



Familial Alzheimer's disease patient-derived neurons reveal distinct mutation-specific effects on amyloid beta

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Abstract

Familial Alzheimer's disease (fAD) mutations alter amyloid precursor protein (APP) cleavage by γ -secretase, increasing the proportion of longer amyloidogenic amyloid- β (A β) peptides. Using five control induced pluripotent stem cell (iPSC) lines and seven iPSC lines generated from fAD patients, we investigated the effects of mutations on the A β secretome in human neurons generated in 2D and 3D. We also analysed matched CSF, post-mortem brain tissue, and iPSCs from the same participant with the *APP* V717I mutation. All fAD mutation lines demonstrated an increased A β 42:40 ratio relative to controls, yet displayed varied signatures for A β 43, A β 38, and short A β fragments. We propose four qualitatively distinct mechanisms behind raised A β 42:40. (1) *APP* V717I mutations alter γ -secretase cleavage site preference. Whereas, distinct presenilin 1 (*PSEN1*) mutations lead to either (2) reduced γ -secretase activity, (3) altered protein stability or (4) reduced *PSEN1* maturation, all culminating in reduced γ -secretase carboxypeptidase-like activity. These data support A β mechanistic tenets in a human physiological model and substantiate iPSC-neurons for modelling fAD.

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Introduction

Familial Alzheimer's disease (fAD) describes a hereditary (high penetrance, autosomal dominant) subgroup of AD that represents less than 1% of all AD cases. Nevertheless, the pathological hallmarks of extracellular amyloid beta (A β) enriched plaques and intracellular neurofibrillary tangles of hyper-phosphorylated tau are shared between fAD and sporadic AD (sAD). fAD-causing mutations in the genes coding for amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) affect the production of A β in the central nervous system (CNS), implicating altered APP cleavage and processing in the AD disease mechanism [1]. Study of these mutations offers a powerful way of interrogating key underlying disease mechanisms for all forms of AD [2].

A β peptides are produced via sequential proteolytic cleavage of APP by β -secretase and then γ -secretase activity [3] (Fig. 1a). Neither of the enzymes that perform this activity (BACE1 and γ -secretase respectively) are limited to proteolysis at a set residue of the APP sequence, but have multiple potential cleavage sites that in turn produce A β peptides of different lengths. BACE1 may cleave APP at Asp1 (of the A β domain), or Glu11, producing the classic or

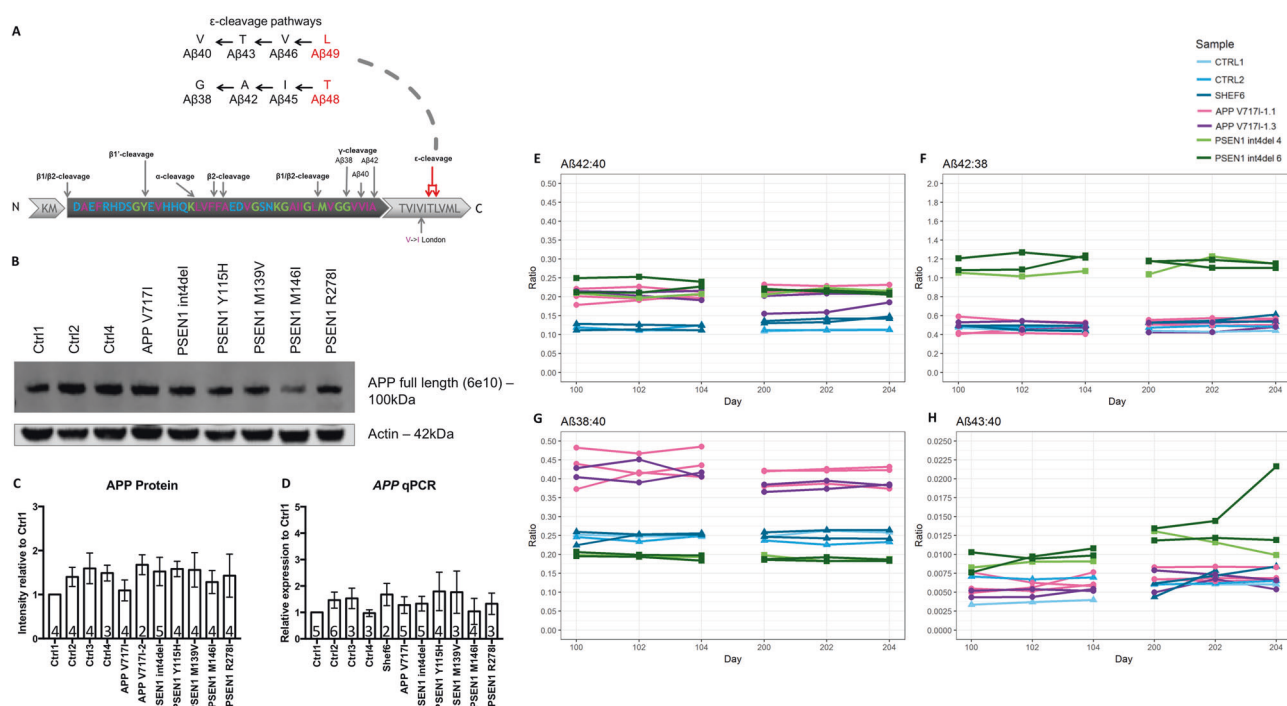


Fig. 1 Ratios act as internal normalisers for relative A β peptide abundance in conditioned media from stem cell models of AD. **a** The A β domain of APP, highlighting the canonical cleavage sites of α -, β -, and γ -secretase within the A β peptide sequence itself, as well as the proposed pathways of carboxypeptidase-like activity following alternative ϵ -cleavage. Letters coloured blue, pink, and green indicate amino acids with hydrophilic, hydrophobic, and amphipathic properties, respectively. **b** Western blotting of full-length APP in iPSC-derived neuronal lysates and **c** quantification of APP western blot band intensities. **d** qPCR expression analysis of APP in iPSC-derived

neurons. Replicates are shown within histogram bars and error bars represent SEM. **e–h** Normalisation of A β peptides measured in cell media using ratios **e** A β 42:40, **f** A β 42:38, **g** A β 38:40 and **h** A β 43:40. **e–h** Display an average percentage coefficient of variance (%CV) < 4% over 6 days and < 7% over 100 days for ratios. For **h** A β 43:40 average %CV was 7.1% over 6 days and 20.7% over 100 days. Data is based on multiple independent inductions per line, specifically APP V717I-1 clone 1 ($n = 3$), APP V717I-1 clone 3 ($n = 2$), PSEN1 int4del clone 4 ($n = 1$), PSEN1 int4del clone 6 ($n = 2$), Ctrl1 ($n = 1$), Ctrl 2 ($n = 1$) and Shef6 ($n = 2$)

N-terminally truncated form of A β , respectively. γ -Secretase performs an initial endopeptidase-like ϵ -cleavage at Leu49 or Thr48, and then continues to make carboxypeptidase-like cleavages which produce shorter fragments [4]. As A β peptides can be truncated both N-terminally and C-terminally, we will hereafter use the convention of referring to peptides cleaved at the canonical N-terminal Asp1 amino acid by the C-terminal truncation (e.g. A β 1–42 as A β 42), while peptides with N-terminal truncations will be directly specified.

The tripeptide hypothesis proposes that γ -secretase cleavage activity occurs in a stepwise manner, every three residues, producing two alternative peptide production pathways: A β 49 > 46 > 43 > 40 and A β 48 > 45 > 42 > 38 [4–6] (Fig. 1a). Many questions still surround this hypothesis, including where A β 39/38/37 and other detectable peptides fit within this model, how rigid these pathways are [7], and whether there may be others [6].

Different fAD-associated mutations are thought to have qualitatively different effects on A β cleavage and processing [8]. APP mutations are proposed to favour processing through the A β 48 pathway, whereas PSEN1/2 mutations are

thought to reduce carboxypeptidase-like activity and lead to an accumulation of longer A β fragments [8]. The A β 42:38 ratio is a potential readout of γ -secretase carboxypeptidase-like cleavage efficiency [9] and the A β 38:40 has been used to compare the theorised A β 49 versus A β 48 dependent pathways [5]. The A β 42:40 ratio has been shown to effectively distinguish fAD and sAD from healthy controls in cerebrospinal fluid (CSF) samples, improving diagnostic accuracy and prediction of AD [10–15]. However, few studies have explored these dynamics in human neurons and at endogenous expression levels.

Induced pluripotent stem cell (iPSC) models are a powerful tool for exploring APP processing in tissue specific cells from individuals with fAD-causing mutations [16]. Yagi et al. found increased levels of secreted A β 42 in neurons with the PSEN1 A246E and PSEN2 N141I mutations [17]. Further work on neurons bearing pathogenic PSEN1 mutations has shown an increased ratio of A β 42:40 [18–23]. Similarly, iPSC-derived neurons with an APP V717I genotype show a raised A β 42:40 ratio [21] as well as increases in A β 42 and A β 38 [24]. APP duplication iPSCs also exhibit high levels of A β 40 [25]. Finally, 3D cerebral organoids provide a

potentially valuable additional model, for example in modelling CSF due to the presence of choroid plexus tissue [26], and early reports suggest that increased amyloid aggregation in 3D cultures [27], as well as an ability to identify candidate proteins involved in AD pathogenesis [28]. However, a comprehensive analysis of the full spectrum of A β peptide production across multiple different fAD mutations to explore the mechanisms proposed by Chávez-Gutiérrez et al. [8] has yet to be performed.

The present study examined the full spectrum of secreted A β peptide ratios in both 2D cortical neurons and 3D cerebral organoids generated from seven fAD patient-derived pluripotent stem cell lines. We present the first analysis of A β 43 in human neurons, and our findings highlight multiple qualitatively distinct fAD mutation-specific effects on APP processing. This study validates the use of in vitro iPSC-derived neuronal models, further develops understanding of APP processing, and provides a basis for stratifying mutation effects with regards to disease modelling and drug screening readouts in heterogeneous patient cohorts.

Materials and methods

Cell culture

Fibroblasts were cultured as previously described [29]. Ethical permission was obtained from the National Hospital for Neurology and Neurosurgery and the Institute of Neurology Joint Research Ethics Committee (09/H0716/64) and informed consent was obtained for all samples. Episomal reprogramming was performed as described by Okita et al. [30], using plasmids #27077, #27078, and #27080 which were obtained from Addgene. Fibroblasts were nucleofected using the Lonza P2 Nucleofection kit (Amaxa) as per manufacturer's instructions. Re-programmed cells were grown on MEFs (ATCC) and nascent iPSC colonies were picked and transferred into feeder-free conditions (essential eight growth media and geltrex matrix (Thermo Fisher)) for further expansion. iPSCs were routinely passaged using ethylenediaminetetraacetic acid (EDTA). Karyotype screens and G-bands analysis were performed on newly generated lines (The Doctors Laboratory, London, UK). Lines were routinely screened for the absence of mycoplasma contamination using the MycoAlert assay (Lonza).

Differentiation to cortical neurons was performed as described in Shi et al. [31], and all reagents were purchased from Thermo Fisher Scientific unless specified. Briefly, iPSCs were grown to 100% confluence and media were changed to neural induction media (N2B27 containing 10 μ M SB431542 (Tocris) and 1 μ M dorsomorphin (Tocris)). N2B27 media consist of a 1:1 mixture of Dulbecco's modified eagle medium F12 (DMEM-F12) and

Neurobasal supplemented with 0.5 \times N2, 0.5 \times B27, 0.5 \times non-essential amino acids, 1 mM L-glutamine, 25U pen/strep, 10 μ M β -mercaptoethanol and 25U insulin. At days 10 and 18, precursors were passaged using dispase and plated in laminin-coated wells (Sigma) in N2B27 media. The final passage was performed at day 35 using accutase, plated at a final density of 50,000 cells per cm² and maintained in N2B27 media until the required timepoint.

Cerebral organoids were produced following the protocol described by Lancaster et al. [26]. Briefly, iPSCs were dissociated to single cells using EDTA followed by accutase, and 9000 cells were seeded in ultra-low attachment U-bottomed 96 well plates to generate embryoid bodies (EBs). EBs were grown in DMEM-F12 supplemented with 20% knockout serum replacement, 3% embryonic stem cell (ESC) quality foetal bovine serum, 1 \times non-essential amino acids and 3.5 μ M β -mercaptoethanol supplemented from day 0 to 4 with rho-associated protein kinase (ROCK) inhibitor at 50 μ M (Y27632 Millipore) and 4 ng/ml basic fibroblast growth factor (FGF)2 (Peprotech). On day 6, the media were changed to neural induction media containing DMEM-F12 1 \times N2 supplement, 1 \times non-essential amino acids, and 1 μ g/ml of heparin. At day 12, EBs were embedded in matrigel (BD), media were changed to N2B27 (as above but with B27 supplement minus vitamin A) and moved to a 5 cm dish. At day 18, EBs were moved to the orbital shaker and fed with N2B27 (containing vitamin A) for the remainder of the experiment.

100 days post neural induction was taken as the time point for mature neurons in both 2D and 3D paradigms.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 15 min and stored in phosphate buffered saline (PBS) at 4 °C until immunostaining was performed. Organoids were fixed for 1 h in 4% paraformaldehyde and infused with 30% sucrose solution overnight at 4 °C before being embedded in optimal cutting tissue compound (OCT) (Bright) and stored at -80 °C. 10 μ m sections were taken on the cryostat and added onto Superfrost slides (VWR). Before staining, OCT was removed via washing in PBS for 20 min. Frozen frontal cortex was sectioned on the cryostat to 10 μ m sections and fixed on the slide for 15 min in 4% paraformaldehyde before immunostaining. After three washes in 0.3% triton-X-100 in PBS (PBST), cells and sections were blocked in 3% bovine serum albumin in PBST. Cells were incubated in primary antibodies in blocking solution overnight (Table 1). After three washes in PBST, secondary antibodies (Alexafluor 488, 568, 594, and 648; Thermo Scientific) were added in blocking solution for one hour in the dark. 4',6-diamidino-2-phenylindole (DAPI) was added to the cells as a nuclear counterstain at 1 μ M and the cells were washed three times in PBST. Cells were mounted on slides using

DAKO mounting media or imaged in PBS. For A β immunofluorescence, after fixation, all samples were treated with formic acid for 5 min for antigen retrieval. Images were captured at room temperature on the Opera Phenix (Perkin-Elmer) or a Zeiss LSM microscope at $\times 20$, $\times 40$, and $\times 63$ magnification using Harmony and Leica LAS software, respectively. No post-hoc manipulation was performed.

Quantitative PCR

RNA was harvested using Trizol reagent (Sigma) as per the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed using Superscript IV (Thermo Fisher) and random hexamers. qPCR was performed using Power Sybr Green (Thermo Fisher) and an MX300P real time PCR cycler (Agilent). Primers used for qPCR are shown in Table 2.

Western blot

Cells were lysed in RIPA lysis buffer containing protease inhibitors (Roche). Lysates were loaded with Protein

Orange G (Li-Cor) and NuPAGE reducing agent and denatured at 95 °C for 5 min. Following centrifugation at 9300 g for 3 min at 4 °C, electrophoresis was conducted with a NuPage 10% Bis-Tris gel in NuPage MES SDS running buffer at 150 V for 1 h. Transfer to nitrocellulose membrane was conducted at 30 volts for 1 h at 4 °C. The membrane was blocked in 3% BSA and incubated with primary antibody overnight (Table 1). The membrane was washed three times with 0.1% PBS-Tween solution and incubated in secondary antibody for 1 h, washed three times in 1 \times PBS and imaged using an Odyssey Fc (Licor Biosciences).

Collection of cell culture media

Cortical neurons and 3D organoids were incubated in N2B27 for 48 h prior to media collection. Media were centrifuged at 2000 relative centrifugal force (RCF) for 5 min at 21 °C, aliquoted at 1 mL into Sarstedt 2 mL PP tubes (cat. 72.694.406), and stored at -80 °C.

Collection of CSF

Anonymised human CSF was provided by the University College London Dementia Research Centre. The CSF used was approved for biomarker research by the National Hospital for Neurology and Neurosurgery/UCLH joint REC (12 LO 1504). CSF samples were collected by lumbar puncture prior to noon according to a standard operating procedure. A volume of up to 20 mL of CSF was collected at ambient room temperature into two 10 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany, cat. 62.9924.284) directly from a 22G spinal needle, without use of a manometer. Samples were centrifuged at 1750 RCF for 5 min at 21 °C, aliquoted to 2 mL tubes (Elkay Laboratory Products, Basingstoke, UK, cat. 021-4204-500) and stored at -80 °C within 1–4 h of collection.

Immunoassays

A β 38/40/42 were measured by electrochemiluminescence (ECL) using a Meso Scale Discovery (MSD) V-PLEX A β peptide panel 1(6E10) kit, according to manufacturer's instructions. Briefly, samples were diluted 1:2 with

Table 1 Antibodies used for immunocytochemistry and western blotting

| Antigen | Company | Host | Dilution |
|----------------------|-----------------------------|------------------|----------|
| NANOG | Cell Signalling Tech D73G4 | Rabbit | 1:500 |
| SSEA4 | Biologend MC-813–70 | Mouse | 1:500 |
| FOXP1 | Abcam Ab18259 | Rabbit | 1:500 |
| pVimentin | MBL International D076–3S | Mouse | 1:250 |
| TUJ1 | Biologend 801201 and 802001 | Mouse and Rabbit | 1:10,000 |
| CTIP2 | Abcam ab18465 | Rat | 1:500 |
| PSD95 | Abcam ab2723 | Mouse | 1:1000 |
| A β | Dako M0872 | Mouse | 1:1000 |
| A β (6e10) | Biologend 803014 | Mouse | 1:1000 |
| A β C terminal | Biologend 802803 | Mouse | 1:1000 |
| MAP2 | Abcam ab5392 | Chick | 1:10,000 |
| PSEN1 C-term | Millipore MAB5232 | Mouse | 1:1000 |
| PSEN1 N-term | Millipore MAB1563 | Rat | 1:500 |
| β -Actin | Sigma | Mouse | 1:10,000 |

Table 2 Oligonucleotide primers used for qPCR

| Gene | Forward | Reverse | Amplicon |
|--------------|------------------------|--------------------------|----------|
| <i>RPL19</i> | CCCACAACATGTACCGGGAA | TCTTGGAGTCGTGGAAGTGC | 180 bp |
| <i>TBR1</i> | AGCAGCAAGATCAAAAGTGAGC | ATCCACAGACCCCTCACTAG | 149 bp |
| <i>CTIP2</i> | CTCCGAGCTCAGGAAAGTGTC | TCATCTTTACCTGCAATGTTCTCC | 129 bp |
| <i>TUBB3</i> | CATGGACAGTGTCCTCAG | CAGGCAGTCGCAGTTTTCAC | 175 bp |
| <i>APP</i> | GGTACCCACTGATGGTAAT | GGTAGACTTCTGGCAATAC | 176 bp |

diluent 35 and added in duplicate to microplate wells coated with mouse monoclonal peptide specific capture antibodies for human A β x-38/x-40/x-42. Samples were incubated with anti-A β antibody (6E10 clone) as the detection antibody conjugated with an electrically excitable SULFO-TAG. Measurements were made using an MSD SECTOR 6000. Concentrations were calculated from ECL signal using a four-parameter logistic curve-fitting method with the MSD Workbench software package.

Lactate dehydrogenase assay

CM samples were thawed at 21 °C for one hour and assayed for lactate dehydrogenase reaction using a Randox LDH P-L 401 kit (Randox, Crumlin, UK). The assay was performed on a Randox Monza according to manufacturer protocol. Briefly, the reaction mix was created by reconstituting lyophilised NADH (0.18 mmol/L) in 3 mL of R1a buffer/substrate (phosphate buffer (50 mmol/L, pH 7.5 and pyruvate (0.6 mmol/L)). After running a blank (de-ionised H₂O), 10 μ L sample/quality control was diluted in 500 μ L reaction mix, vortexed and analysed.

Immunoprecipitation mass spectrometry

A β peptides were immunoprecipitated using A β -specific antibodies coupled to magnetic beads [32]. Briefly, 4 mg of the anti-A β antibodies 6E10 and 4G8 (Signet Laboratories, Dedham, MA, USA) were separately added to 50 mL each of magnetic Dynabeads M-280 Sheep Anti-Mouse IgG (Invitrogen, Carlsbad, CA, USA). The 6E10 and 4G8 antibody-coated beads were mixed and added to the samples to which 0.025% Tween20 in phosphate-buffered saline (pH 7.4) had been added. After washing, using the KingFisher magnetic particle processor, the A β peptides were eluted using 100 μ L 0.5% formic acid. Mass spectrometry measurements were performed using a Bruker Daltonics UltraFleXtreme matrix-assisted-laser-desorption/ionisation time-of-flight/time-of-flight (MALDI TOF/TOF) instrument (Bruker Daltonics, Bremen, Germany). All samples were analysed in duplicate. It should be noted that although relative quantification of A β peptides is valid, this does not represent a reflection on absolute abundance due to different ionisation efficiencies and peptide hydrophobicity.

Statistical analysis

Statistical analysis was performed in R (version 1.1.456), graphs were created using the ggplot2 package. Mean concentrations for each biomarker were calculated, within group variation was calculated as standard error of the mean

(SEM). For time course experiment data, variation between different inductions of the same line was calculated by coefficient of variance. Data normality was assessed by histogram, qq-plot, and Shapiro–Wilk test. Given non-normal data distribution, pairwise comparisons between Non-AD controls and each fAD genotype were conducted by Mann–Whitney U test (alpha 0.05). Spearman's rank correlation coefficient was conducted to assess the relationship between cell media concentrations of A β peptides or total tau and LDH, A β ratios and age of onset, and Non-AD and fAD line A β peptide spectra.

The use of 7 fAD lines, together with 5 controls, represents sufficient statistical power for a false discovery rate of 0.2 [33]. For comparison of biomarkers in different tissues/fluids of the same patient the sample size was limited to one, therefore it was not possible to conduct tests of statistical significance between sample types.

Assay variation

Percent coefficient of variance (CV) of the intra- and inter-assay variability respectively were A β 38 (4.7%, 13.3%), A β 40 (5.9%, 9.4%), A β 42 (4.7%, 12.6%), A β 43 (7.4%, 13.2%), T-tau (7.9%, 12.6%), calculated from concentrations of an internal control CSF sample. The intra- and inter-assay CV was calculated according to ISO 5725-2 standards [34].

Results

To explore A β processing in vitro, we employed a 2D cortical differentiation protocol to a panel of five control lines (four control iPSCs and one human embryonic stem cell line), hereafter referred to as non-AD, and seven fAD patient-derived lines consisting of two independent *APP* V717I patient lines and five *PSEN1* mutation lines (int4del, Y115H, M139V, M146I, and R278I, see Table 3 and Fig. S1). All lines generated cortical neurons with a similar efficiency, quantified by similar population expression of cortical layer markers *TBR1* and *CTIP2* after 100 days of differentiation (Fig. S1). *APP* expression was not altered at the protein or RNA level by the presence of fAD mutations (Fig. 1b–d).

Consistent A β ratios over time and between independent inductions

Absolute concentrations of secreted A β 42, A β 40, A β 38 (using ECL), and A β 43 (using ELISA) were quantified in 2D iPSC-derived neuronal conditioned media (CM) from three non-AD and two fAD iPSC lines. Measurements were taken at three successive 48-h intervals at 100 days of differentiation

Table 3 Cell lines employed in this study

| Cell line | Mutation | Sex | Age of onset | Age at biopsy | APOE genotype | Origin |
|---------------|--|-----|--------------|---------------|---------------|--------------------|
| Ctrl1 | Cognitively normal | M | - | 78 | 3/3 | Dr Tilo Kunath |
| Ctrl2 | Cognitively normal ND41886 | M | - | 64 | 2/3 | Coriel repository |
| Ctrl3 | Cognitively normal RBi001-a | M | - | 45–49 | 3/3 | Sigma Aldrich |
| Ctrl4 | Cognitively normal SIGi1001-a-1 | F | - | 20–24 | 3/4 | Sigma Aldrich |
| Shef6 | Human embryonic stem cell line – no known mutation | F | - | - | 3/3 | UK Stem Cell Bank |
| APP V717I 1 | APP London mutation (2 clones) | M | 49 | 58 | 4/4 | StemBance |
| APP V717I 2 | Unrelated APP London mutation. Presymptomatic. (1 clone) | F | - | 47 | 3/3 | Generated in house |
| PSEN1 int4del | Intron 4 deletion in PSEN1 (2 clones) | F | 47 | 47 | 3/3 | StemBance |
| PSEN1 Y115H | PSEN1 Y115H (1 clone) | M | 34 | 39 | 3/3 | Generated in house |
| PSEN1 M139V | 2 Clones | F | 34 | 45 | 2/3 | StemBance |
| PSEN1 M146I | 1 Clone. Presymptomatic. | M | - | 38 | 3/3 | StemBance |
| PSEN1 R278I | 2 Clones | M | 58 | 60 | 2/4 | Generated in house |

and again at day 200. We have previously characterised A β profiles through neuronal differentiation and day 100 represents a timepoint when neurogenesis is largely complete and a neuronal A β secretome is detectable [35].

Secreted A β 43, A β 42, A β 40, and A β 38 did not correlate to levels of cell death. This was in contrast to secreted tau that displayed a strong correlation to cell death (Fig. S2). Although acceptable measurement consistency (<20% coefficient of variance (CV)) was observed within each induction over the course of 6 days, a large degree of variability (>20% CV) was observed over longer time periods (day 100 versus day 200) (Fig. S2). Furthermore, different iPSC clones of the same reprogrammed fibroblast line and independent inductions of the same iPSC line were highly variable, in some cases even over the course of 6 days.

However, when iPSC-neuron-derived A β peptides were analysed as a ratio to the most abundant product, A β 40, results were highly consistent when comparing distinct clones from the same patient, independent neural inductions, and different time points from the same induction (Fig. 1e–h). Thus, A β production ratios appear tightly regulated within a narrow physiological range, which remains stable over substantial periods of time once cells have reached maturity 100 days post-induction. Employing A β ratios overcomes experimental variability through internal normalisation, allowing meaningful comparisons between fAD lines.

A β ratios are consistent in different biosamples of the same individual patient donor

To further investigate the consistency of A β ratios in patient-derived material, we compared relative A β levels in iPSC conditioned media, iPSC-neuronal lysates, CSF, and

post-mortem brain tissue homogenate from the same patient donor (APP V717I-1) (Fig. 2a–c and Fig. S1).

Results showed that A β 38:40 levels were highly consistent between samples (Fig. 2b). Notably, these data were increased compared with Non-AD CM (Fig. 1g). Unfortunately, A β 38 levels were below the detection threshold in brain homogenate. A β 42:40 ratios were less consistent between the different cellular compartments and cellular states (Fig. 2a). Interestingly, the A β 42:40 ratio secreted by cultured neurons closely matched that observed in the soluble fraction of matched cortical tissue. A comparative reduction in CSF may be consistent with preferential deposition of A β 42 into insoluble amyloid plaques, thus affecting downstream CSF ratios. Deposition of amyloid into dense-core and diffuse plaques was observed in post-mortem brain of this individual, but not in our in vitro models (Fig. 2c). Results from cultured neuron lysates, representing soluble intracellular and membrane bound A β , show depletion of A β 42:40 equivalent to that of CSF compared to that secreted into culture media.

These data promote the use of A β ratios to compare different cell states and show the value of iPSC-based disease modelling.

Mutation-specific effects on APP cleavage, highlighting relative increments in A β 42 and A β 43

We expanded our investigation to include additional iPSC lines and samples derived from 3D cerebral organoids. The expression of APP was largely consistent between 2D and 3D differentiation paradigms, with 3D organoids showing increased variability likely due to the increased cellular diversity and heterogeneity of this system (Fig. S1). Estimates of total levels of A β peptides in the CM were made relative to

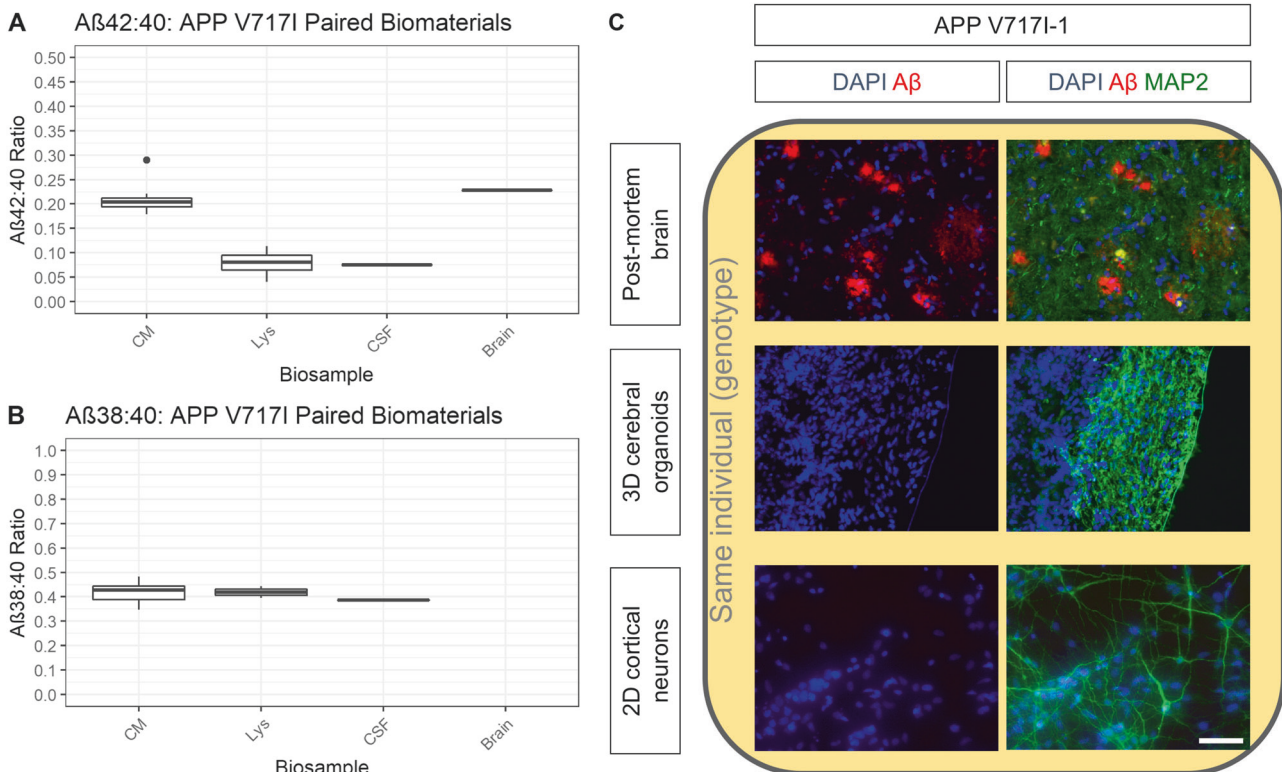


Fig. 2 Aβ ratios are consistent between matched in vitro and in vivo samples from the same patient donor. **a** Aβ42:40 and **b** Aβ38:40 measured in conditioned media ($n = 12$), cell lysates ($n = 8$), lumbar CSF ($n = 1$) and brain tissue homogenate ($n = 1$) from the same

individual. **c** Post-mortem tissue, 3D cerebral organoids and 2D iPSC-neurons from the same individual were immunostained for Aβ and MAP2

cell pellet protein content (Fig. S2). The data displayed non-significant trends, such as increased Aβ42 and decreased Aβ38 released from fAD neurons, however a large degree of variability was shown. Because of this variability, investigations were focused on the ratios of Aβ peptides.

In both 2D and 3D cultures, Aβ42:40 was increased in all fAD mutations, to approximately twice that of controls (Fig. 3a). The *PSEN1* R278I mutation displayed the smallest increase in Aβ42:40.

Aβ42:38, a putative biomarker for γ-secretase cleavage efficiency, was significantly increased in all *PSEN1* mutation lines versus non-AD (Fig. 3b). Specifically, *PSEN1* int4del and *PSEN1* Y115H demonstrated similar Aβ42:38, whereas *PSEN1* M139V, *PSEN1* M146I, and *PSEN1* R278I exhibited decreasing changes versus non-AD. *APP* V717I mutant neurons also showed a small, yet significant, increase in Aβ42:38. 3D cultures closely followed the results of their 2D counterparts in the fAD lines, although greater variability between 3D and 2D control lines was observed.

Aβ38:40 is a proposed marker of γ-secretase cleavage pathway. Interestingly, this ratio was able to distinguish neuronal lines based on mutation status (Fig. 3c). Compared to non-AD cells Aβ38:40 was significantly increased in *APP* V717I, whilst in *PSEN1* mutations the ratio was

unchanged versus non-AD in *PSEN1* Y115H, *PSEN1* M146I and *PSEN1* R278I, and decreased in *PSEN1* int4del, *PSEN1* M139V. Once more results from 3D cultures closely aligned with those of 2D.

Ratios of Aβ43 to other Aβ peptides have rarely been described in the literature. Results showed that in comparison to non-AD, all *PSEN1* mutations, except *PSEN1* M146I, demonstrated increased Aβ43:40 (Fig. 3d). The magnitude of increase in *PSEN1* R278I is particularly noteworthy given the comparatively small degree of change in the Aβ42:40 ratio we observed in this line, and the previous reports of elevated Aβ43 in this mutation [36, 37]. *APP* V717I was not observed to differ from non-AD. Results for Aβ42:43 and Aβ38:43 generally mirror results for Aβ38:40. These ratios compare products on the two cleavage product pathways (Fig. 3c, e, f, see also Fig. 1a) where ratios were raised in *APP* V717I versus non-AD. This was not the case for *PSEN1* mutations, where changes in the Aβ production pathway shift are not supported (Fig. 3c, e, f).

When Aβ peptide ratios were compared with age of onset for each genotype, the Aβ42:40 ratio showed negative trend (Fig. S3), depicting younger age of onset with higher Aβ42:40 ratios. Aβ43:40 showed a weak

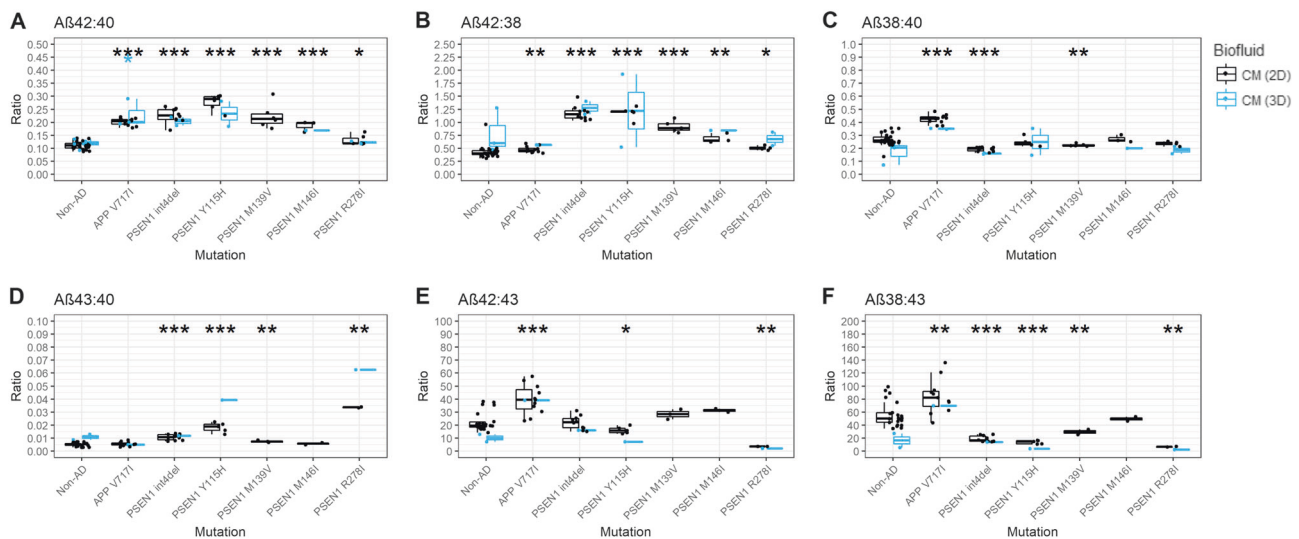


Fig. 3 fAD neurons display mutation-specific A β profile differences. Conditioned media was collected at 100 days post-neuronal induction for analysis. Results from the ratios **a** A β 42:40, **b** A β 42:38, **c** A β 38:40, **d** A β 43:40, **e** A β 42:43, **f** A β 38:43 are displayed. 2D data were generated from multiple inductions per line, specifically *APP* V717I-1 clone 1 ($n = 7$), *APP* V717I-1 clone 3 ($n = 3$), *APP* V717I-2 ($n = 2$), *PSEN1* Int4del clone 4 ($n = 5$), *PSEN1* Int4del clone 6 ($n = 5$), *PSEN1*

Y115H ($n = 6$), *PSEN1* M139V ($n = 6$), *PSEN1* M146I ($n = 3$), *PSEN1* R278I ($n = 6$). Control data were generated from the following inductions: Ctrl1 ($n = 5$), Ctrl2 ($n = 6$), Ctrl3 ($n = 7$), Ctrl4 ($n = 6$), and SHEF6 ($n = 4$). 3D data consisted of two inductions of each line, except *APP* V717I-1 clone 3, SHEF6, and M139V for which no data is available. Significance levels: * = <0.05, ** = <0.01, *** = <0.001

positive trend (Fig. S3), suggesting that A β 43 is less well correlated to age of onset. Combining A β 42 and A β 43 relative to A β 40 and A β 38 did not enhance correlation, suggesting that A β 42 shows the strongest link with age of onset. It should be noted that none of these correlations reached significance, likely due to the low-sample size.

In summary, our panel of fAD iPSC-derived neurons display a series of mutation-specific alterations in the relative production of different A β peptides, highlighting varied size and scale of changes in γ -secretase endopeptidase and carboxypeptidase-like cleavage of APP. The *APP* V717I mutation specifically alters the endopeptidase cleavage pathway, with A β 38:40 and A β 42:43 results reinforcing the proposed shift in product lineages [8]. *PSEN1* mutations lead to higher relative proportions of either A β 42 or A β 43 via reduced carboxypeptidase-like activity, and may implicate a role for A β 43 in AD pathogenesis.

Mass spectrometry highlights varied reduction in γ -secretase activity relative to other secretases as a result of fAD mutations

To investigate the secretome of A β peptides and to infer different activities of the α -, β -, and γ -secretases, MALDI TOF/TOF mass spectrometry was performed on A β peptides immunoprecipitated from 2D iPSC-neuronal supernatants. Given that A β profiles were consistent in 2D and 3D

cultures, only media from 2D cultures were analysed. As demonstrated in Fig. 1, using ratios of A β peptides to A β 40 acts as an internal normalisation to represent the data, and therefore was utilised to probe the mass spectrometry data.

Mass spectrometry confirmed the broad findings for relative amounts of A β 38/40/42 generated using the MSD immunoassay previously described, although A β 43 was not detectable with this method of analysis (Supplementary Table 1). A β 42:40 was significantly raised in all fAD mutations except *PSEN1* R278I (Fig. 4a), which had been the mutation where this ratio was least altered in the immunoassay. A β 42:38 was significantly increased versus non-AD in all *PSEN1* mutations except *PSEN1* Y115H (although a clear tendency for increase, proportional to the immunoassay results, was observed) and R278I where the level of significance in the immunoassay had once again been weak (Fig. 4b). A β 38:40 was significantly increased in the *APP* V717I line versus non-AD, unchanged versus non-AD in *PSEN1* Y115H, *PSEN1* M146I and *PSEN1* R278I, and decreased in *PSEN1* int4del and M139V (Fig. 4c), mirroring the immunoassay results.

Moving beyond A β 38/40/42, mass spectrometry highlighted several interesting patterns among less well studied peptides. A β 39 ratios displayed a similar, though not identical, pattern to A β 38 whereby specifically *APP* V717I showed increased A β 39:40 relative to non-AD and *PSEN1* mutant neurons (Fig. 4d). A β 38 is often used as the final fragment in the A β 48 > 45 > 42 > 38 pathway, although A β 39 is reported as an alternative fragment generated from A β 42

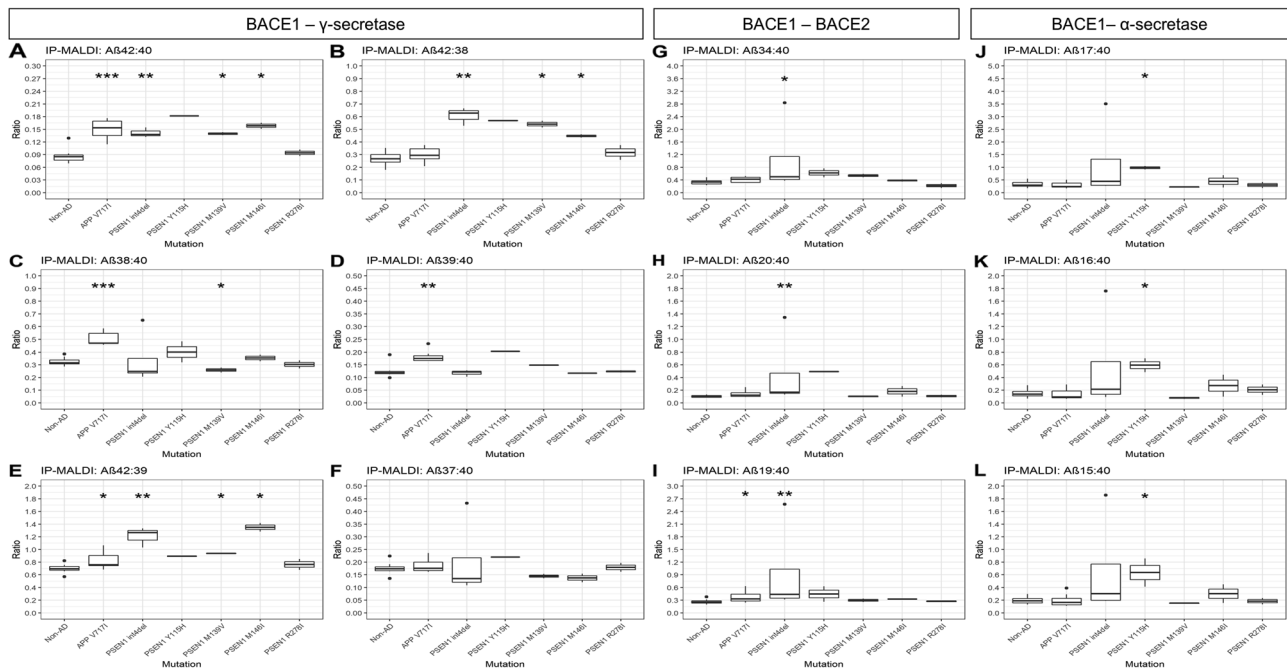


Fig. 4 Mutation specific differences of Aβ secretomes from multiple proteolytic pathways. Results from mass spectrometric analysis of cell media for Aβ peptides generated by **a–f** BACE1 and γ-secretase activity **g–i**, BACE1 and BACE2 activity, and **j–l** BACE1 and α-secretase activity normalised as ratios. Mean data were generated at day 100 from multiple independent inductions per line, specifically non-AD (*n* = 10) consisting of pooled data of Ctrl1 (*n* = 2), Ctrl2 (*n* =

2), Ctrl3 (*n* = 2), Ctrl4 (*n* = 2), and Shef6 (*n* = 2). fAD data were generated from the following, *APP* V717I-1 clone 1 (*n* = 5), *APP* V717I-1 clone 3 (*n* = 2), *PSEN1* int4del clone 4 (*n* = 2), *PSEN1* int4del clone 6 (*n* = 2), *PSEN1* Y115H (*n* = 2), *PSEN1* M139V (*n* = 2), *PSEN1* M146I (*n* = 2), and *PSEN1* R278I (*n* = 2). Significance levels: * = < 0.05, ** = < 0.01, *** = < 0.001

[6]. Aβ42:39 was found to be raised significantly in a subset of *PSEN1* mutant neurons as well as *APP* V717I versus non-AD (Fig. 4e), supporting findings of Aβ42:38 by MSD.

No differences in Aβ37:40 were observed between non-AD and any fAD mutation (Fig. 4f), indicating that the disease mechanism that results in impaired γ-secretase carboxypeptidase-like efficiency mainly affects the processing of longer Aβ peptides, which is in line with the proposed mechanistic model for fAD [38].

Relative to the γ-secretase-dependent peptide Aβ40, *PSEN1* int4del neurons exhibited significantly raised levels of BACE1–BACE2 products (Aβ19/20) and BACE1–BACE1/BACE2 products (Aβ34) (Fig. 4g–i) [35, 39, 40], as well as a non-significant skew toward increased α-secretase products (Aβ15/16) (Fig. 4k, l), and Aβ17, attributed to either γ-secretase cleavage [41] or endothelin-converting enzyme cleavage [42] (Fig. 4j). Similarly, *PSEN1* Y115H significantly increased BACE1–α-secretase products and displayed a tendency for increased BACE1–BACE2 products (Fig. 4g–l). These effects were not evident in other fAD neurons, which displayed grossly similar secretomes to non-AD samples (Fig. S3).

Finally, N-terminally truncated Aβ peptides (in ratio to Aβ40) in fAD cell lines did not significantly differ from non-AD. The exceptions to this were the *APP* V717I and *PSEN1* M139V lines, which displayed increased Aβ2–40:40

(*p* = 0.01 and *p* = 0.04, respectively) as well as *PSEN1* M139V that also exhibited a decrease in Aβ11–40:40 (*p* = 0.03), (Fig. S3). Generally, similar mutation-specific effects were seen for Aβ11–x as for Aβ1–x peptides described above that would be worth pursuing with greater sample sizes; e.g. carboxypeptidase-like defects caused by *PSEN1* mutations and endopeptidase effects in *APP* mutant cells. For example, the fAD lines tended to increase Aβ11–42 in ratio to Aβ11–40 (Supplementary Table 1 and Fig. S3), an effect previously observed by Siegel et al. [43].

Together these data report on fAD mutation dependent effects on APP. Mass spectrometry data reinforce the findings that *APP* mutations alter endopeptidase cleavage and that *PSEN1* mutations reduce γ-secretase carboxypeptidase-like activity. Additionally *PSEN1* int4del and Y115H mutations appear to display a greater deficiency in γ-secretase activity than other *PSEN1*-mutation bearing neurons, shown by an increase in α- and β-secretase-dependent products relative to γ-secretase-dependent peptides.

γ-Secretase protein levels are altered in a subset of PSEN1 mutant lines

PSEN1 mutations have been shown to alter γ-secretase stability [38, 44], and so we investigated total PSEN1 protein levels in our human neuronal model using western blotting

(Fig. 5a). Neurons harbouring the *PSEN1* mutation R278I displayed a band at 46 kDa; relating to full length *PSEN1* that has not undergone autocatalysis and maturation [9, 36, 37]. As a result, this line exhibited reduced mature *PSEN1* levels (Fig. 5b). Despite proper maturation, the *PSEN1* mutations M139V and M146I showed a high degree of variability in *PSEN1* protein levels. In a subset of neural inductions, *PSEN1* levels were considerably lower than control neurons, however, this was inconsistent and did not reach significance (Fig. 5a, b). *PSEN1* int4del and Y115H lines showed consistent *PSEN1* protein levels that were similar to *APP* V717I mutant neurons and non-AD lines (Fig. 5a).

These data suggest that mature *PSEN1* protein levels are variably altered when harbouring M139V, M146I or R278I amino acid substitutions. These lines showed A β profiles that were most similar to control secretomes (Fig. 4 and S3). The *PSEN1* mutant lines that showed the greatest reduction in γ -secretase-dependent A β peptides, int4del and Y115H, displayed *PSEN1* protein levels similar to controls. These data suggest that three alternative mechanisms behind *PSEN1* partial loss of function, lack of *PSEN1* protein maturation, lack of *PSEN1* stability and reduced catalytic activity of γ -secretase.

Discussion

In this study, we systematically investigated the profile of A β species using in vitro, patient-derived stem cell models of fAD. The main finding was that different fAD mutations have qualitatively distinct effects on APP processing and A β production by γ -secretase; affecting the APP ϵ -cleavage pathway and carboxypeptidase-like activity in different ways (summarised in Table 4). Other key findings were that employing ratios of each A β peptide relative to A β 40 provides a robust internal normalisation to overcome inherent variability between cultured cell lines and between inductions of the same line. Additionally, A β ratio profiles in cultured cortical neurons, CSF and brain tissue from the same patient with an fAD genotype and diagnosis were compatible with the current understanding of AD-associated A β brain deposition and clearance. Our work shows that fAD mutation effects are consistent between 2D cortical neurons and 3D cerebral organoids and we provide the first investigations into A β 43 as well as smaller A β peptides in iPSC neuronal models without overexpression.

The data presented offer a human neuronal validation of two hypotheses advocated by Chavez-Gutierrez et al. [8].

Fig. 5 *PSEN1* protein levels are variably altered in a subset of *PSEN1* mutant neurons.

a Representative western blot of 3 control neuron lysates and 6 fAD lysates. The asterisk depicts immature, full length *PSEN1* protein at 46 kDa.

b Quantification of independent neuronal lysates, replicates are depicted within histogram

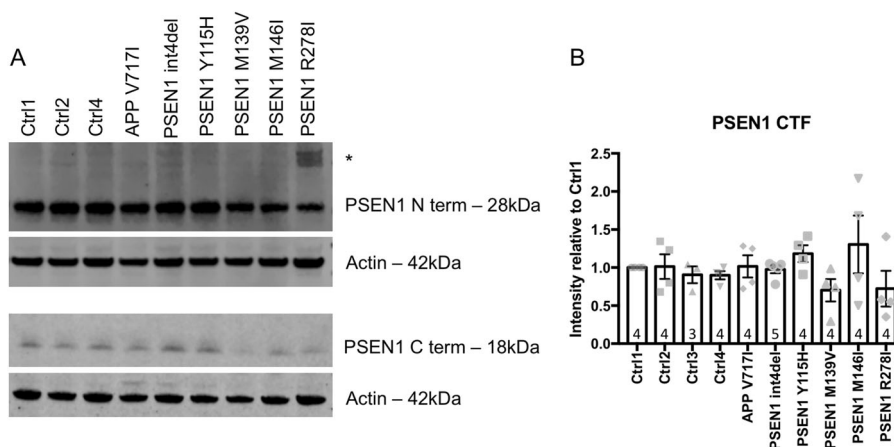


Table 4 Summary of phenotypes in each mutation line

| Mutations | A β 42:40 | A β 42:38 | A β 38:40 | A β 43:40 | γ -sec | β -sec | α -sec |
|----------------------|-----------------|-----------------|-----------------|-----------------|----------------|--------------|---------------|
| <i>APP</i> V717I | ↑↑↑ | ↑↑ | ↑↑↑ | | Endo (↑42,↓43) | | |
| <i>PSEN1</i> int4del | ↑↑↑ | ↑↑↑ | ↓↓↓ | ↑↑↑ | Carboxy | ↑ | |
| <i>PSEN1</i> Y115H | ↑↑↑ | ↑↑↑ | | ↑↑↑ | Carboxy | | ↑ |
| <i>PSEN1</i> M139V | ↑↑↑ | ↑↑↑ | ↓↓ | ↑↑ | Carboxy | | |
| <i>PSEN1</i> M146I | ↑↑↑ | ↑↑ | | | Carboxy | | |
| <i>PSEN1</i> R278I | ↑ | ↑ | | ↑↑ | Carboxy | | |

The number of arrows represent *p* value significance levels from Fig. 3 (↑ = <0.05, ↑↑ = <0.01, ↑↑↑ = <0.001). γ -Sec indicates the nature of C-terminal cleavage effect on tripeptide cleavage pathways ('42' or '43' representing the A β 48,45,42,38 and A β 49,46,43,40 pathways, respectively), with 'Endo' representing changes to endopeptidase-like activity predisposing to one pathway over another, and 'Carboxy' representing decreased carboxypeptidase-like activity affecting both pathways. The β -sec and α -sec columns depict findings from Fig. 4 displaying a relative increase in fragments dependent on each secretase. Sec—secretase, endo—endopeptidase activity, carboxy—carboxypeptidase-like activity

Firstly, that pathogenic *APP* mutations favour increased γ -secretase $A\beta_{48} > 45 > 42 > 38$ ϵ -cleavage. The concomitant increase in $A\beta_{38:40}$, $A\beta_{42:40}$, and $A\beta_{42:43}$ observed in *APP* V717I cells is consistent with this idea. Interestingly, this pattern extended to $A\beta_{39:40}$, $A\beta_{39}$ being the true tripeptide postcedent of $A\beta_{42}$ [6]. Study of $A\beta_{39}$ has been largely limited to models of oligomerisation [45, 46] and further investigation is called for.

The second tenet substantiated by our data is that mutations in *PSEN1* lead to inefficient γ -secretase carboxypeptidase activity. This predisposes neurons to the accumulation of longer $A\beta$ fragments, demonstrated by consistently increased $A\beta_{42:40}$ alongside increased $A\beta_{42:38}$ in *PSEN1* mutation cell lines. The observed increase in $A\beta_{43:40}$ reinforces the idea of reduced carboxypeptidase efficiency on the $A\beta_{49} > 46 > 43 > 40$ pathway, and it is interesting that different mutations specifically affect one pathway or both. Reduced carboxypeptidase activity can be further explained by three distinct mechanisms. Firstly, reduced γ -secretase activity is suggested for *PSEN1* int4del and Y115H lines, potentially due to their location near the substrate docking domain [47, 48]. Secondly, incomplete maturation of PSEN1 protein with R278I mutations leads to reduced levels of mature protein [36, 37]. Finally, *PSEN1* M139V and M146I mutations lead to variably altered PSEN1 levels, consistent with altered protein stability [38, 44] and supported by studies of PSEN1 abundance in brain tissue of early onset AD patients [49, 50]. This variability in PSEN1 protein levels is likely to reduce the pool of functional γ -secretase enzyme. This finding demonstrates the advantage of using a more physiological model, such as iPSC-derived neurons, as protein instability could explain why the pathogenic M139V protein shows close to wild-type biochemical enzyme kinetics [8]. Reduced carboxypeptidase activity is especially relevant, given the recent suggestion that shorter $A\beta$ peptides may be protective, meaning reductions in $A\beta_{38}$ and $A\beta_{40}$ could lie behind certain *PSEN1*-associated pathology [51]. It remains unclear why *PSEN1* mutations affect the carboxypeptidase-like activity of γ -secretase without an apparent change to endopeptidase activity, but can be explained due to enzyme-substrate interaction destabilisation as proposed by Szaruga et al. [38].

Three findings described by the data add to the complexities of the tripeptide hypothesis. Firstly, *PSEN1* int4del and M139V mutations reduce $A\beta_{38:40}$, potentially suggesting that alterations to the ϵ -cleavage pathway. It is important to note that altered γ -secretase efficiency between the two tripeptide pathways can also explain these findings. Secondly, *APP* V717I mutations may lead to small yet significant increases in $A\beta_{42:38}$ and $A\beta_{42:39}$, suggesting that reduced carboxypeptidase activity in these neurons. Thirdly, $A\beta_{37:40}$ ratios were not significantly altered where

this might have been expected in the PSEN1 lines, suggesting either that the effect of reduced γ -secretase carboxypeptidase-like efficiency may diminish beyond a focal point in the APP C-terminal sequence or that $A\beta_{37}$ is γ -secretase independent.

Given the divergence of different phenotypes in different lines, the use of additional lines in future work will provide added validation; for example, investigating extra C-terminal PSEN1 mutations and alternative APP mutation bearing lines. Larger studies may make it possible to correlate the functional outcome of the different APP processing defects to clinical symptoms. For example, whether mutations pre- and post-codon 200 of *PSEN1* could predict age of onset [52]. Despite these limitations, our study represents one of the largest series of fAD lines, thereby allowing comparisons of the effects of different mutations on the $A\beta$ secretome.

Conclusion

This study employed multiple patient-derived iPSC-neurons to model APP processing and $A\beta$ production in the context of fAD *APP* and *PSEN1* mutations. Ratios of secreted $A\beta$ peptide fragments revealed consistent fundamental differences between mutations, leading to quantitatively and qualitatively divergent $A\beta$ secretomes, explained by three partial loss-of-function mechanisms and one ϵ -cleavage pathway shift. Common cellular functional outcomes of these changes are needed to further our understanding of the disease mechanism and these patient differences are a fundamental consideration for clinical trials involving γ - and β -secretase inhibitors. This work demonstrates that iPSCs offer a valuable model to investigate underlying cellular dysfunction that results from inherited fAD mutations; representing early changes that may progress with age to neurodegeneration.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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