Elevated 4R-tau in Astrocytes From Asymptomatic Carriers of the *MAPT* 10+16 Mutation

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Short report

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Abstract

The *MAPT* 10+16 intronic mutation causes frontotemporal lobar degeneration (FTLD) by increasing expression of four-repeat (4R)-tau isoforms. We investigated the potential role for astrocytes in the pathogenesis of FTLD by studying the expression of 4R-tau. We derived astrocytes and neurons from induced pluripotent stem cells from two asymptomatic 10+16 carriers and, compared to controls, showed persistently increased 4R:3R-tau transcript and protein ratios in both cell types. However, beyond 300 days culture, 10+16 neurons showed less marked increase of this 4R:3R-tau transcript ratio compared to astrocytes. Interestingly, throughout maturation, both 10+16 carriers consistently displayed different 4R:3R-tau ratios at transcript and protein levels. Our study shows elevated levels of 4R-tau in astrocytes implicating glial cells in the pathogenic process and also suggests a cell-type-specific regulation and may inform and help on treatment of preclinical tauopathies.

Introduction

The four-repeat (4R) tauopathies are a group of neurodegenerative disorders including progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and frontotemporal lobar degeneration (FTLD) with neuronal and glial tau pathology consisting of intracellular 4R-tau aggregates. The microtubule-associated protein tau gene (*MAPT*) exon 10+16 intronic mutation (IVS10+16C>T) causes autosomal dominant FTLD by increased incorporation of *MAPT* exon 10 resulting in excess levels of the more fibrillogenic 4R-tau isoforms in astrocytes and neurons [1]. However, the underlying processes that contribute to neurodegeneration are unclear [2]. Glial cells, including astrocytes, play an important role in 4R tauopathy pathogenesis [3,4,5] and focus on these cells in pre-symptomatic cases could lead to identification of biomarkers for early pre-clinical detection of tauopathies [4]. Induced pluripotent stem cell (iPSC) technology by minimally invasive derivation of patient cells can be used to develop cell models to study underlying disease mechanisms and identify decisive factors in other frontotemporal dementia cases [6]. In this unique study we assessed the long-term expression of 4R-tau mRNA and protein in iPSC-derived astrocytes and neurons from asymptomatic carriers of the 10+16 *MAPT* mutation and affected post-mortem brain.

Results

After 140 days *in-vitro* (DIV), both astrocytes and neurons derived from the asymptomatic *MAPT* exon 10+16 carrier (S1) showed elevated 4R-tau mRNA (exon 10⁺) relative to 3R-tau mRNA (exon 10⁻) when compared to controls (Fig 1a). Semi-quantitative analysis of *MAPT* mRNA at different time-points showed significantly elevated 4R-tau mRNA in astrocytes from both asymptomatic cases (S1 and S2) compared to controls ($p=0.001$) and these differences persisted over 100, 200 and 300 DIV ($p=0.0034$) (Fig 1b). At much later time-points (370-620 DIV), it is also striking that though astrocytes maintain the increased 4R:3R-tau mRNA ratio in 10+16 neurons decreases with time (Fig 2). On agarose gels, we observed an intermediate band of about 380bp between those for the 3R and 4R-tau mRNA bands (305 bp and 397bp, respectively). To resolve this, we used the fluorescent FAM-labelled forward primer with

capillary electrophoresis and demonstrated that this is a heteroduplex artefact, as previously described [7,8]. Of note is that during the course of the maturation of the astrocytes, the consistently increased 4R:3R-tau ratio in the 10+16 cases was clearly less pronounced with the astrocytes from S2 (Fig 1b).

The increased 4R-tau in the 10+16 astrocytes is also seen at protein level at 300 DIV. Similar to mRNA levels, this is less pronounced with the astrocytes from S2 (Fig 3). In the control astrocytes, the major isoform is the embryonic 0N3R-tau, with the 10+16 astrocytes showing increased 0N4R-tau (Fig 3a). Interestingly, although the corresponding 10+16 neurons at the same time-point show increased 0N4R-tau compared to controls (Fig 3b), this is to a lesser extent than in the astrocytes. As with the astrocytes, the ratio of increased 4R:3R-tau is lower in the neurons from the S2 asymptomatic 10+16 case compared to S1. Western blot of cortical tissue from post-mortem brain of a 10+16 FTLD case also demonstrated increased 4R-tau (Fig 3c).

Discussion

This unique observational study of iPSC derived astrocytes and neurons from two 10+16 mutation carriers identifies astrocytic phenotypes that may help understand disease pathogenesis in FTLD. In addition to intraneuronal neurofibrillar pathology, the 4R tauopathies, including PSP, CBD, and FTLD-tau with exon 10 splicing mutations, are characterised by additional glial 4R-tau pathology, including tufted astrocytes and oligodendroglial coiled bodies. It is unclear if this is due to endogenous upregulation of tau expression, uptake of external tau, or both. In this study, we have shown that astrocytes derived from iPSCs from two carriers of the 10+16 mutation have increased 4R-tau which is known to lead to tauopathy. We demonstrated tau expression in both 10+16 and control astrocyte cultures, with detectable and increasing 4R-tau mRNA during maturation in 10+16 cell lines. The increased 4R-tau production in 10+16 astrocytes persisted at all the time-points of our analysis. As we have previously reported, neurons also had increased 4R:3R-tau mRNA ratio compared to controls [9], but with time, the ratio decreased, whilst in astrocytes, the increased ratio in astrocytes was maintained. This may reflect an autonomous astrocytic role in the evolving 4R-tau pathogenesis of 10+16 FTLD, rather than passive uptake of abnormal tau protein from neurons. Nevertheless, several questions remain unresolved and other factors may be involved, including differences in genetic and/or epigenetic factors as well as environmental influences driving post-transcriptional processes, and the interaction between neurons and glia *in vivo* [10,11].

To support the findings at mRNA level, we also demonstrated increased 4R-tau protein in the 10+16 astrocytes and neurons compared to controls, which was mirrored in frontal cortex from a brain with 10+16 FTLD. It is of interest that one of the 10+16 carriers (S2) consistently showed less pronounced increases in 4R-tau mRNA and protein compared to the other carrier (S1). This variable expressivity of the mutation could be due to differences in genetic background between the two carriers. For example, S1 is homozygous for the *MAPT* H1/H1 haplotype that is the strongest genetic risk factor for PSP and CBD whereas, S2 is heterozygous, H1/H2 [9]. It is plausible that the protective H2 haplotype exerts an epistatic effect on its opposing mutant allele. Previous postmortem work in the 10+16 brains has also shown

significant variability in the distribution, type, and severity of pathological abnormalities not only between cases but also within the same brain [12]. Therefore, our findings raise the possibility that underlying genetic factors play a role in influencing the functional effect of the 10+16 mutation on *MAPT* exon 10 splicing and can affect expressivity and cellular and clinicopathological phenotype, as described in other tauopathies [3,5].

This study suggests a role of cell autonomous astrocytic involvement in the pathogenic process of 10+16 FTLT, and further studies to identify mechanisms specific to astrocytes are required to confirm these results and to better understand the sequence of cell-specific events. In addition, it paves the way for a future preclinical detection of tauopathies to target early pathogenic pathways which in turn offers new therapeutic avenues to explore.

Methods

iPSCs from two unrelated, asymptomatic female carriers (S1 and S2) with the IVS10+16C>T mutation and 3 healthy control cell lines were used as described previously [9]. The iPSC were differentiated into neurons and astrocytes following adapted previously established protocols [13,14,15] and assessed at the same time points during maturation. All astrocyte cell lines were derived from 3 inductions. The purity of all astrocyte cell lines was confirmed by immunocytochemistry (ICC) with antibodies against glial fibrillary acidic protein (GFAP 1:1000) (Dako) and solute carrier family 1 glial high affinity glutamate transporter (EAAT1 1:1000) (Abcam). Nuclei were stained with DAPI. (Suppl Fig 1). Astrocytes were analysed from 140 days in-vitro (DIV). Five images were taken in each specific antibody staining for each sample. Image J software was used for cell recognition with specific threshold for cellular shape and for the counting cells positive for each specific astrocyte marker. The number of positive staining iPSC-derived astrocytes in 10+16 carriers and controls were higher than the 95% in both cases (Suppl Fig1b). Astrocyte-specific function was characterized using fluorescence measurements of Fura-2AM obtained on an epifluorescence inverted microscope and $[Ca^{2+}]$ was monitored in single cells using excitation light provided by a xenon arc lamp and the beam passing monochromator at 340 and 380 nm (Cairn Research, Kent, UK). The number of cells showing an increase in the ratio 340/380 were counted and divided by the total number of astrocytes. All cell lines were tested using this method. Typically 50-200 cells were analysed per experiment in a total of 17 experiments in control cells and 21 in mutants (Suppl Fig2). The ATP-induced calcium response in $45\pm7\%$ in control and $54\pm7\%$ in 10+16 subjects' iPSC-derived astrocytes. The Shapiro-Wilk test confirmed normal distribution. Total of n=2 unpaired t-test ($p=0.3$) and one-way ANOVA with Bonferroni post-hoc was performed and OriginPro 2019 software was used for this analysis. No statistically significant differences were found between them (controls, mutants and primary astrocytes (ScienCell) with $p=0.98$, $p=1$ and $p=1$, respectively), and the percentage of responding cells were also similar to the those obtained when analysing the ATP-induced calcium response of commercial human primary astrocytes ($33\pm9\%$, $n=2$). Thus, with immunocytochemistry and calcium response to ATP we were able to confirm that our cell lines faithfully reproduce astrocyte identity and functionality.

The total tau antibody (Dako; 1:1,000) was used in astrocytes and neurons at 140 DIV for the identification of endogenous tau (Suppl Fig3) and the ICC was also repeated for astrocyte cell lines after 300 days in culture. DAPI was used for nuclear staining in all samples. No differences in morphology or tau expression were found between controls and 10+16 carriers at this stage.

Tau mRNA and protein levels were also analysed at 140, 200 and 300 DIV. Post-mortem brain tissue (Brodmann area 9) from an affected *MAPT* exon 10+16 mutation carrier and an age-matched healthy control were also included. The mRNA from cells and post-mortem brains were extracted using TRIzol™ (Life Technologies) and cDNA derived using SuperScript III first strand kit (Invitrogen) following standard protocols. For PCR analysis of the derived cDNAs for *MAPT* exon 10 splicing, we used primers for flanking exons: Forward (exon 9): 5'-GTCAAGTCCAAGATCGGCTC-3' and reverse (exon 13): 5'-TGGTCTGTCTTGGCTTTGGC-3, and amplification of GAPDH cDNA used to normalise the amount of protein between samples [16]. Noting the presence of an additional band with agarose electrophoresis *MAPT* cDNA PCR products, we carried out fluorescent PCR with the *MAPT* forward primer (above) 5'-6-FAM-tagged, followed by denaturing capillary electrophoresis in a 3730XL (Applied Biosystems). We used Gene Map software (Thermofisher) and Image Studio Lite 5.2 (Li-Cor Biosciences) for analysis and quantification. The mRNA analysis at late time-points (370-620 DIV) also showed an intermediate band in the agarose gel (~380bp) between the bands for 3R- and 4R-tau mRNA. Using PCR with the fluorescent FAM-labelled forward primer followed by capillary electrophoresis, the 380bp band was not detected (Suppl Fig 4), confirming previous observations of a heteroduplex artefact, and was excluded in all analyses.

A Shapiro-Wilk test was used to assess normality of distribution and when variables were not normally distributed data were subjected to a log transformation. A two-way mixed design ANOVA and a two-way mixed repeated measures ANOVA was used to compare the results of the outcome variable (4R) with time (100, 200 and 300 DIV) as within-subjects variables and group (case versus control) as a between-subjects variable for 3 repetitions and 4 repetitions separately. The mRNA 4R-tau expression in astrocytes at different time points showed statistically significant differences between carriers and controls and 4R-tau expression did not show significant differences over time (140, 200 and 300 DIV) or the interaction between disease group and time (Suppl Table 1).

The mRNA results for expression in 3R-tau showed no significant differences among case/controls, over time or with the interaction between group and time (Suppl Table 2). Of note is that during the course of the maturation of the astrocytes we identified a decreased number of cells in cultured as days in-vitro increased. The cell culture for a prolonged period of time indicates a progressive maturation of tau protein [9] but at the same time the number of samples available for analysis was smaller at later stages.

For protein analysis, cells were lysed with Complete Lysis-M, EDTA-free buffer (Roche), with added cOmplete protease and phoSTOP phosphatase inhibitors (Roche). The lysates were dialysed into a 50mM Tris-HCl, pH 7.5 buffer and subsequently dephosphorylated using lambda protein phosphatase (New England Biolabs) as described previously [17]. Samples were analysed by Western blot using the

following antibodies: Total tau (Dako; 1:1,000) and GAPDH (Invitrogen; 1:10,000) and respective infrared IRDye 800CW and 680RD secondary antibodies followed by acquisition and quantification on the Odyssey Fc Infrared Imaging System (Li-Cor Biosciences). Quantification of the protein bands were performed using the software Prism8 (GraphPad) and the statistical analysis used was ANOVA with post-hoc Tukey (Suppl Fig 5). Significant differences in 4R-tau levels between S1 and controls and S2 samples were identified (Suppl Table 3)

List Of Abbreviations

4R: four-repeat

3R: three-repeat

PSP: progressive supranuclear palsy

CBD: corticobasal degeneration

FTLD: frontotemporal lobar degeneration

MAPT: microtubule-associated protein tau

iPSC: induced pluripotent stem cells

ICC: immunocytochemistry

GFAP: glial fibrillary acidic protein

EAAT1: solute carrier family 1 glial high affinity glutamate transporter

DIV: days *in-vitro*

Declarations

Ethics approval and consent to participate:

Ethical permission for this study was obtained from the National Hospital for Neurology and Neurosurgery and the Institute of Neurology joint research ethics committee (study reference 09/H0716/64).

Consent for publication: Not applicable

Availability of data and materials:

All data generated or analysed during this study are included in this published article [and its supplementary information files]

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions:

Núria Setó-Salvia – experiments (ICC, PCR, mRNA, protein, WB), analysis, writing, figures.

Noemi Esteras – writing, functional analysis (calcium signalling).

Rohan de Silva – writing, experiments (dialysis).

Eduardo De Pablo-Fernandez - statistical analysis.

Charles Arber- statistical analysis, cell culture.

Christina E Toomey – experiments (mRNA and protein extraction in healthy and 10+16 brains).

James M Polke – experiments (heteroduplex analysis and detection).

Huw R Morris –writing and patient recruitment.

Jonathan D Rohrer –writing.

Rickie Patani – iPSC-derived astrocyte original protocol for characterization and maturation.

Selina Wray – concept and provision of cell lines.

Thomas T Warner – concept, writing.

All authors read and approved the final manuscript.

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Figures

Figure 1

The mRNA 4R:3R-tau levels in controls and 10+16 asymptomatic carriers at 140 – 300 DIV. a. The mRNA 4R-tau levels were analysed between astrocytes and neurons at 140 DIV. Ratios of 4R-tau relative to 3R-tau expression in astrocytes with or without mutation were higher compared with neurons at same time-point. b. Astrocytes at different DIV were analysed to determine the levels of 4R-tau expression between

controls and subjects. Astrocytes with the 10+16 mutation expressed elevated 4R:3R-tau mRNA ratio compared to controls and these values were consistent in the different time-points.

Figure 2

Analysis of the 4R:3R-tau mRNA between astrocytes and neurons cell lines after 370-620 DIV. Standard and fluorescent PCR of mRNA tau expression was performed to confirm the intermediate band (asterisk) as heteroduplex artefact and discard from quantification. The two PCR products at 397bp and 305bp correspond to 4R (exon 10+) and 3R-tau (exon 10-), respectively. An affected 10+16 post-mortem brain

tissue and a healthy age-matched donor samples were included in the analysis. The astrocytes (A) (with and without mutation) showed an increased 4R:3R-tau mRNA ratio compared with neurons (N) at same time-point. The 3R and 4R-tau expression pattern differed also between controls and 10+16 carrier cell lines.

Figure 3

Protein analysis of total tau derived-astrocytes and neurons at 300 DIV and brain donor samples. a. Differences in tau protein levels were identified in both astrocyte cell line carriers. The S1 sample showed increased 0N4R isoform compared to S2. b. Increased 0N4R isoform was also seen in neurons, although S1 still showed an increased pattern of 0N4R compared to S2. c. Frontal cortex samples from a 10+16 brain donor and control brain also showed increased 0N4R band in the 10+16 brain sample compared to control.

Supplementary Files

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