Using stem cell models to understand early-onset Alzheimer's Disease and its progression

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Abstract

Alzheimer's disease (AD) is the most common form of dementia, making it an urgent health priority globally. Understanding the cellular processes that drive rapid disease progression remains a major challenge. Studying rare genetic forms of AD has thus far been critical to gaining insight into the underlying biology of the disease mechanisms. AD is characterised by progressive changes in the brain associated with two major proteins, amyloid-β (Aβ) and tau. This project investigated whether aggressive familial AD mutations could provide human cell models in which early tau pathology is more readily detectable. The central aim was to compare patient-derived neurons carrying either the PSEN1 L166P or R278i mutations with healthy control neurons and determine whether they showed altered levels of pTau217, a phosphorylated form of tau associated with AD pathology.

Patient-derived induced pluripotent stem cells (iPSCs) were characterised and differentiated into cortical neurons, while rapid NGN2-mediated differentiation was used to generate i3 neurons. pTau217 was measured by Western blotting alongside total tau and actin. In cortical neurons, pTau217 appeared slightly increased in L166P and R278i lines, although the pTau217:total tau ratio was not clearly altered, while total tau also appeared slightly elevated. In i3 neurons, pTau217 was measured and detected for the first time, but showed no clear increase in L166P compared with control.

Together, these findings support the use of patient-derived neuronal models to investigate early tau changes in familial AD and suggest that the observed phenotype may depend on the neuronal model used. The work provides a basis for further investigation of how PSEN1-associated amyloid abnormalities progress towards downstream tau pathology.

Introduction and Background

AD is the most common cause of dementia, affecting approximately 750,000 people in the UK and 50 million worldwide (Peel, 2025). It is a progressive neurodegenerative disorder in which neuronal dysfunction and loss lead to worsening cognitive decline. Despite its prevalence, the mechanisms that drive disease progression remain incompletely understood and the development of effective treatments has proved difficult. More than 200 AD drug programmes have failed over the past decade, reflecting challenges including late

intervention, poor patient stratification and limitations in how well conventional animal models reproduce human disease.

Two proteins are central to AD pathology: Aβ and tau. Aβ peptides are generated through processing of amyloid precursor protein (APP), with disease-associated changes favouring longer, more aggregation-prone forms. Tau normally helps maintain neuronal structure and function, but in AD it becomes abnormally modified, including through excessive phosphorylation, and can ultimately aggregate (Shi et al., 2012). Importantly, tau pathology correlates closely with neuronal loss and disease severity. While amyloid abnormalities are thought to occur relatively early, how these upstream changes lead to downstream tau dysfunction remains a major unresolved question.

This project therefore focused on tau rather than re-characterising a fairly well-established amyloid phenotype. In particular, we investigated pTau217, tau phosphorylated at the amino acid threonine 217. pTau217 is strongly associated with AD-related tau pathology and provides a defined molecular readout with which to ask whether abnormal tau phosphorylation is already present in experimental disease models (Kavanagh et al., 2025). We sought to measure and compare pTau217 levels in 3 cell types – L166P, R278i and control neurones. The two former represent mutations in PSEN1. Mutations in PSEN1 cause AD with nearly total genetic penetrance, because they cause dysfunction in presenilin-1, a core component of the γ-secretase complex responsible for processing APP (Ziff et al., 2025). The L166P mutation causes an exceptionally aggressive, early-onset form of familial AD and produces a markedly abnormal amyloid profile, with symptoms reported in late twenties (Alzforum.org, 2022). Its severe phenotype makes it a potentially useful, accelerated model in which downstream tau changes may become detectable within an experimentally accessible timeframe and measurable in-vitro. The R278i mutation (Alzforum.org, 2011) was included as an additional patient line, allowing tau phosphorylation to be considered across more than one disease-associated PSEN1 background.

Studying these early changes directly in patients is difficult because molecular pathology can develop long before symptoms become apparent. Patient-derived induced pluripotent stem cells (iPSCs) offer a way around this problem. Adult patient cells can be reprogrammed into a stem-cell state via defined protocols including the expression of certain transcription factors (Wray et al., 2012) and subsequently differentiated into human cortical neurons and glia – particularly relevant because cortical neurons are most heavily affected in AD, preserving the disease-causing genetic background while allowing pathology to be studied experimentally. This can be done in two ways: 1) ‘slow-grown’ cortical neuronal models, which are grown over 80 days (Shi et al., 2012), alongside 2) rapidly-generated i3 neurons. i3 neurons use inducible expression of the neuronal transcription factor NGN2 to produce relatively uniform cortical-like excitatory neurons on a much shorter timescale, providing a reproducible and scalable system for molecular experiments (Fernandopulle et al., 2018). Importantly, this study was the first time that i3 neurones were generated from L166P patient-derived iPSCs and tested for tau pathology.

The central question was therefore whether PSEN1-mutant neurons show abnormal pTau217 compared with control neurons. Detecting such a change would suggest that these human neuronal models reproduce not only upstream defects in APP processing but also a downstream feature of AD-associated tau pathology, helping to define how faithfully they capture early disease progression.

Methods

i) iPSC culture and neuronal modelling

Human iPSCs were maintained under sterile tissue-culture conditions. This involved thawing frozen cell stocks, feeding cultures with fresh growth medium, monitoring cell morphology and density, and passaging or splitting cells as cultures expanded. New L166P cells were grown on Geltrex-coated plates; cells were passaged using EDTA; ROCK inhibitor was added after thawing or replating to improve cell survival.

These procedures allow stem cells to remain healthy and undifferentiated until they are required for an experiment. Practical work included preparation of culture media, sterile handling within a tissue-culture hood, cell freezing and subsequent recovery of stored cells.

Cortical neurons were generated as previously described (Arber et al., 2019). Briefly, iPSCs were pooled to form a monolayer (at 0DIV – days in vitro) and underwent 12 days of induction to form a neuroepithelial sheet. Cells were then lifted and replated (12DIV) to allow for the growth of neocortical progenitor cells. This process was repeated approximately twice weekly until 30DIV where cells were passaged to single cells and plated for long-term culture and differentiation. Cells were harvested after 100DIV for use in these experiments.

Cells were also prepared for NGN2-mediated neuronal differentiation. Briefly, iPSCs were transfected with a PB-TO-hNGN2 (Piggybac-Tet-ON) plasmid vector and transposase in order to insert NGN2 into the cell genome. Cells underwent antibiotic selection to purify for successfully transfected cells. Once a cell line with stable NGN2 vector expression was established. NGN2 expression was induced with doxycycline to drive rapid neuronal differentiation. The cells are subsequently transferred into neuronal maturation conditions, with supportive factors such as BDNF, allowing them to develop into assay-ready neurons over approximately 2–3 weeks.

Cell cultures were additionally examined using microscopy and immunocytochemistry. Immunocytochemistry uses antibodies to label particular proteins within cells, allowing cellular identity and morphology to be visualised.

ii) Western blotting and detection of pTau217

The central experimental technique was Western blotting, which allows specific proteins to be detected and compared within complex cell samples. Proteins were first extracted from neuronal lysates and separated by SDS-PAGE gel electrophoresis. SDS unfolds the proteins

and gives them a similar negative charge, so when an electric current is applied they move through the gel mainly according to size, with smaller proteins travelling further than larger proteins. The separated proteins were then transferred from the gel onto a membrane, producing an accessible copy of the protein distribution that could be probed using antibodies.

Before antibody incubation, the membrane was blocked to cover areas of the membrane where no protein had been transferred. Without this step, antibodies could bind directly and non-specifically to the membrane, creating background signal that could obscure the protein of interest. We performed several blots and used different blocking solutions including milk in PBST and bovine serum albumin (BSA) as well as different transfer membranes including nitrocellulose and polyvinylidene fluoride (PVDF) in an attempt to obtain a cleaner, more specific signal. This was done because milk contains phosphoproteins, including casein, which can sometimes increase background or interfere with phospho-specific antibodies.

Detection occurs through a two-antibody system. The primary antibody is designed to recognise a particular part, or epitope, of the target protein – in this project, against pTau217, total tau and the housekeeping protein actin. These antibodies are produced in a host species such as rabbit or mouse. After the primary antibody has bound its target, a fluorescently labelled secondary antibody is added which is recognises antibodies from the species in which the primary antibody was raised. The fluorescent label on the secondary antibody allows bound protein to be visualised as a band, with greater fluorescence generally indicating a greater amount of target protein.

Protein samples ran at 150V for 2.5h and transfer was performed on ice at 30V for 1h in order to be probed for pTau217, and for total tau, which detects tau regardless of its phosphorylation state. Measuring both was important because a stronger pTau217 signal could result either from greater phosphorylation at T217 or simply from a greater amount of tau protein overall. Comparing pTau217 with total tau therefore provides a more meaningful measure of changes in phosphorylation. Actin was additionally used as a housekeeping or loading control, because its relatively stable abundance in all types of cells allows differences in the amount of total protein loaded between lanes to be taken into account.

Running (left) and transfer (right) of Western blots.

iii) Validating antibody specificity

A major methodological challenge was demonstrating that a band produced by a pTau217 antibody genuinely represented phosphorylated tau. Despite optimisation, antibodies can sometimes bind non-specifically to unintended proteins, meaning a signal at approximately the expected molecular weight is not by itself definitive proof of protein identity. To address this, dephosphorylation experiments were performed.

Samples were treated with lambda (λ) phosphatase, an enzyme that removes phosphate groups from proteins. Proteins were diluted in lysis buffer and combined with phosphatase buffer, $MgCl_2$ and λ-phosphatase before centrifugation and incubation to allow dephosphorylation to occur. The reaction was then stopped by heating the samples at 95°C. If the antibody is specific for pTau217, removal of phosphate groups should markedly reduce or eliminate its signal compared with the untreated sample. This provided an important control for distinguishing genuine pTau217 detection from non-specific antibody binding.

Brain lysate from familial AD tissue was also introduced as a positive control because it was expected to contain AD-associated phosphorylated tau, and at a higher banding position. This provided an additional reference against which neuronal samples and antibody performance could be assessed.

iv) Complementary experimental approaches

The project also involved a range of techniques that provided wider context for studying neurodegeneration.

Brain tissue donated from patients who suffered with various neurodegenerative conditions was sectioned, stained and examined by microscopy to visualise pathological changes in conditions including Alzheimer’s and various forms of dementia. Different stains and antibodies can reveal structures or cell types within tissue, allowing cellular pathology to be considered alongside molecular measurements from cultured cells. For example, Congo red staining was used to identify Aβ plaques in Alzheimer's disease brain tissue.

RNA-based experiments were also undertaken. RNA was extracted from samples and converted into complementary DNA (cDNA) which can be analysed using polymerase chain reaction (PCR). PCR selectively amplifies particular DNA sequences, allowing researchers to investigate which forms of a gene transcript are present.

This was applied to tau splicing. Tau can exist in several different molecular forms, or isoforms, generated when its RNA transcript is processed in different ways. In particular, the balance between 3R and 4R tau isoforms is biologically important and can be disrupted in some neurodegenerative disorders. PCR experiments were therefore used as a complementary way to investigate tau biology at the RNA level. We also tested primers for exon 6 – the site of the presenilin mutation in L166P cells.

Together, these approaches provided a broader view of how disease can be studied across stem-cell, protein, RNA and human tissue levels, while the direct research question remained focused on pTau217 in the L166P neuronal model.

Brain samples being fixed and stained for microscopy analysis

Results

i) iPSC characterisation

Immunocytochemistry (ICC) staining and brightfield microscopy analysis was used to confirm identity of patient-derived and control iPSC cultures as well as stored, previously grown cortical neurones, ensuring they were successfully established, maintained and expanded throughout the project. Stem cell markers DAPI (nuclear marker), NANOG (nuclear pluripotency transcription factor), OCT3/4 (nuclear pluripotency transcription factor) and SSEA4 (pluripotency cell-surface antigen) were used individually and together to confirm that iPSC reprogramming was successful (see Figures 1&2). The cells retained the expected stem-cell morphology during expansion and could be repeatedly passaged and recovered for downstream experiments. This provided the starting material required for neuronal differentiation and subsequent analysis of tau pathology.

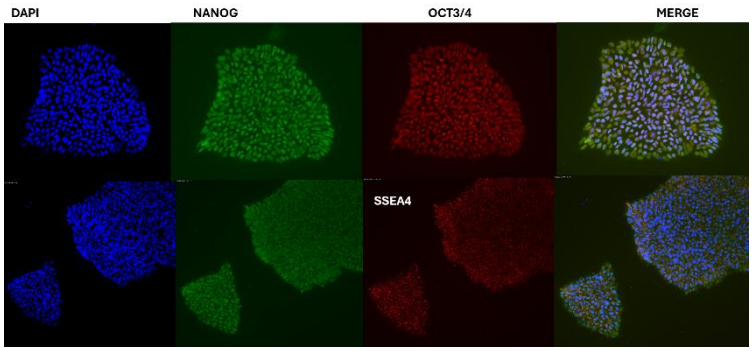


Figure 1: Immunocytochemistry (ICC) staining and imaging of stem cells. DAPI, NANOG, OCT3/4 SSEA4 represent stem cell markers, confirming that iPSC reprogramming was successful.

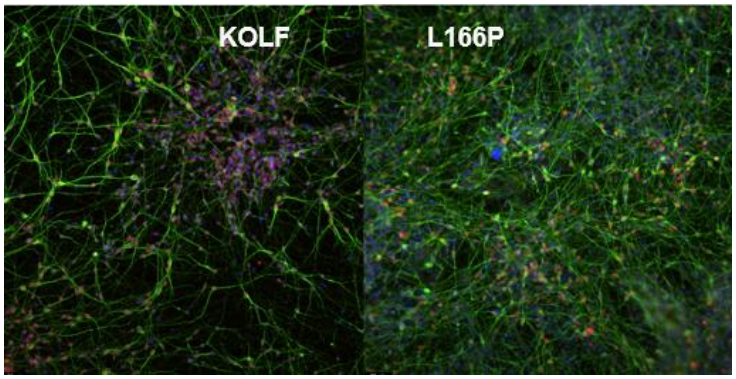


Figure 3: ICC confirming neuronal identity of KOLF and L166P cortical neurones (100 days-in-vitro (DIV).

Key:

DAPI (nuclear marker)

TUJ1 (axonal marker)

NeuN (nuclear maker for neuronal maturity (Tsefou et al., 2026))

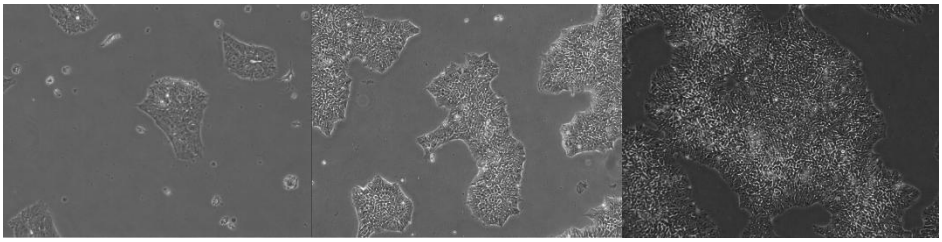
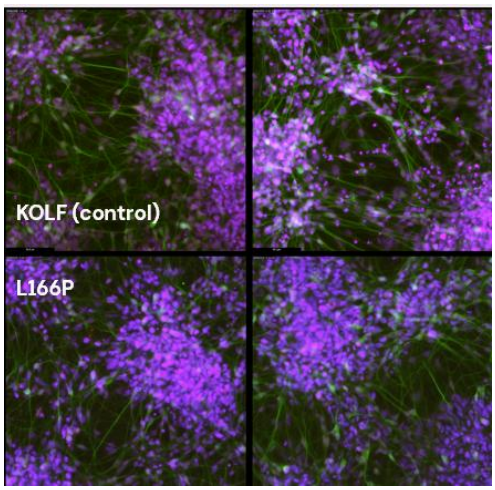


Figure 2: Figure 2: Brightfield microscope imagery of iPSCs for 3 days of growth, (10x, 20x, 40x)

ii) Neuronal induction and characterisation

This project represents the first time that i3 neurones were generated from L166P patient-derived iPSCs successfully. Cells were expanded to the required confluency, selected for the NGN2 construct and induction was initiated using doxycycline following Fernandopoulle et al. (2018) protocol (see Figure 3).

Figure 3: ICC confirming neuronal identity of KOLF (control) and L166P i3 neurones



iii) pTau217 antibody validation

pTau217 antibody was validated using untreated and dephosphorylated samples. The CST (#51625 Cell Signalling Technology) pTau217 antibody produced a clear band at the expected tau molecular-weight region in untreated samples, while the corresponding signal was completely absent following dephosphorylation (treatment with lambda-phosphatase). This loss of signal after removal of phosphate groups confirmed that antibody binding was phosphorylation-dependent, supporting its specificity for pTau217. Familial Alzheimer's disease brain lysate was included as a positive control and produced a clear pTau217 band. This confirmed that the Western blot assay was capable of detecting pTau217 when it was present. Together, the positive-control signal and complete loss of signal following dephosphorylation validated the CST antibody for subsequent analysis of pTau217 in the neuronal models

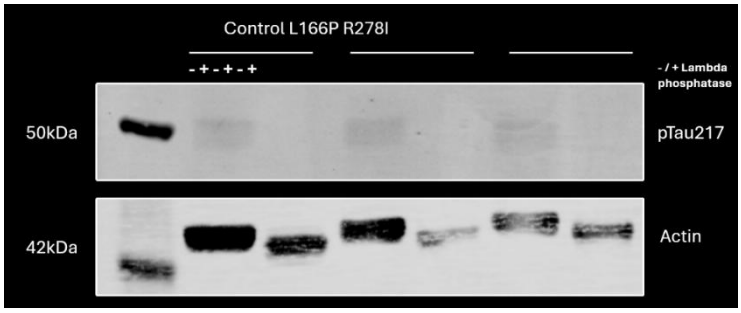


Figure 4: pTau217 antibody is specific to phosphorylated tau as shown by abolition of signal when cortical neuronal samples are treated with Lambda-Phosphatase

iv) Comparison of pTau217 expression in Cortical Neurones

Protein lysates from mutant and control neurons were analysed using antibodies against pTau217, total tau and a housekeeping protein actin, allowing pTau217 levels to be assessed relative to overall tau/protein abundance. Quantification was performed by dividing pTau217 by actin, averaging controls and subsequently dividing patient by control to find signal relative to control.

Western blotting confirmed that pTau217 was detectable in cortical neurones derived from both control and PSEN1-mutant iPSC lines. Quantification across multiple Western blots showed that normalised pTau217 abundance was higher on average in both L166P and R278I

cortical neurones compared with controls, with the largest increase observed in L166P. However, there was considerable variability between samples. When pTau217 was normalised to total tau, the ratio was broadly similar across control, L166P and R278i neurones. In contrast, total tau abundance itself was higher on average in both mutant lines relative to controls. Thus, the increased absolute pTau217 signal in the mutant cortical neurones was accompanied by increased total tau, rather than a clear increase in the proportion of tau phosphorylated at T217 (see Figures 5&6).

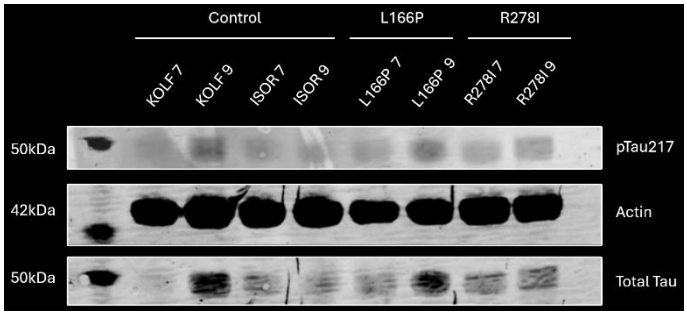


Figure 5: Western blot showing pTau217, Actin and total Tau expression in control and patient-derived cortical neuronal samples

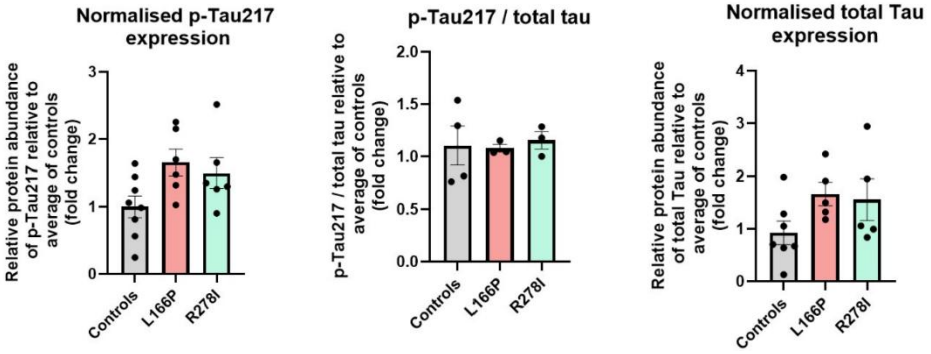
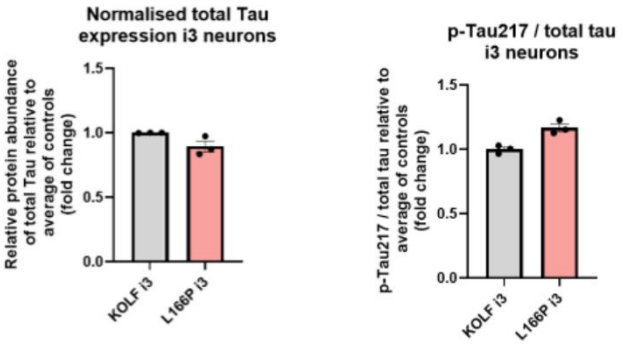


Figure 6: Figure 6: Quantification of cortical Western blot imaging

v) Comparison of pTau217 expression in i3 Neurones

Western blotting also detected pTau217 in i3 neurones from both the control KOLF line and the patient-derived L166P line. Actin was detected consistently across samples and was used as a loading control. Quantification of three independent inductions showed that normalised pTau217 levels were very similar between control and L166P i3 neurones**.** L166P showed only a small increase relative to the control average (see Figures 7&8).

Figure 7: Quantification of i3 Western blot



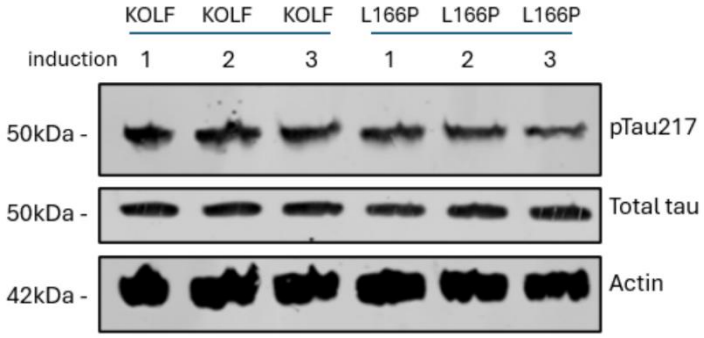


Figure 8: Western blot showing pTau217, Actin and total Tau expression in control and patient-derived i3 neuronal samples

Discussion and Conclusion

This project investigated whether patient-derived neuronal models carrying the rare AD mutations PSEN1 L166P and R278I reproduce changes in pTau217, a disease-relevant form of phosphorylated tau. The results establish that pTau217 can be detected in both conventionally differentiated cortical neurones and rapidly generated NGN2/i3 neurones, supporting the use of these models to investigate early tau pathology in vitro. In cortical neurones, pTau217 levels appeared slightly increased in L166P and R278I samples relative to controls. However, the pTau217:total tau ratio was not clearly altered, while total tau itself appeared marginally elevated in both mutant lines. This suggests that the higher absolute pTau217 signal may partly reflect increased overall tau abundance rather than a selective increase in phosphorylation at T217. The variability between samples means that these observations should currently be interpreted as trends rather than definitive mutation-associated differences, and further work is needed to conclusively say that ptau217 is always higher in mutation-carrying neurones.

An important part of the study was establishing that the assay itself was capable of reliably detecting pTau217. This required substantial optimisation. Initial cortical-neuron blots produced inconsistent or weak signal, making it difficult to distinguish genuine pTau217 detection from non-specific antibody binding. Different pTau217 antibodies were therefore compared: the Fisher antibody did not produce sufficiently convincing signal, whereas the CST antibody gave much clearer detection and was taken forward for subsequent experiments. The work therefore illustrates how assay development and validation can form a substantial part of establishing a new disease model, particularly when relatively little previous work has examined pTau217 in these neuronal systems.

The NGN2/i3 experiments provided a complementary model. Initial probing produced little detectable pTau217 despite strong actin signal, but after the membrane was stripped and detection conditions were altered, including changing from PBS- to TBS-based conditions, pTau217 could be detected, based on similar studies performed with NGN2-induced neurones

(Lagomarsino et al., 2021). Quantification across three inductions showed very similar normalised pTau217 levels in L166P and control i3 neurones, with no clear increase associated with L166P. This contrasts with the small increase observed in cortical neurones and suggests that the expression of disease-associated tau phenotypes may depend on the neuronal model and its maturation state. Rapid NGN2 induction offers the important advantage of producing relatively uniform neurones quickly and at scale, but further research is needed to see whether these cells reproduce all molecular features present in more slowly differentiated cortical cultures. Importantly, the cells used in this project were 18 days old, which may mean they cannot fully show disease markers. The lack of significance in the result does not exclude future significance entirely, and a key next step would therefore be a time-course experiment, examining pTau217 and total tau at multiple stages of neuronal maturation in i3 samples rather than at a single endpoint.

These findings also fit within broader work using patient-derived fAD models to understand the sequence of molecular events linking PSEN1-associated amyloid abnormalities to downstream tau pathology. The value of the present study is that it extends this work beyond established amyloid phenotypes to ask whether downstream tau changes can already be detected in human neuronal models. The absence of a large or consistent increase in pTau217 is itself informative. It may indicate that increased T217 phosphorylation is not an immediate consequence of the PSEN1 mutations in isolated neurones, or that additional time, cellular ageing or interactions with other brain cell types are required before a stronger tau phenotype emerges. Furthermore, it is well established that tau (MAPT) is a developmentally regulated protein/gene, however it is not clear whether the pTau217 epitope is itself regulated during development (Sposito et al., 2015). The aforementioned time-course experiments to better understand pTau217 expression levels throughout the generation of the cortical and i3 neurons would be informative to this end.

Functional analysis beyond protein level quantification would provide insights into further pathology caused by pTau217. An imaging-based assay could complement Western blotting by determining whether pTau217 changes in its cellular distribution, even when total abundance changes only slightly, and could reveal heterogeneity between individual neurones that is lost when whole cultures are analysed together. Lactate dehydrogenase (LDH) release, could additionally test whether molecular tau changes are accompanied by altered cell viability or neurotoxicity, and would aid in placing this research within a wider context of disease progression by linking molecular changes in tau to downstream effects on neuronal function, survival and molecular pathomechanisms.

The models also remain focused, though simplified, representations of AD. Neuronal cultures lack the interactions with astrocytes, microglia and other cellular processes that contribute to inflammation, protein clearance and neurodegeneration in patients. Extending the system to multicellular models would therefore provide a more complete picture of how PSEN1 mutations influence tau pathology.

Overall this study demonstrates that patient-derived iPSCs carrying rare PSEN1 familial AD mutations can be used to generate neuronal models that express detectable pTau217. Cortical

neurones carrying L166P and R278I showed small increases in pTau217 and total tau relative to controls, although the pTau217:total tau ratio was not clearly altered, while L166P i3 neurones showed no obvious increase in normalised pTau217.

Together, the findings suggest that early tau phenotypes may be subtle and dependent on the neuronal model used. Cortical differentiation provides a model in which potential disease-associated differences can be examined, while NGN2/i3 neurones provide a rapid and scalable platform for future experiments. Further replication, longer maturation and inclusion of ageing and multicellular features will be important for determining how faithfully these models reproduce the progression from PSEN1-associated amyloid abnormalities to downstream tau pathology in patients.

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