Neurobiology of Disease 85 (2016) 1–10

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Reduced number of axonal mitochondria and tau hypophosphorylation in mouse P301L tau knockin neurons

Teresa Rodríguez-Martín [a], Amy M. Pooler [a,1], Dawn H.W. Lau [a], Gábor M. Mórotz [a], Kurt J. De Vos [a,2], Jonathan Gilley [b], Michael P. Coleman [b], Diane P. Hanger [a,*]

[a] King's College London, Institute of Psychiatry, Psychology & Neuroscience, Maurice Wohl Clinical Neuroscience Institute, Department of Basic and Clinical Neuroscience, London, SE5 9NU, UK
[b] Signalling Programme, The Babraham Institute, Cambridge CB22 3AT, UK

ARTICLE INFO

Article history:
Received 16 July 2015
Revised 28 September 2015
Accepted 8 October 2015
Available online 11 October 2015

Keywords:
Dementia
Tau
Mitochondria
Axonal transport
Phosphorylation
Membrane

ABSTRACT

Expression of the frontotemporal dementia-related tau mutation, P301L, at physiological levels in adult mouse brain (KI-P301L mice) results in overt hypophosphorylation of tau and age-dependent alterations in axonal mitochondrial transport in peripheral nerves. To determine the effects of P301L tau expression in the central nervous system, we examined the kinetics of mitochondrial axonal transport and tau phosphorylation in primary cortical neurons from P301L knock-in (KI-P301L) mice. We observed a significant 50% reduction in the number of mitochondria in the axons of cortical neurons cultured from KI-P301L mice compared to wild-type neurons. Expression of murine P301L tau did not change the speed, direction of travel or likelihood of movement of mitochondria. Notably, the angle that defines the orientation of the mitochondria in the axon, and the volume of individual moving mitochondria, were significantly increased in neurons expressing P301L tau. We found that murine tau phosphorylation in KI-P301L mouse neurons was diminished and the ability of P301L tau to bind to microtubules was also reduced compared to tau in wild-type neurons. The P301L mutation did not influence the ability of murine tau to associate with membranes in cortical neurons or in adult mouse brain. We conclude that P301L tau is associated with mitochondrial changes and causes an early reduction in murine tau phosphorylation in neurons coupled with impaired microtubule binding of tau. These results support the association of mutant tau with detrimental effects on mitochondria and will be of significance for the pathogenesis of tauopathies.

Introduction

The microtubule-associated protein tau is expressed mainly in neurons where it stabilises microtubules and is involved in neurite outgrowth (Hernandez et al., 2002; Lee and Rook, 1992). The microtubule-binding domain of tau is located in its carboxy-terminal half and tubulin binding is regulated by tau phosphorylation state (Bramblett et al., 1993; Busciglio et al., 1995). The amino-terminal projection domain of tau interacts with components of the plasma membrane and is also affected by tau phosphorylation state (Bramblett et al., 1993; Busciglio et al., 1995; Pooler et al., 2012).

The human *MAPT* gene is located on chromosome 17 and comprises 16 exons. Exclusion or inclusion of exon 10 gives rise to tau isoforms with three (3R) or four (4R) microtubule binding repeats (Andreadis et al., 1992; Goedert et al., 1989). In the developing brain, 3R tau isoforms predominate, whereas in adult human brain 3R and 4R tau are expressed in approximately equal amounts. Mutations in *MAPT* cause frontotemporal dementia with parkinsonism linked to tau mutations on chromosome 17 (FTDP-17T) (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998), characterised by intraneuronal aggregates of insoluble, highly phosphorylated tau. FTDP-17T and other neurodegenerative diseases with CNS tau aggregates are collectively referred as tauopathies (Ballatore et al., 2007; Gallo et al., 2007). Disease-associated mutations in *MAPT* occur as exonic missense mutations (e.g. P301L), silent mutations (e.g. N279N), or intronic mutations that affect exon 10 splicing regulatory elements and thereby alter the 4R/3R tau isoform ratio (D'Souza et al., 1999; Grover et al., 1999; Spillantini et al., 1998). However, not all of the known mutations in

Abbreviations: DIV, days in vitro; E18, embryonic day 18; EGFP, enhanced green fluorescent protein; GSK-3, glycogen synthase kinase-3; PBS, phosphate-buffered saline; PP, protein phosphatase; SDS, sodium dodecyl sulphate.

* Corresponding author at: King's College London, Institute of Psychiatry, Psychology & Neuroscience, Department of Basic and Clinical Neuroscience, Maurice Wohl Clinical Neuroscience Institute, (Room 1.24), 125, Coldharbour Lane, London SE5 9NU, UK.
E-mail addresses: teresa.rodriguez@kcl.ac.uk (T. Rodríguez-Martín), amy.pooler@rd.nestle.com (A.M. Pooler), dawn.lau@kcl.ac.uk (D.H.W. Lau), gabor.morotz@kcl.ac.uk (G.M. Mórotz), k.de_vos@sheffield.ac.uk (K.J. De Vos), jon.gilley@babraham.ac.uk (J. Gilley), michael.coleman@babraham.ac.uk (M.P. Coleman), diane.hanger@kcl.ac.uk (D.P. Hanger).
[1] Present address: Department of Cognitive Health and Ageing, Nestlé Institute of Health Sciences, EPFL Innovation Park, Bâtiment H, 1015 Lausanne, Switzerland.
[2] Present address: Department of Neuroscience, Sheffield Institute for Translational Neuroscience (SITraN), University of Sheffield, Sheffield, S10 2HQ, UK.
Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2015.10.007

MAPT result in altered tau splicing and furthermore, the molecular mechanisms that link these mutations to the observed pathological and clinical features of the tauopathies are not well understood.

Many transgenic mouse lines that model tauopathies have been generated by overexpression of either wild-type or FTDP-17T mutant tau (reviewed in Denk and Wade-Martins, 2009; Noble et al., 2010). Axonal degeneration and transport impairments have been described in several of these mouse models, with more frequent mature filamentous tau pathology occurring in mice overexpressing mutant tau. However, differences in the expression of exogenous tau due to the use of heterologous promoters, and an imbalance in tau isoform expression by overexpression of individual isoforms of human tau, are significant limitations in many of these models. For example, P301L or P301S tau expressed under the control of different promoters including prion (Lewis et al., 2000), Thy 1 (Allen et al., 2002; Terwel et al., 2005) and calcium-calmodulin kinase II (Santacruz et al., 2005), each result in different tau expression patterns and variable phenotypic outcomes.

We created a transgenic tau knock-in (KI) mouse expressing physiological levels of murine tau and harbouring mutant P290L tau, equivalent to human P301L tau (Gilley et al., 2012). We used this mouse line to investigate the impact of P301L tau on FTDP-17T-associated tau pathology and neural dysfunction (Gilley et al., 2012). Overt tau pathology was not observed and interestingly, we found that the overall level of tau phosphorylation was reduced in adult KI-P301L mice (Gilley et al., 2012). However, these transgenic mice exhibited age-dependent changes in mitochondrial axonal transport.

Mitochondria are highly dynamic organelles that undergo continuous bi-directional movements, combined with frequent fission and fusion events (Schulz et al., 2012). Dysregulation of mitochondrial activity and transport is associated with a number of age-related neurodegenerative disorders (De Vos et al., 2008; Exner et al., 2012; Lin and Beal, 2006). Recent findings also implicate defective mitochondrial function and dynamics induced by amyloid beta-peptide and/or tau in the pathogenesis of Alzheimer's disease (Amadoro et al., 2014; Eckert et al., 2013; Manczak and Reddy, 2012).

To gain insight into the mechanisms underlying the axonal transport defect observed in KI-P301L mice, we characterised the effects of tau on axonal mitochondrial transport in primary cortical neurons and investigated tau phosphorylation. We found that the total number of mitochondria in axons was reduced and the volume of individual motile mitochondria was significantly increased in neurons derived from KI-P301L mice. We also found that tau hypophosphorylation observed in adult mouse brain was recapitulated in cultured cortical neurons. Our results suggest that physiological expression of mutant P301L tau decreases tau phosphorylation and reduces the number of axonal mitochondria at a very early stage of development, supporting the association of mutant tau with dysregulation of mitochondrial activity.

Material and methods

Mouse maintenance and generation

All mice were bred and housed in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986. KI-P301L mice were generated in a C57BL/6 background by (Gilley et al., 2012). Briefly, the human FTDP-17 P301L mutation was targeted to the homologous codon in exon 10 of the mouse *Mapt* gene — P290 in 2N4R murine tau. Correct targeting of the knockin allele was verified by Southern blotting and mice were genotyped by Southern blotting or PCR. Expression and normal splicing of *Mapt* messenger RNA derived from the "P301L" tau knockin allele was confirmed by RT-PCR (Gilley et al., 2012).

Cortical neuronal culture and transfection

Embryonic day 18 (E18) wild-type and P301L tau-expressing mouse cortical neurons were cultured as described previously (Cuchillo-Ibanez et al., 2008). Cortical neurons were transfected using either calcium phosphate (Promega, Madison, USA) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturers' instructions.

Time-lapse imaging and analysis of mitochondria in transfected neurons

E18 rat cortical neurons 7 DIV were co-transfected with plasmids expressing enhanced green fluorescent protein (EGFP) or DsRed-Mito (Invitrogen, Carlsbad, CA, USA) and time-lapse images were recorded after 24–48 h. Coverslips were placed in a sealed chamber and the cells were maintained at 37 °C using an objective heater (Tempcontrol 37–2, Zeiss, Jena, Germany) and "The Box" Microscope Temperature Control System (Life Imaging Services Basel, Switzerland) on the stage of an Axiovert 200 M Zeiss microscope equipped with a Lambda LS Xenon-Arc light source (Sutter Instrument Company, Novato, CA, USA). Images of mitochondrial movement were collected at 3 s intervals for 10 min using a Plan-ApoChromat 40 × 1.4 NA oil immersion objective, an EGFP/DsRed filter set (Chroma Technology Corp., Rockingham, VT, USA) and an AxioCam MRm camera. Image analysis was performed with ImageJ developed by Wayne Rasband (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). EGFP expression enabled identification of transfected neurons and tracing of axons from the neuronal cell body to the growth cone, as we reported previously (Morotz et al., 2012). Mitochondrial kinetic parameters were assessed using Difference Tracker, a programme consisting of 2 plugins for the ImageJ software (Andrews et al., 2010). The contrast and despeckle functions were applied to all images. The difference filter plugin was used with minimum difference 10 and difference frame onset 4, and for the mass particle tracker plugin the settings for the initial flexibility and subsequent flexibility were 25 and 20, respectively. Quantitation and statistical analyses (Student t-test) were performed using Excel and Prism software (GraphPad Software Inc., La Jolla, CA, USA). Image analysis for parameters (angle, average size, circularity, perimeter, solidity, primary axis and secondary axis) of all mitochondria (stationary and motile) was performed using ImageJ.

Gel electrophoresis and Western blots

10^6 neurons per well of a 6-well plate were rinsed with phosphate-buffered saline (PBS) at 4 °C and cells were scraped into hot (2×) Laemmli sample buffer. Proteins were separated on 10% (*w*/*v*) sodium dodecyl sulphate (SDS) polyacrylamide gels and transferred to nitrocellulose. Membranes were probed with antibodies to total tau (rabbit polyclonal, DAKO, Glostrup, Denmark) and monoclonal Tau5 (Sigma-Aldrich, Gillingham, Dorset, UK), phosphorylated tau (polyclonal pT231 and pS404, Cell Signalling, Danvers, MA, USA and PHF1, a kind gift from Professor Peter Davies, Albert Einstein College of Medicine, New York), dephosphorylated tau (monoclonal Tau1, Millipore, Billerica, MA, USA), PP1 and PP2 (Santa Cruz, Dallas, Texas, USA), Opa1 (BD Transduction Laboratories), Mfn2 and monoclonal β-actin (both from Sigma, Gillingham, Dorset, UK). Antigens were visualised using an Odyssey scanner (Li-Cor Biosciences, Lincoln, Nebraska, USA).

Microtubule binding assay

Assays for microtubule binding of tau were performed as described previously (Rodriguez-Martin et al., 2013). Wild-type and KI-P301L-expressing 12 DIV cortical neurons were rinsed in warm PBS, and suspended in warm microtubule-stabilising buffer (80 mM PIPES/KOH pH 6.8, 1 mM GTP, 1 mM $MgCl_2$, 1 mM ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 0.5% (*w*/*v*) Triton X-100, 30% (*v*/*v*) glycerol), containing 1 mM phenylmethylsulfonylfluoride, Complete protease inhibitor (Roche, Basel, Switzerland), 0.5 μM okadaic acid (Calbiochem Billerica, MA, USA) and 10 μM taxol (Sigma-Aldrich, Gillingham, Dorset, UK). Cell suspensions were centrifuged at 5000 *g* for 10 min at ambient temperature and an aliquot of the supernatant was retained as the post-nuclear lysate (input). The remaining post-nuclear