Amyloid precursor protein processing in human neurons with an allelic series of the PSEN1 intron 4 deletion mutation and total presenilin-1 knockout

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Mutations in presenilin-1 (PSEN1), encoding the catalytic subunit of the amyloid precursor protein-processing enzyme γ-secretase, cause familial Alzheimer’s disease. However, the mechanism of disease is yet to be fully understood and it remains contentious whether mutations exert their effects predominantly through gain or loss of function. To address this question, we generated an isogenic allelic series for the PSEN1 mutation intron 4 deletion; represented by control, heterozygous and homozygous mutant induced pluripotent stem cells in addition to a presenilin-1 knockout line. Induced pluripotent stem cell-derived cortical neurons reveal reduced, yet detectable amyloid-beta levels in the presenilin-1 knockout line, and a mutant gene dosage-dependent defect in amyloid precursor protein processing in PSEN1 intron 4 deletion lines, consistent with reduced processivity of γ-secretase. The different effects of presenilin-1 knockout and the PSEN1 intron 4 deletion mutation on amyloid precursor protein-C99 fragment accumulation, nicastrin maturation and amyloid-beta peptide generation support distinct consequences of familial Alzheimer’s disease-associated mutations and knockout of presenilin-1 on the function of γ-secretase.
Introduction

Amyloid precursor protein (APP) is cleaved by γ-secretase, the catalytic subunit of which consists of presenilin-1 (PSEN1) or presenilin-2 (PSEN2), to produce amyloid β (Aβ). Mutations in APP and PSEN1/2 that cause familial Alzheimer’s disease (fAD) are believed to alter this interaction, increasing the relative proportion of aggregation-prone Aβ species (Ryan et al., 2016), and forming the basis of the amyloid cascade hypothesis (Hardy and Allsop, 1991).

PSEN1 and PSEN2 are alternate catalytic subunits of γ-secretase, a tetrameric protein complex also containing nicastrin (NCSTN), PSEN enhancer (PEN2) and alternate subunits anterior pharynx 1a/b (APH1a/b; De Strooper, 2003). γ-Secretase serves as a membrane protease, cleaving numerous substrates (Haapasalo and Kovacs, 2011) that include the products of β-secretase and α-secretase cleavage of APP (C99 and C83, respectively). Cleavage of C99 by γ-secretase occurs through an initial endopeptidase-like activity followed by subsequent carboxypeptidase-like cleavages to generate shorter Aβ fragments (Takami et al., 2009; Matsumura et al., 2014). In addition, γ-secretase-independent activities for PSEN1 have been described, such as a chaperone activity crucial for the glycosylation and maturation of NCSTN (Leem et al., 2002).

PSEN1 mutations have been shown to consistently reduce the carboxypeptidase-like activity of γ-secretase, leading to the accumulation of longer, more amyloidogenic Aβ species, such as Aβ42 and Aβ43 (Chávez-Gutiérrez et al., 2012; Szaruga et al., 2015; Arber et al., 2019). This can be evidenced as an increased Aβ42:38 ratio (Takami et al., 2009; Matsumura et al., 2014). The PSEN1 intron 4 deletion mutation (L113_I114insT; hereafter referred to as int4del) describes the deletion of a guanine nucleotide in the splice donor region of PSEN1 after exon 4 leading to three alternative transcripts; one coding a full-length protein with an insertion of an additional threonine in the PSEN1 protein, and two shorter transcripts with premature stop codons (De Jonghe et al., 1999). The long transcript was shown to be responsible for elevated Aβ42 generation (De Jonghe et al., 1999). We and others have previously shown that this mutation increases the Aβ42:38 ratio in patient-derived iPSC-neurons and potentially reduces overall γ-secretase activity (Moore et al., 2015; Arber et al., 2019).

There has been contention over the question of whether mutant PSEN1 alleles confer predominantly gain or loss of function (Veugelen et al., 2016; Xia et al., 2016). In order to further investigate the molecular mechanisms of the PSEN1 int4del mutation in a human neuronal system, we used CRISPR/Cas9 gene editing to produce an isogenic allelic series from patient-derived iPSCs. The
series is represented by isogenic control (wild type) cells, heterozygous and homozygous mutation-bearing cells, as well as PSEN1 knockout cells. We find that iPSC-derived cortical neurons maintain Aβ generation in PSEN1 knockout cells and display a mutant gene dosage-dependent phenotype on APP/Aβ processing and Aβ42 generation. The data support distinct effects of fAD-associated mutations and PSEN1 protein knockout.

Materials and methods

Cell culture

The acquisition of patient fibroblasts for the generation of iPSC was approved by the National Hospital for Neurology and Neurosurgery and Institute of Neurology Joint Research Ethics Committee (Study Title: Induced pluripotent stem cells derived from patients with familial Alzheimer’s disease and other dementias as novel cell models for neurodegeneration, Reference: 09/H0716/64).

All reagents are from ThermoFisher unless specified. PSEN1 int4del iPSCs were obtained from StemBanc and cultured in Essential 8 media on Geltrex substrate and passaged using 0.5 mM EDTA, with the exception of cultured in Essential 8 media on Geltrex substrate and...
Western blotting

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Roche). Lysates were denatured in NuPage LDS buffer and loaded onto 4–12% precast polyacrylamide gels in MES running buffer (NuPage/ThermoFisher). Proteins were transferred to a nitrocellulose membrane, blocked in 3% bovine serum albumin and blotted using antibodies in Table 2. Images were taken and analysed on an Odyssey Fc (LiCor Biosciences).

**Aβ-Electrochemiluminescence**

Forty-eight hours conditioned media were collected from neuronal cultures and centrifuged to remove cell debris. Aβ42, Aβ40 and Aβ38 were quantified simultaneously using the Meso Scale Discovery V-Plex Aβ peptide panel kit (6E10) by electrochemiluminescence. Samples were diluted 1:1 with diluent 35 and measurements were made on the MSD Sector 6000. Aβ concentrations in the cell media were normalized to cell pellet protein concentration, measured using BioRad BCA assay.

**Statistical analysis**

Data analysis was performed in Microsoft Excel and GraphPad Prism 7. Samples were compared via one-way ANOVA with subsequent post hoc Tukey’s multiple comparisons test (*P > 0.05, **P > 0.01, ***P > 0.001, ****P > 0.0001). Error bars on histograms show ± standard deviation of the mean and independent experimental replicates are shown via numbers within histograms.

**Data availability**

The authors confirm that all the data supporting the findings of this study are available within the article and readily available upon request. For ANOVA analyses, exact P-values, F-values and degrees of freedom are presented in the Supplementary material.

**Results**

**Generation of PSEN1 int4del allelic series**

CRISPR/Cas9 gene editing was used to generate an isogenic series of iPSC lines from a patient-derived PSEN1...
int4del iPSC line (Fig. 1). In order to generate an allelic series, a PAM site 6 base pairs upstream of the mutation was selected, recognizing both mutant and wild-type alleles. This enables both homology-directed repair from the ssODN (single-stranded oligodeoxynucleotide) and template-free repair of the pathogenic variant in the same CRISPR/Cas9 transfection (Shen et al., 2018). For increased efficiency of homology-directed DNA repair, the ssODN template was modified to contain phosphorothioate moieties (Renaud et al., 2016).

Following an initial screen of 800 iPSC colonies by RFLP (see Materials and methods section), Sanger sequencing was used to confirm the generation of; (i) an isogenic control cell line, (ii) an unedited line, (iii) a homozygous int4del line and (iv) a PSEN1 knockout line (Fig. 2A). The knockout line was a compound heterozygous, which contained a 4 and a 25 base pair deletion; each leading to a reading frame shift (Supplementary Fig. 1). The allelic series was screened and found to be free from off-target nucleotide changes at five most likely genomic sites (see Materials and methods section) and pluripotency was confirmed via the expression of OCT4 and SSEA4 (Fig. 2B). Karyotype stability was tested and no significant aberrations were found (Supplementary Fig. 2).

iPSCs were subjected to cortical differentiation, generating the cell type affected by fAD (Shi et al., 2012). All lines generated neurons with a similar efficiency, as evidenced by the expression of the deep-layer cortical marker TBR1 by immunocytochemistry and the population expression level of TUBB3, TBR1 and CTIP2 by qPCR (Fig. 2C and D). Finally, to confirm the mutation status of the iPSC-derived neurons, cDNA was analysed by PCR to depict aberrant splicing of PSEN1 in mutation-bearing neurons (Fig. 2E; Tysoe et al., 1998). In addition to the full-length L113_I114insT encoding transcript (374 bp), heterozygous and homozygous PSEN1 int4del lines show evidence of one short transcript produced by aberrant splicing (193 bp).

**Figure 1 Scheme for the gene editing of patient-derived PSEN1 int4del iPSCs. (A) Strategy for the generation of isogenic cells using CRISPR/Cas9-editing of an iPSC line from an individual carrying the PSEN1 int4del mutation. (B) Genomic positioning of the editing site, showing ssODN repair arm (purple), mutation site (purple) and sgRNA (green) with PAM site (red). sgRNA (single guide RNA, for CRISPR/Cas9 targeting); RFLP (used for screening); ssODN (single-stranded oligodeoxynucleotide, for homology-directed repair).**
Interestingly, PSEN2 showed similar expression in all cell lines, suggesting that loss of PSEN1 does not result in compensation by up-regulation of this alternate β-secretase subunit. Expression of APP and BACE1 was not significantly altered based on mutation status.

At the protein level, the expected absence of PSEN1 protein was confirmed in the knockout line (using antibodies that recognize the N- or C-terminus of PSEN1; Fig. 3B). The PSEN1 knockout line also showed a reduced maturation of NCSTN, evidenced as a relative lack of the larger band that represents the glycosylated protein (Leem et al., 2002). This phenotype was not seen in lines containing the mutation (Fig. 3C). Further characterization of cortical differentiation was performed using qPCR analysis 100 days post-neural induction to assess expression of neuronal marker TUBB3 and cortical layer markers TBR1 and CTIP2. Numbers within histogram represent the number of independent neural inductions. (E) PCR analysis of PSEN1 splicing in cDNA from day 100 neurons using primers recognizing exons 3 and 5 of PSEN1 mRNA. The full-length transcript is depicted at 374 bp and one short transcript caused by aberrant splicing is evident at 193 bp in mutation-bearing neurons (we do not see evidence of a second aberrantly spliced band at 111 bp).

**Aβ is produced in knockout cells and disease-associated processing defects are dependent on mutant gene dosage**

To investigate the molecular effects of the PSEN1 int4del mutation, we analysed the Aβ profiles of the neuronalcondition media 100 days post neural induction. PSEN1 knockout cells produced less Aβ compared with other lines, reaching significance for Aβ38 (Fig. 4A–C). The levels of Aβ remained within detection limits, which are in contrast to neurons treated with the γ-secretase inhibitor DAPT, where Aβ was at or below the detection threshold. Expression of four Aβ degrading enzymes remains consistent between genotypes (Supplementary Fig. 3), suggesting that the detection of Aβ is not a result of reduced degradation.

Knockout cells produced a non-significant increase in the Aβ42:40 and Aβ42:38 ratios and a significantly reduced Aβ38:40 ratio when compared with isogenic

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**Figure 2 Characterization of gene-edited iPSC and neurons.** (A) Sanger sequencing was used to confirm the generation of a PSEN1 knockout (i), a corrected wild-type PSEN1 line (ii), an unedited heterozygous mutant line (iii) and a homozygous PSEN1 int4del mutation line (iv). (B) Immunocytochemistry was performed on all iPSC lines following CRISPR/Cas9 editing to confirm pluripotency with the pluripotency markers OCT4 (red) and SSEA4 (green). Scale bar 100 μm. (C) Successful differentiation of iPSC into neurons was characterized 50 days post-induction by immunocytochemistry for the neuronal marker TUJ1 (red) and deep-layer cortical neuronal marker TBR1 (green). Scale bar 25 μm. (D) Further characterization of cortical differentiation was performed using qPCR analysis 100 days post-neural induction to assess expression of neuronal marker TUBB3 and cortical layer markers TBR1 and CTIP2. Numbers within histogram represent the number of independent neural inductions. (E) PCR analysis of PSEN1 splicing in cDNA from day 100 neurons using primers recognizing exons 3 and 5 of PSEN1 mRNA. The full-length transcript is depicted at 374 bp and one short transcript caused by aberrant splicing is evident at 193 bp in mutation-bearing neurons (we do not see evidence of a second aberrantly spliced band at 111 bp).
control cells (Fig. 4D–F). These changes are analogous to the changes witnessed between the wild type and the heterozygous int4del line.

The heterozygous and homozygous int4del lines showed increased total levels of Aβ42 and reduced production of Aβ38 in a gene dosage-dependent manner (Fig. 4A–C). This corresponds to similar stepwise mutant gene dosage-dependent changes to Aβ42:40, Aβ42:38 and Aβ38:40 (Fig. 4D–F). In each instance, the homozygous line was significantly different from the patient-derived heterozygous line.

Discussion

We successfully generated an isogenic allelic series of the PSEN1 mutation int4del with which to investigate the molecular mechanisms of fAD mutation-dependent effects on APP processing. We found that PSEN1 knockout cells produce low levels of Aβ and that PSEN1 int4del heterozygous and homozygous cells produce a stepwise increase in longer, disease-associated Aβ peptides.

It is intriguing that PSEN1 knockout neurons produce small amounts of Aβ. This is in contrast to γ-secretase-inhibited cells where Aβ species are often below the detection limit but in agreement with the detection of around one-fifth of total Aβ levels in mouse PSEN1 knockout primary neurons (De Strooper et al., 1998). It is noteworthy that Aβ is barely detectable after γ-secretase administration to patients during clinical trials (Gillman et al., 2010). We provide evidence for no compensatory up-regulation of PSEN2 at either the transcriptional or protein level, which could potentially be due to alternate subcellular compartmentalization (Sannerud et al., 2016). These data suggest that PSEN2 may produce low levels of Aβ in neurons. Alternatively, the lack of compensation by PSEN2 in PSEN1 knockout neurons, taken together with the reduced functional γ-secretase
pool, seen via NCSTN immaturity, promotes the hypothesis that alternative Aβ-generating enzymes are contributing to Aβ production in PSEN1 knockout human neurons. This hypothesis is reinforced as Aβ is detectable in PSEN1 and PSEN2 double knockout mice (Wilson et al., 2002). It should be noted that in non-neuronal cells, knockout of either PSEN1 or PSEN2 does not alter total Aβ generation, whereas double knockout drastically reduces total Aβ (Lessard et al., 2019). Candidates for C-terminal Aβ degrading activity include matrix metalloproteinase 2 or 9 (MMP2/9; Hernandez-Guillamon et al., 2015) and cathepsin B (Mueller-Steiner et al., 2006).

The finding that PSEN1 knockout cells have a relative increase in Aβ42 compared with Aβ38 means that variability in PSEN1 expression levels could contribute to altered Aβ profiles in Alzheimer’s disease.

It is important that int4del homozygous cells display an additional, stepwise increase in disease-associated Aβ profiles compared with the patient-derived lines. This equates to a linear increase in total Aβ42 production. Our data demonstrate a mutant gene dosage-dependent effect in Aβ42 generation reinforcing similar findings with the PSEN1 exon 9 mutation (Woodruff et al., 2013), the PSEN1 M146I mutation (Paquet et al., 2016) and recently 7 fAD mutations investigated by genome editing (Kwart et al., 2019). The PSEN1 knockout neurons and mutation-bearing neurons show different phenotypes with respect to quantitative production of Aβ and, taken together with the dissimilar effects on C99 accumulation and NCSTN maturation, these data argue against a simple loss-of-function mechanism for PSEN1 mutations. In agreement with these findings and in contrast to fAD-causing mutations, PSEN1 loss-of-function mutations have been found to cause acne inversa rather than dementia (Wang et al., 2010).

The fact that PSEN1 int4del homozygosity does not lead to altered NCSTN maturation, suggests that the effect of the mutation acts on γ-secretase as a whole and
not on the activity of PSEN1 itself. This is reinforced by recent crystal structure findings (Yang et al., 2019; Zhou et al., 2019) and mechanistic studies (Szaruga et al., 2017; Petit et al., 2019) whereby FAD mutations appear to destabilize substrate to holo-enzyme complex interaction to release longer Aβ fragments before complete enzymatic processing, rather than altering the PSEN1 subunit activity.

The fact that APP protein is significantly increased in the isogenic control compared with the parental PSEN1 intr4del heterozygous line is intriguing. In our previous work, total APP is not significantly altered in the patient-derived line versus unrelated controls (Arber et al., 2019). We believe the slight increase in APP total levels in the corrected line may directly relate to the correction of the mutation.

In conclusion, data from this isogenic human neuronal allelic series reinforce the findings that FAD-associated mutations in PSEN1 lead to accumulation of Aβ by reducing the processivity of APP by γ-secretase. Mutations reduce carboxypeptidase-like activity, releasing longer amyloidogenic Aβ peptides in a gene dose-dependent manner. PSEN1 knockout cells generate Aβ and also show distinct substrate processing alterations from intr4del homozygous cells, potentially separating γ-secretase-dependent and independent functions of PSEN1 and arguing against a simple loss-of-function mechanism. These findings support a destabilization of γ-secretase-substrate interaction by the mutation; information that is valuable for the design of novel therapeutics.

Supplementary material

Supplementary material is available at Brain Communications online.

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Competing interests

The authors report no competing interests.

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