodetection was performed with specific secondary antibodies conjugated with horseradish peroxidase (HRP) and the ECL-plus chemiluminescent detection system (GE Healthcare Life Sciences). Western blot results were visualized and quantified using the ImageQuant™ LAS4000 digital imaging system and the ImageQuant™ TL software (GE Healthcare Life Sciences, Buckinghamshire, UK). Independent protein preparations and western blot experiments were performed three times.

**Statistical analyses**

For the rare variants analysis, power calculation was performed within the SKAT framework in R (version 3.1.2). A logistic test for dichotomous traits with a SKAT-O model was used (target sequence: 3545 bp, causal variant percentage = 40%, protective variant percentage = 10%, Maximal OR = 5). Under these conditions both patient-control cohorts (> 1300 individuals) reached the power requirement level of 80% with a 0.05 significance level, since the total sample...
cohort required for this level is at least 925 individuals. Gene-based burden analysis of rare variants (minor allele frequency (MAF) < 1%) was performed with SKAT-O in R, version 3.1.2, using the SKAT package. Adjustment was applied because the sample size was < 2000. A two-sided $p$ value < 0.05 was considered significant. Odds ratio (OR) and 95% confidence interval (CI) were calculated using an allelic Fisher’s exact test. To investigate the effect of $DPP6$ rare variants on AAO, disease duration (DD) and family history (FH), we tested the patients with available records of the specific phenotypical traits. Patients carrying a $DPP6$ variant were compared to the non-carrier patients’ cohorts.
Patients with a pathogenic mutation in known causal genes were excluded. A two-tailed non-parametric Mann–Whitney U test was applied to test for an effect on AAO and DD in GraphPad Prism 6 (La Jolla, CA, USA). A $\chi^2$-test was applied to test for enrichment in familial load in carriers of DPP6 rare variants compared to non-carrier patients. For the mRNA expression studies, three qRT-PCR experiments were independently normalized as previously described [80] and the results were pooled. The non-parametric two-tailed Mann–Whitney U test was used to compare the expression of total DPP6 levels between patients, carriers of DPP6 variants and control individuals in GraphPad Prism 6 (La Jolla, CA, USA). A two-tailed $p$ value < 0.05 was considered significant. Western blot results were quantified using the ImageQuant™ TL software (GE Healthcare Life Sciences, Buckinghamshire, UK). Relative protein expression levels between mutation carriers and control individuals were analyzed using a two-tailed Student’s $t$ test. A $p$ value < 0.05 was considered significant. For the protein stability assay, the normalized LUC values of six experiments were pooled per variant and compared to the wild-type levels using a non-parametric Kruskal–Wallis test in combination with a post hoc Dunn’s test in GraphPad Prism 6 (La Jolla, CA, USA).

Results

Family 1270

Earlier, the overall clinical picture of dementia in the multi-generational family 1270 was reported to be compatible with AD (Fig. 1, Fig. S1) [65, 76]. However, at the time of diagnosis of the index patient and of the affected relatives, cerebrospinal fluid (CSF) biomarkers and amyloid brain imaging were not available. Also, there was no autopsied brain available to obtain a definite diagnosis of dementia subtype based on neuropathology. Previous genetic analyses in the linked locus 7q36 in family 1270 [65] identified seven variants in five different genes: ATP-binding cassette subfamily F, member 2 (ABCF2); N-acetylgalactosaminyltransferase 11 (GALNT11); DPP6; PAX transcription activation domain interacting protein (PAXIP1) and Engrailed 2 (EN2), which were segregating on the disease haplotype in family 1270 [65]. Most of these variants were synonymous and only the PAXIP1 p.A660 variant was absent from control individuals [65]. Current public genetic data (e.g., ExAc) [40], showed different nucleotide changes in PAXIP1, leading to the same silent variant (p.A660), making it unlikely that the PAXIP1 variant has a deleterious effect. Further, we found no evidence for aberrant splicing of PAXIP1 in lymphoblast cells of patients [65].

WGS analysis of single nucleotide variants in the 7q36 locus

In family 1270 (Fig. S1), we performed paired-end WGS on high molecular weight genomic DNA of four patients in three different sibships of generation III, i.e., patients III-12, III-38, III-41, and III-48 (Fig. 1). On average 95.5% of the genome sequence had reliable diploid calls in all four patients. The genetic variants were annotated and good quality [68], rare or heterozygous non-coding variants (<1% in 1000 Genome Project) [2], that were shared between the four patients, were further investigated. In the linked locus, between STR markers D7S636 and D7S559 [65], variant selection retained 79 non-coding variants. Validation and segregation analysis in family 1270 retained 38 non-coding variants that co-segregated in family 1270 on the disease haplotype. Genotyping of the variants in control individuals, showed that four of the variants were unique to family 1270 (Fig. 1). Variant 1 (chr7:g.153577081 A>G), variant 2 (chr7:g.153737600 C>T; rs567013292), and variant 3 (chr7:g.153744958 G>T) are all located in intron 1 of DPP6 (Figs. 1, 2). Variant 4 (chr7:g.155325040 G>C) mapped in an intergenic region, 27.3 kb distal of the closest gene SHH (Fig. 2), which is located within the linked locus. 908 kb downstream of DPP6. None of the four variants had a high disruptive potential based on the ENCODE [69] annotation.

To exclude that coding variants outside the linked locus were causing the disease, we extracted the exome from the WGS data and analyzed the presence of rare and/or novel coding non-synonymous variants. Heterozygous variants, shared by the four patients, were selected (UTRs, synonymous and non-synonymous), and filtered based on quality [68] and frequency similar as for the non-coding variants. Only the rare/novel (<1% in 1000 Genome Project) [2] variants in known protein coding genes, that predicted to impact...
the protein sequence (non-synonymous), were validated. This selection generated nine non-synonymous variants (Table S1), but none co-segregated with disease in family 1270.

**An inversion of 4 Mb disrupting the DPP6 sequence was detected in the linked 7q36 locus**

Investigation of the variants within the linked region evidenced the presence of inverted low copy repeats (LCRs) in the 7q36 locus. In silico simulation of amplification of primers, designed to validate these variants, showed PCR products that aligned in opposite orientations at two loci on chromosome 7, separated from each other by about 4 Mb. Local alignment of the DNA regions between 149,169,800 and 154,794,690 bp on chromosome 7 (hg19), confirmed the presence of inverted paralogous low copy repeats (IP-LCRs) with > 98% sequence homology and located ca. 4 Mb apart (Fig. 2). Bioinformatics analysis of the WGS data of the four patients identified a paracentric (sub-telomeric) inversion in the q-arm of chromosome 7 of about 4 Mb (inv(7)) (Fig. S2) with the inversion breakpoints located within the IP-LCRs regions (Fig. 2). The distal breakpoint is located within the linked locus at 7q36.2, in intron 1 of
dipeptidyl-peptidase 6 (DPP6), and it is predicted to disrupt the coding sequence of the gene (Fig. 2). The proximal inversion breakpoint is located outside the linked locus, in an intergenic region between proximally ATP6V0E2 at 122 kb and distally ACTR3C at 243 kb (Fig. 2). These results were confirmed by direct long-read WGS on PromethION performed for patient III-48. The sequence run generated 21.2 Gbase of data resulting in a median coverage of 6×. The npInv inversion caller [71] independently identified an inversion at chr7:149,704,610–153,786,893 confirming the previous findings. The inversion at 7q36.2, with one breakpoint in intron 1 of DPP6, was not detected in publicly available WGS data of 209 unrelated individuals, all sequenced with the same short-read sequencing technology. Also, the inversion was not present in long-read WGS of ten unrelated dementia patients and control persons, generated by us using the Oxford Nanopore PromethION sequencer.

**In vitro visualization of the 7q36 inversion**

We successfully visualized the inv(7) in lymphoblast cells of patient III-48 (Fig. 3) using directional genomic hybridization (dGH™) [66]. The inverted chromosomal fragments inherently possess an opposite 5' → 3' orientation, resulting in a jump of fluorescent hybridization signal from one sister chromatid to the other. We also confirmed that this structural
variation was absent in two healthy relatives who did not carry the disease haplotype (III-23 and III-39).

Fig. 4 RNA and protein expression levels of DPP6 missense variants. a Scatter plot of the DPP6 mRNA expression levels in patients (n = 3) compared to control individuals (n = 4). Each circle (patients) or square (control individuals) represents a single measurement; the graph reports the mean ± standard error of the mean (s.e.m.). **p value = 0.0096. b DPP6 mRNA expression level results of three experiments for each of the variants (grey bars) compared to averaged data of four controls (black bar). c Western blot of DPP6 carriers and control individuals and e western blot of Kv4.2 in DPP6 variant carriers and control individuals. d, f Quantification of the expression levels of DPP6 (d) and Kv4.2 (f), obtained from pooling two independent protein preparations within the same western blot experiment. Quantifications are shown for each of the missense variants (grey bars) compared to control individuals (n = 3, black bar). The relative protein expression is reported as average ± standard deviation of three independent protein preparations and quantifications per sample *p < 0.05, **p < 0.005

Rare variants in DPP6 are associated with neurodegenerative dementia

To better understand the genetic contribution of DPP6 to NBD, we performed massive parallel gene resequencing of
Rare variants alter DPP6 and Kv4.2 expression levels in brain tissue of patients

Frozen autopsy brain was only available of three patients carrying a DPP6 missense variant. These patients had a probable clinical diagnosis of primary progressive aphasia (PPA, logopenic variant), FTD (behavior variant) plus Paget’s disease of the bone, and FTD plus ALS (bulbar type) (Table S4). The neuropathological diagnoses were AD (DR414), FTLD-TDP type D (DR40) and FTLD-ALS type B (DR1152) (Fig. S3). Patient DR40 also carried a causal VCP mutation, p.R159H (Table S4). For all three DPP6 variants p.P509R, p.R47L, and p.D596N, we observed significantly reduced mRNA levels (p = 0.0096, Fig. 4a, b), and a marked decrease in DPP6 protein expression levels in the p.P509R and p.R47L carriers (p = 0.03) (Fig. 4c, d). Moreover, the protein levels of the potassium channel Kv4.2, binding partner of DPP6, were also severely reduced (Fig. 4e, f).

Rare variants in the extracellular domain of DPP6 destabilize the protein and alter its membrane expression

In silico modeling of the missense variants located in the extracellular domain of DPP6, predicted a destabilizing effect, measured in positive values of free Gibbs energy, for 7/10 (70%) of the missense variants found in patients only (Table 1, Table S3, Fig. 5). The three variants (p.V220I, p.G269R and K570N), found in controls only, were all predicted to stabilize the protein (ΔΔG < −1). A similar stabilizing effect was detected for the variant p.A778T found in both patients and controls. Furthermore, investigation of the intramolecular interaction with functional residues showed that two missense variants (p.R322H and p.D569N), detected only in patients, could have a deleterious effect on protein glycosylation, either because of the conformational location nearby the canonical glycosylated residue, as p.R322H is in the vicinity of the glycosylation site N319, or because of the amino acid change itself (p.D569N), which could compete for the glycosylation with the closely localized glycosylation site N566 (Fig. S5). Similar to p.R322H, the variant p.K571Q, present only in patients, could compromise the glycosylation of residue N173 (Fig. S6).

Since DPP6 is known to localize on the plasma membrane, we monitored DPP6 stability as changes in DPP6 abundance on the plasma membrane due to folding properties or stability issues or retention in one of the organelles (e.g., endoplasmic reticulum) in the presence of missense variants. To this end, we generated C-terminally Nano-Glo® HiBiT tagged DPP6 constructs and compared wild-type against its missense variants in HEK293T cells. The wild-type construct fused with the PEST sequence, promoting an accelerated degradation, was used as an internal control and the p.Q230* construct was used as a negative control. Of the 14 modeled missense variants located in the extracellular domain of DPP6 (Table 1, Table S2, Fig. 5a), we observed a significant reduced expression (p value < 0.001, Fig. 5) on the plasma membrane for 5/10 (50%) of the variants found in patients only (Table 1, Fig. 5). Their CADD scores range from 15.06 for p.E208Q to 34 for p.R247H. The latter variant showed the most drastic reduction of the plasma membrane expression of DPP6 next to the p.Q230*, which was not expressed at all. A significant reduction was observed for one variant (p.G269R) found in controls only. No significant differences were recorded for the variants starting from amino acid position 570. Western blot analysis of the overall DPP6 protein expression showed that the detected differences in DPP6 plasma membrane expression levels were not due to direct changes in total protein abundance (Fig. S7). Moreover, immunofluorescence staining showed proper protein localization on the plasma membrane (Fig. S8).

Discussion

The 4 Mb inversion at 7q36 is disrupting DPP6 causing dementia in family 1270

Our family-based genetic and genomic investigation evidenced the presence of a 4 Mb paracentric inversion at 7q36, segregating on the disease haplotype and explaining the linkage in family 1270. This chromosomal rearrangement was detected by two independent sequencing technologies. Furthermore, the direct long-read sequencing on PromethION, mapped the two breakpoints on chr7:149,704,610–153,786,893. The inversion is likely triggered by the presence of inverted paralogous LCRs (IP-LCRs) with high (>98%) homology and located 4 Mb apart. This notion is supported both by our DNA local alignments and the data of a genome-wide IP-LCRs search,
Genetic association of DPP6 in dementia

To better understand the genetic contribution of DPP6 to NBD and to support haploinsufficiency as the mode of action, we re-sequenced the coding region of DPP6 and identified nonsense and frameshift variants, and several missense and short indels that were scattered over the whole DPP6 gene in patients. The p.Q230* and p.E79Gfs*9 variants, were found in a FTD and FTD-ALS patient who were 67 and 76 years at inclusion in the FTD cohort. These PTC variants likely lead to DPP6 haploinsufficiency through nonsense-mediated decay (NMD) of their mutated transcript. While we did not have brain tissue of the two PTC carriers, an independent study confirmed NMD of a DPP6 PTC variant with 41% reduction of DPP6 transcript levels in the cortex of a definite FTLD patient supporting the loss of DPP6 as the underlying biological mechanism [62].

Since we lacked biosamples to confirm transcript degradation under physiological conditions, mutation modeling of the p.Q230* PTC variant located in the extracellular domain of DPP6, supported the loss of DPP6 protein. In controls, the missense and indel variants were mainly clustering in the non-conserved intracellular protein domain (exon 1) while PTC variants were not observed. The carriers of DPP6 variants observed only in patients, had an average onset age of 62.9 (n = 12, range 44–77) and an inclusion age of 66.1 (n = 22, range 50–81) years, which is comparable to the ages and age range observed in family 1270, which is 66.8 ± 7.4 years (n = 13; range 47–77). Highly variable onset ages have been reported in patients and families with mutations leading to loss-of-protein, i.e., in GRN [10] in FTD and in ATP-binding cassette subfamily a member 7 gene (ABCA7) [74] in AD. These genes also show a wide spectrum of mutations having different effects on expression and found over a larger onset age range from early- to late-onset [8, 74]. The GRN and ABCA7 findings indicate that these mutations have different risk contributions to disease that can vary from high to low penetrance [56, 79] which could be valid also for DPP6 missense variants. Since the extracellular domain of DPP6 is highly structured, missense variants in this domain could affect the protein conformation, its function or the interaction with additional proteins. Additional studies are needed to further understand how these variants act. In our study, we did not identify a correlation between DPP6 rare variants and a specific phenotypic trait including age at onset, disease duration and family history. However, taken the small number of patients in our cohort and the rarity of DPP6 variants, the analysis was likely underpowered and additional studies will be needed. Furthermore, the SKAT-O analysis showed an enrichment of DPP6 rare variants in both AD and FTD patients, with a stronger significance level in FTD (p = 0.006), in which we also identified the PTC variant carriers (0.3%, 2/614). This is in line with an independent genome-wide association study on whole genome sequencing on TDP-43 (TAR
DNA binding protein 43) pathology [62]. Two common SNPs in intron 1 of DPP6 showed genome-wide significant association (rs4726389 \( p = 4.63 \times 10^{-8}, OR = 2.45 \) and rs118113626 \( p = 4.88 \times 10^{-08}, OR = 2.48 \) [62]. Moreover, one PTC carrier was identified amongst the FTLD-TDP43 patients and never in controls, thus matching our findings [62].

**Functional and expression analyses support DPP6 loss as disease mechanism**

Understanding the effect of missense variants, compared to PTC mutations, is not trivial. We used the Nano-Glo® HiBiT assay to further characterize the missense variants we identified in DPP6. We monitored the changes in DPP6 abundance on the plasma membrane, as in silico analysis predicted differences in protein stability due to alterations in folding properties in 7/10 variants found in patients only in the extracellular domain. The in vitro modeling showed that five variants (p.E208Q, p.R274H, p.R322H, p.H357R and p.509R) identified in patients only, destabilize the protein leading to a reduced level on the plasma membrane, suggesting a loss of function. This was also detected in one variant (p.G269R) found in a control person, suggesting that the missense variants might have a different risk contribution and that the mode of action of missense variants can involve different mechanisms, e.g., the protein function, interaction with other proteins not investigated by this assay. The predictions and the in vitro experiment did not completely overlap, stressing the need for in vitro validation in support of in silico prediction analyses. The in vitro assay correlates with the DPP6 protein levels measured in brain of two carriers of a missense variant. In fact, DR414, carrier of p.S509R, showed reduced DPP6 brain protein expression, in accordance to the protein destabilization detected in vitro. While in the brain tissue of DR1152, carrier of the p.D569N, there were no evident reductions, in agreement with the in vitro assay, suggesting a possible alternative mode of action of this specific variant, for example protein glycosylation. This is supported by the alterations detected in brain expression levels of the K,4,2 for this variant carrier. The fact that 50% of the patient-specific variants that we modeled in vitro, have a deleterious effect is a relevant consideration to make, because of the highly structured extracellular domain of DPP6 [72], which is crucial for the protein expression and function, with the different protein domains of DPP6 responsible for its protein localization [44]. In light of this, we suggest a loss of function effect also for these missense variants located in the extracellular domain.

In terms of protein function, DPP6 belongs to the dipeptidyl-peptidase protein family, but lacks protease activity because of a serine into aspartic acid change in the catalytic peptidase domain [72]. By binding, most likely, at the permeation and gating modules [35] of the potassium channel K,4,2, DPP6 enhances its expression and regulates its gating properties [35, 51, 52, 73] and it is known to control the dendritic excitability of the hippocampal neurons [73] and the neuronal plasticity [36]. DPP6 and K,4,2 directly interact in a multimeric protein complex, in which K,4,2 is additionally bound to the auxiliary potassium channel interacting proteins (KChIP) [33]. Reduced potassium and persistent enhanced sodium currents converge to produce neuronal hyperexcitability [81]. A recent study on Dpp6 knockout (KO) mice suggested a structural function in the formation of filopodia, the precursor of the dendritic spines, and in cellular stability through the binding to the extracellular matrix, thus directly affecting dendritic arborization, spine density and synaptic function [46]. Furthermore, DPP6 loss has been shown to determine memory and learning impairments in young Dpp6-KO mice [45]. Patients affected by anti-DPPX syndrome, with autoantibodies targeting DPP6 and causing reduced protein expression, have memory deficits and neuronal hyperexcitability, features that are improved when DPP6 expression levels are increased [27]. Taken together our data and these studies point toward a deleterious effect of DPP6 loss and support its contribution to dementia.

**Conclusions**

Our investigations show that the loss of DPP6 can occur on different levels: the genomic level, with the inversion disrupting the coding sequence; the genetic level with the identification of PTC and deleterious missense variants as well as the protein level, where different variants show a spectrum of alteration in the cellular surface expression. The genetic association with rare variants is an additional line of genetic evidence to link DPP6 to dementia. Our assays to model the missense variants suggest that not all variants identified are deleterious, like the variants located in the α/β hydrolase domain (e.g., p.A778T). This is not unexpected, knowing that widely accepted causal genes for dementia are known to harbor benign variants [9].

The involvement of DPP6 in diverse and independent cellular pathways including neurogenesis and neuronal excitability, could explain why loss of DPP6 was associated with autosomal dominant microcephaly with mental retardation [43] and other neurodevelopmental disorders, including Gilles de la Tourette syndrome (TS) [63] and autism spectrum disorders (ASD) [47]. Heterozygous loss of DPP6 may not represent a single cause of severe intellectual disability but it is likely a susceptibility factor to this phenotype [63]. Currently the link between neurodevelopment and
neurodegeneration is unclear, but parallels between the two mechanisms have been proposed [82].

Alterations in the homeostasis of neuronal firing [24] and early neuronal network dysfunctions [58, 84] are emerging concepts in neurodegenerative brain diseases. The results of our genomic, genetic, expression and modeling analyses, provide direct evidence to support the involvement of DPP6 loss in dementia, with loss of function variants (PTC, inversion) having a higher penetrance and disease impact and missense variants having a variable risk contributions to disease from high to low penetrance [56, 79]. Additional studies are needed to fully understand the role of these variants in the disease etiology. Our findings on DPP6, as a novel genetic factor in dementia, provide supportive evidence to the emerging concept that neuronal hyperexcitability and alteration of the homeostasis of neuronal firing represent a relevant disease mechanism warranting further investigation.

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