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Mislocalization of neuronal tau in the absence of tangle pathology in phosphomutant tau knockin mice

Jonathan Gilley [a,*], Kunie Ando [b], Anjan Seereeram [c], Teresa Rodríguez-Martín [c], Amy M. Pooler [c], Laura Sturdee [a], Brian H. Anderton [c], Jean-Pierre Brion [b], Diane P. Hanger [c], Michael P. Coleman [a,1]

[a] Signalling Programme, The Babraham Institute, Cambridge, UK
[b] Laboratory of Histology, Neuroanatomy and Neuropathology, Faculty of Medicine, ULB Neuroscience Institute, Université Libre de Bruxelles, Brussells, Belgium
[c] Department of Basic and Clinical Neuroscience (PO37), Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK

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ABSTRACT

Hyperphosphorylation and fibrillar aggregation of the microtubule-associated protein tau are key features of Alzheimer's disease and other tauopathies. To investigate the involvement of tau phosphorylation in the pathological process, we generated a pair of complementary phosphomutant tau knockin mouse lines. One exclusively expresses phosphomimetic tau with 18 glutamate substitutions at serine and/or threonine residues in the proline-rich and first microtubule-binding domains to model hyperphosphorylation, whereas its phosphodefective counterpart has matched alanine substitutions. Consistent with expected effects of genuine phosphorylation, association of the phosphomimetic tau with microtubules and neuronal membranes is severely disrupted in vivo, whereas the phosphodefective mutations have more limited or no effect. Surprisingly, however, age-related mislocalization of tau is evident in both lines, although redistribution appears more widespread and more pronounced in the phosphomimetic tau knockin. Despite these changes, we found no biochemical or immunohistological evidence of pathological tau aggregation in mice of either line up to at least 2 years of age. These findings raise important questions about the role of tau phosphorylation in driving pathology in human tauopathies.

1. Introduction

Neurodegenerative disorders termed tauopathies are characterized by aggregation of microtubule-associated protein tau into neurofibrillary tangles (NFTs). Among these, Alzheimer's disease (AD) is the most common, but mutations in the *MAPT* gene encoding tau have yet to be implicated as a causative factor. Instead, *MAPT* mutations are associated with a familial tauopathy, frontotemporal dementia with parkinsonism linked to tau mutations on chromosome 17 (FTDP-17T), which is an early-onset, autosomal-dominant disorder (Wolfe, 2009). Although there is currently no clear unifying mechanism to encompass the effects of all the different FTDP-17T mutations, which include disruption of tau microtubule binding and alterations in the proportion of tau isoforms with 3 or 4 microtubule-binding domain (MTBD) repeats (3R or 4R tau), ultimately they all promote the formation of somatodendritic NFTs.

Tau is a phosphoprotein and over 40 serine, threonine, and tyrosine residues have been identified as sites of physiological or disease-associated phosphorylation (Hanger et al., 2009). Although a variety of kinases have the potential to phosphorylate these sites, the number of true in vivo tau kinases is probably more limited (Hanger et al., 2009). Phosphorylation of tau is less extensive in adult brain than fetal brain, but tau does become relatively hyperphosphorylated in NFTs and this occurs at many of the same sites that are phosphorylated during early development (Brion et al., 1993; Johnson and Stoothoff, 2004). However, because developmental phosphorylation of tau does not promote filamentous aggregation, the precise relationship of tau hyperphosphorylation to the pathological process leading to tauopathy remains unclear.

Many mouse models have been created to investigate the role of tau dysfunction in neurodegeneration, but the majority involve transgenic expression of nonphysiological levels of wild-type

* Corresponding author at: Signalling Programme, The Babraham Institute, Babraham Research Campus, Cambridge, CB22 3AT, UK. Tel.: +44 122 349 6634.
E-mail address: jon.gilley@babraham.ac.uk (J. Gilley).
[1] Present address: John van Geest Centre for Brain Repair, Robinson Way, Cambridge, UK.

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human tau isoforms or human tau isoforms bearing FTDP-17T mutations in the presence of endogenous murine tau (Denk and Wade-Martins, 2009; Noble et al., 2010). Although tau phosphorylation is often increased in these mice, assessing its impact separately from the effects of disease mutations, increased expression, and/or altered tau isoform ratios is not possible. Overexpression or activation of known tau kinases, such as glycogen synthase kinase-3β and cyclin-dependent kinase 5, increases tau phosphorylation and can also enhance somatodendritic aggregation of endogenous murine tau or transgene-expressed wild-type or mutant human tau in mice (Ahlijanian et al., 2000; Cruz et al., 2003; Noble et al., 2003; Spittaels et al., 2000; Terwel et al., 2008); however, these kinases are relatively promiscuous meaning that any experimental outcome cannot be conclusively attributed to specific tau phosphorylation sites, or even to their effects on tau alone.

More recently, transgenic mice expressing a permanently pseudohyperphosphorylated phosphomimetic human tau mutant, referred to as PHP tau, were generated to address the role of tau hyperphosphorylation more directly (Hundelt et al., 2011). Surprisingly, expression of PHP tau did not promote significant tau aggregation, NFT formation, or neurodegeneration in these mice, but this could have been due to a relatively low level of PHP tau expression and/or the unexpectedly limited effect that specific subset of modified sites appeared to have on normal tau function.

Here, we have generated two novel, complementary knockin mouse lines to investigate further the impact of phosphorylation of tau at multiple sites on both its normal physiological function and its potential role in driving pathological change. One exclusively expresses a stably pseudohyperphosphorylated tau mutant in which 18 phosphomimetic negative charges have been introduced at specific sites via glutamate substitutions, whereas its counterpart exclusively expresses phosphodefective tau in which incorporation of alanines at the same sites prevents phosphorylation. Comparison between the two should allow us to distinguish potentially phosphorylation-dependent effects from unrelated structural effects of the mutations. Sixteen of the modifications are concentrated in the proline-rich domain (PRD), located centrally in tau in a region containing an unusually high density of potential sites of proline-directed phosphorylation, with the two other modifications located in the first MTBD. Most of the modifications are known sites of phosphorylation in AD brain tau (Hanger et al., 2009) and/or are sites that have been shown to be phosphorylated in murine tau in wild-type and human APP transgenic mice (Morris et al., 2015). Although the average phosphate content of hyperphosphorylated insoluble tau in AD brains shows interindividual variation of between 3 and 15 mol phosphate per mol of tau (Kopke et al., 1993; Ksiezak-Reding et al., 1992), the range at the single molecule level, likely within a highly heterogeneous pool of tau species, is probably even greater. If the overall amount of tau phosphorylation correlates with pathogenicity, then the phosphomimetic tau mutant, with 18 pseudophosporylated sites, might be expected to broadly replicate the effects of some of the most extensively hyperphosphorylated AD-associated tau species. The mutants may also provide additional insight into the role of phosphorylation of residues within the specific domains targeted in this study, both of which are key regulatory regions involved in protein interactions (Ittner and Gotz, 2011; Kolarova et al., 2012; Mietelska-Porowska et al., 2014). Crucially, our phosphomimetic mutant also has a greater pseudophosphorylation load than PHP tau (10 substitutions), and the complement of modifications is largely nonoverlapping (Hundelt et al., 2011).

We find that some key properties of tau are altered in a largely phosphorylation-dependent manner in these mice, including reduced interactions with microtubules and membranes. These are consistent with established in vitro effects of phosphorylation at a variety of sites in tau, including some of those mutated here, although this particular combination of sites has not previously been studied in any context. We also see significant mislocalization of both tau mutants, with strong reductions in axonal tau and increases in somatodendritic tau in some neuron types in both lines. Structural changes in the PRD, rather than pseudophosphorylation, may thus be key, although there is also some evidence for earlier and more pronounced changes in the phosphomimetic tau knockin. Notably, despite these observed changes in tau properties, we could not detect pathological aggregation of tau in either line. We discuss the implications of these results with respect to the role of extensive tau phosphorylation on normal tau function and in neurodegenerative diseases.

2. Materials and methods

2.1. Generation of knockin mice expressing phosphomimetic and phosphodefective tau

Serine and threonine codons in human *MAPT* exon 9 (within a plasmid containing the whole *MAPT* coding region) were mutated to glutamate or alanine by QuikChangeII site-directed mutagenesis (Stratagene, La Jolla, CA, USA). Polymerase chain reaction (PCR) cloning was used to generate the targeting vectors (Fig. 1A) for each knockin (identical bar the mutations) using pEasy-Flox as a backbone (a gift from Profs. W. Müller and K. Rajewsky). Briefly, the 5′ arm (5′ to the Neo^r cassette) was generated by ligation of the mutant human *MAPT* exon 9 (either with 9E18 or 9A18 mutation sets) to sequences flanking the mouse *MAPT* exon 9, using restriction sites and flanking sequences introduced into the PCR primers so that flanking intronic regions remained unaltered. The 3′ arm was cloned as a single fragment. The flanking mouse genomic regions were amplified by PCR from C57BL/6J mouse bacterial artificial chromosomes (Children's Hospital, Oakland Research Institute). Linearized targeting vector was electroporated into C57BL/6-Thy1.1-derived Bruce 4 embryonic stem (ES) cells by the Babraham Institute Gene Targeting Facility. Correctly targeted G418-resistant clones for each knockin were identified by Southern blot analysis of *Spe*I-digested genomic DNA using flanking 5′ and 3′ probes (positions shown in Fig. 1A) and were injected into C57BL/6J-Tyrc-2J (albino) blastocysts and transferred to pseudopregnant women (C57BL/6JOlaHsd × CBA/Ca). Resulting chimeric male offspring were mated to C57BL/6J-Tyrc-2J women and germ line transmission of the mutant allele assessed by Southern blotting. Cre recombinase-mediated excision of the floxed neomycin-resistance cassette (Neo^r) was performed by mating to C57BL/6J deleter mice bearing a ubiquitously expressed X-linked Cre transgene (kindly provided by Miguel Constancia). Mice were PCR genotyped for presence of the knockin alleles using primers 5′-TTGGGGTCACCCTACTGACTTC-3′ and 5′-GACTGAGGAATGGTTCACAGCC-3′ (primer locations and representative results are shown in Fig. 1A and B). Normal Mendelian ratios were obtained from heterozygote crosses. Mice were bred to homozygosity so that mice expressing only each tau mutant were studied. All mice were bred, housed, and used in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986, under Project Licenses PPL 80/1778, 80/2254, and 70/7620.

2.2. Cell culture and transfection

Superior cervical ganglia (SCGs) obtained from P1 or P2 mouse pups were plated whole onto 3.5-cm tissue culture dishes precoated with poly-L-lysine (20 μg/mL for 1–2 hours; Sigma) and laminin (20 μg/mL for 1–2 hours; Sigma) for SCG explant cultures. For dissociated SCG cultures, ganglia were incubated in 0.025%