

Change in tau phosphorylation associated with neurodegeneration in the ME7 model of prion disease

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Abstract

Hyperphosphorylation of the microtubule-associated protein tau is a significant determinant in AD (Alzheimer's disease), where it is associated with disrupted axonal transport and probably causes synaptic dysfunction. Although less well studied, hyperphosphorylation has been observed in prion disease. We have investigated the expression of hyperphosphorylated tau in the hippocampus of mice infected with the ME7 prion agent. In ME7-infected animals, there is a selective loss of CA1 synapse, first discernable at 13 weeks of disease. There is a potential that dysfunctional axonal transport contributes to this synaptopathy. Thus investigating hyperphosphorylated tau that is dysfunctional in AD could illuminate whether and how they are significant in prion disease. We observed no differences in the levels of phosphorylated tau (using MC1, PHF-1 and CP13 antibodies) in detergent-soluble and detergent-insoluble fractions extracted from ME7- and NBH- (normal brain homogenate) treated animals across disease. In contrast, we observed an increase in phospho-tau staining for several epitopes using immunohistochemistry in ME7-infected hippocampal sections. Although the changes were not of the magnitude seen in AD tissue, clear differences for several phospho-tau species were seen in the CA1 and CA3 of ME7-treated animals (pSer^{199–202} > pSer²¹⁴ > PHF-1 antibody). Temporally, these changes were restricted to animals at 20 weeks and none of the disease-related staining was associated with the axons or dendrites that hold CA1 synapses. These findings suggest that phosphorylation of tau at the epitopes examined does not underpin the early synaptic dysfunction. These data suggest that the changes in tau phosphorylation recorded here and observed by others relate to end-stage prion pathology when early dysfunctions have progressed to overt neuronal loss.

Introduction

Active bi-directional transportation of organelles along the axon between the cell body and the synapse is essential for neuronal signalling and survival [1]. Synaptic components, and their precursors, are transported anterogradely by kinesin motors moving along MTs (microtubules), whereas the dynein–dynactin complex mediates retrograde axonal transport, from synapse to the cell body [2,3]. Tau is an MT-associated protein that binds to and stabilizes MTs in axons. This ensures MT-dependent axonal transport of vesicles and organelles by motor proteins [4], but, upon phosphorylation, tau affinity for MTs is reduced [5,6], leading to a decrease in MT stability which cumulatively leads to loss of function, aggregation of tau and formation of NFTs (neurofibrillary tangles) [7]. Phosphorylation is sufficient to induce this loss of function as dephosphorylation of pathological tau by phosphatases restores the MT-stabilizing activity of tau [8]. In addition, hyperphosphorylated and/or aggregated

species of tau may exert direct toxic effects on neurons [9]. Hyperphosphorylated tau and paired helical filaments are a characteristic of AD (Alzheimer's disease) [10], as well as some prion diseases [11]. Also, there is a reported increase in tau protein and phospho-tau in cerebrospinal fluid in sporadic CJD (Creutzfeldt–Jakob disease) patients [12–14].

In animals inoculated with ME7 prion, the ensuing disease involves an early synaptic degeneration independent of subsequent cell loss [15]. It may be that, during disease progression, pathological misfolded PrP (prion protein), PrP^{Sc} (scrapie isoform of PrP), results in blockade of axonal transport. Indeed, past studies have also suggested a molecular interaction between tau protein and PrP [16]. Nevertheless, transgenic mice overexpressing four-repeat tau with a reported impairment of axonal transport displayed comparable incubation times with those in control animals following intraneural infection with scrapie [17]. There was also no change in the incubation time after peripheral prion infection in mice with a heterozygous mutation of dynein, a motor protein involved in the transport of axonal cargo along the cytoskeleton [18]. Studies in BSE (bovine spongiform encephalitis)-infected bovine prion protein (Bo-PrP) transgenic animals reported an increase in hyperphosphorylated tau [19]. Together, these studies imply

Key words: Alzheimer's disease, ME7, neurodegeneration, phosphorylation, prion, tau.

Abbreviations used: AD, Alzheimer's disease; BSE, bovine spongiform encephalitis; CJD, Creutzfeldt–Jakob disease; GFAP, glial fibrillary acid protein; MT, microtubule; NBH, normal brain homogenate; PrP, prion protein; Bo-PrP, BSE-infected bovine PrP; PrP^{Sc}, scrapie isoform of PrP; 3×Tg-AD, triple-transgenic AD.

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that tau contributes directly to disease or might act as a useful marker defining key stages in disease progression. We have taken advantage of our observation that synapse loss occurs relatively early in ME7 disease to investigate whether changes in tau are implicated in this early pathology. Using previously described protocols for Western blotting and immunocytochemical investigation of tau [20,21], we found data to suggest that tau is not causative in early disease mechanism, but rather reflects aspect(s) of end-stage prion disease pathology.

ME7 model

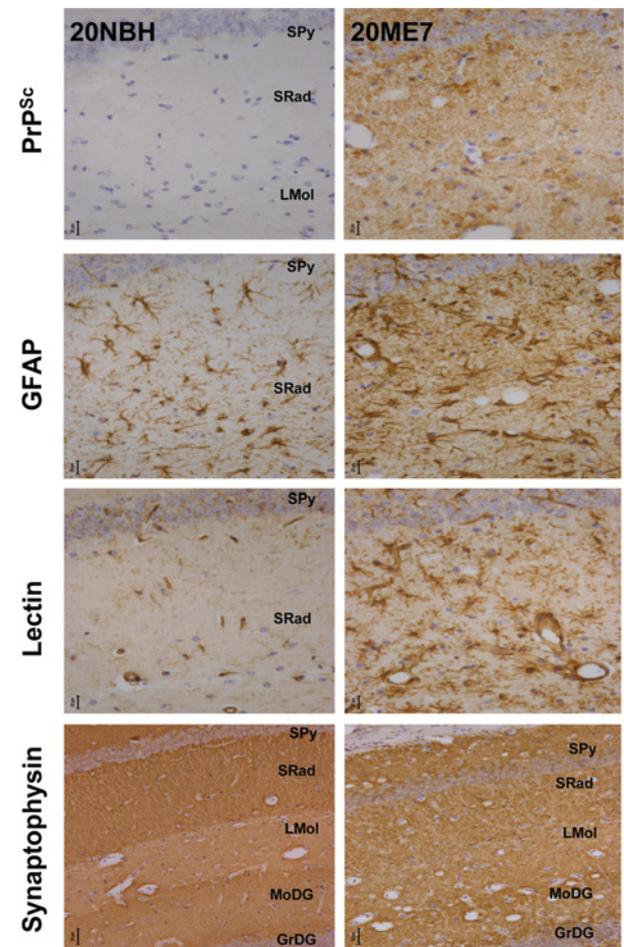
Before commencing analyses of tau biochemistry and immunohistochemistry, neuropathological examination was performed on brains used for the study described below. This revealed spongiform changes, synaptic degeneration, astrogliosis and abundant PrP immunoreactivity in hippocampal extracts and sections from ME7- when compared with NBH- (normal brain homogenate) treated animals. These comparisons used animals at 13 and 20 weeks after inoculation to define relatively early and late stages of disease (Figure 1) [20,21]. Immunostaining with 6H4 monoclonal anti-PrP antibody reveals PrP^{Sc} deposits at the late stage of disease, anti-GFAP (glial fibrillary acid protein) antibody staining shows large numbers of activated astrocytes, and tomato lectin staining shows microglia with altered morphology. Anti-synaptophysin staining depicts increasing disorganization of synaptic architecture and decreased expression of presynaptic marker protein in ME7-compared with NBH-treated animals. In a similar way, Western blot analysis of brain homogenates from ME7-compared with NBH-treated animals revealed accumulation of PrP^{Sc}, decreased immunoreactivity for synaptophysin (synapse loss) and increased GFAP (astrocytosis) (results not shown) [20–22].

Tau biochemistry

Hippocampal homogenates from NBH- and ME7-treated animals described above were separated into detergent-soluble and detergent-insoluble fractions by ultracentrifugation in RIPA buffer containing 1% Nonidet P40 as described in [20]. The insoluble pellet is enriched in tau aggregates and such insolubility is a characteristic of authentic AD-derived tau filaments [23,24]. In addition, hyperphosphorylated tau has reduced MT binding and is released into the soluble pool. This is associated with a destabilized MT network and leads to deficits in the neuronal transport. Thus investigating the proportion of various tau species in these fractions from control and diseased brains gives distinct biochemical correlates of dysfunctional tau. We used a phosphorylation-independent anti-(total tau) antibody to immunoblot detergent-soluble and detergent-insoluble tau extracted from the hippocampus of NBH- and ME7-treated animals at 13 and 20 weeks. Tau resolved as a band of 45–50 kDa (Figure 2A). This pattern was identical with that detected with anti-phospho-tau antibodies PHF-1 (epitope at

Figure 1 | Immunohistochemical findings in ME7- compared with NBH-treated animals

Coronal section of the dorsal hippocampus reveals widespread PrP-immunoreactivity (6H4 immunostaining), pronounced astrogliosis (GFAP immunostaining), evidence of microgliosis (tomato lectin immunostaining) and synaptic degeneration (synaptophysin immunostaining). Scale bars, 20 μ m, except for synaptophysin: 50 μ m. In the PrP-immunohistochemistry panels, note the evidence of diffuse and focal PrP deposits. In the synaptophysin-immunostaining panels, note the loss of discrete laminae with late-stage disease. Nuclei were counterstained with haematoxylin. GrDG, granular layer dentate gyrus; LMol, molecular layer; MoDG, molecular layer dentate gyrus; SPy, stratum pyramidale; SRad, stratum radiatum.



residues 396/404), MC1, a conformation-dependent antibody (amino acids 7–9 and 313–322), and CP13 (epitope at residue 202), which were gifts from Dr Peter Davies (Albert Einstein College of Medicine, New York, NY, U.S.A.) (Figures 2B–2D). The immunoblots were quantified and are presented as normalized relative pixel intensities (Figures 2E–2H) [21,25]. Total tau content measured in both detergent-insoluble and detergent-soluble fractions did not differ in NBH-compared with ME7-treated animals at 13 or 20 weeks, but we observed a higher level of detergent-soluble tau compared with detergent-insoluble tau at 20 weeks. Phosphorylation of

tau as measured by three antibodies (CP13, MC1 and PHF-1) showed no difference between NBH- and ME7-treated animals at either time point (Figure 2E-H). However, as with the total tau, we observed a higher level of phospho-tau immunoreactivity in the detergent-soluble tau fractions compared with detergent-insoluble tau at 20 weeks.

Tau histology

We immunostained coronal sections adjacent to those shown in Figure 1 with anti-phospho-tau antibodies and revealed numerous tau-positive cellular profiles in the hippocampus. As a control for disease-associated hyperphosphorylated tau, we examined the triple-transgenic AD (3×Tg-AD) mouse model. These mice harbour the mutant genes for amyloid precursor protein_{SWE}, for presenilin 1_{M146V} and for tau_{P301L}, and show temporal- and region-specific tau pathology, closely resembling that seen in the human AD brain [26]. We found that several brain regions were tau-immunopositive in the 3×Tg-AD animals as reported previously [26] (Figure 3, and results not shown). In the 3×Tg-AD mice, the neurons were intensely labelled by markers of tau pathology PHF-1, pSer²¹⁴ (epitope at residue 214 (results not shown), and in particular pSer^{199–202} antibody (epitope at residues 199–202). There is abundant pSer^{199/202} staining in the hippocampus and, in the case of CA1 neuronal somata, the staining extends into dendritic processes. This is consistent with the mis-targeting of tau upon hyperphosphorylation, as seen in AD.

Sections from NBH- and ME7-treated animals at 13 and 20 week were treated in parallel, and comparative staining was carried out with the same antibodies. We observed a clear increase in the pattern and intensity of tau accumulation in ME7- compared with NBH-treated animals at 20 weeks (Figures 3A–3F). The NBH- and ME7-treated animals at 13 weeks lacked these changes (results not shown), suggesting that they were selective to late-stage pathology. Much of the staining in ME7-treated animals is atypical relative to tau pathology in 3×Tg-AD animals. In particular, ME7-treated animals showed an anomalous presence of phospho-tau-immunoreactive neuritic profiles, which were unlike those of the 3×Tg-AD positive control (Figures 3A–3F, arrowheads). PHF-1 antibody showed an increased staining in ME7-treated animals relative to NBH. This increase was sparsely scattered in CA1 and CA3 and restricted to 20 weeks (Figures 3A–3B, and results not shown). The anti-pSer²¹⁴ antibody stains primarily CA1 hippocampal neuronal somata in ME7-treated animals (Figure 3C) and CA3 (Figure 3D) but not in NBH-treated animals. There are also tau-immunopositive cells in the molecular layer that may be non-neuronal (bottom arrowhead). The anti-pSer^{199/202} antibody stains more scattered neurons in ME7- compared with NBH-treated animals (Figures 3E and 3F, arrowheads). There is some evidence of pSer^{199/202} staining in the neuronal somata of NBH-treated animals at 20 weeks (Figures 3E and 3F, arrowheads), but in ME7-treated animals, the staining is increased in the neuronal somata, neuropils and across all hippocampal subfields and their layers, including those

relatively deficient in neuronal cell bodies (Figures 3E and 3F, arrowheads). Overall, the pattern of immunoreactivity was different with all anti-phospho-tau antibodies, but there was a consistent increase in the immunohistochemically detected phospho-tau intermediates in the ME7-infected tissue.

Tau and prion disease

Hyperphosphorylation of tau and abrogation of its physiological function [27,28] can lead to alteration of cellular transport of essential materials to and from the synapses [29,30]. This altered transport may lead to progressive degeneration initiated at the distal end of the processes before moving retrogradely towards the cell body [31]. The appearance of phosphorylated tau, well known in AD and Pick's disease, is also apparent in prion diseases [10,11]. In the latter case, this could conceivably contribute to or be used experimentally to highlight underlying transport deficits. Phospho-tau deposition was assessed in detergent-soluble and -insoluble hippocampal extracts and histologically in hippocampal subregions using the phospho-dependent tau antibodies. The total tau antibody revealed no differences in the abundance of detergent-soluble and -insoluble tau in NBH- compared with ME7-treated animals. Western blotting using PHF-1, MC1 and CP13 (Figure 2), anti-pSer²¹⁴ and anti-pSer^{199/202} (results not shown) antibodies revealed that insoluble tau was phosphorylated at multiple sites in both NBH- and ME7-treated animals at 13 and 20 weeks, but no differences were observed between disease and control. In contrast, immunohistology suggests that PHF-1, pSer²¹⁴ and, most particularly, immunoreactivity to the pSer^{199/202} epitopes were more evident in ME7-treated than NBH-treated animals at 20 weeks. Even though there was a clear difference in the phospho-tau staining in ME7-infected tissue, it was clearly more restricted than seen in the 3×Tg-AD tissue. Indeed, the failure to detect differences in phospho-tau epitopes in ME7 hippocampal extracts biochemically suggests that the changes observed are rather restricted. There are some caveats inherent to quantification of the Western blot data as both the MC1 and PHF-1 antibodies show greater specificity towards pathological tau on histological sections than in Western blots [32,33]. Also, the use of Western blots for quantification averages in a crude mixture of changing cell types, masking more discrete changes revealed by immunohistochemistry. The brain regional distribution of phospho-tau in ME7-treated animals paralleled the topography of astrogliosis and PrP^{Sc} deposition observed in these animals [21], and some of the staining associated with ME7-infected tissue suggests that it might be associated with non-neuronal cells. Thus the rather restricted changes of phospho-tau staining in ME7 disease provides three lines of evidence that suggest it is not associated with the early synaptic dysfunction in prion disease. First, it appears only at late time points of disease some time after the initiation of synapse loss. Secondly, it is associated with degenerating neurons and/or non-neuronal cells that are reactive to neuronal loss. Thirdly, the ME7-induced

Figure 2 | For legend see facing page

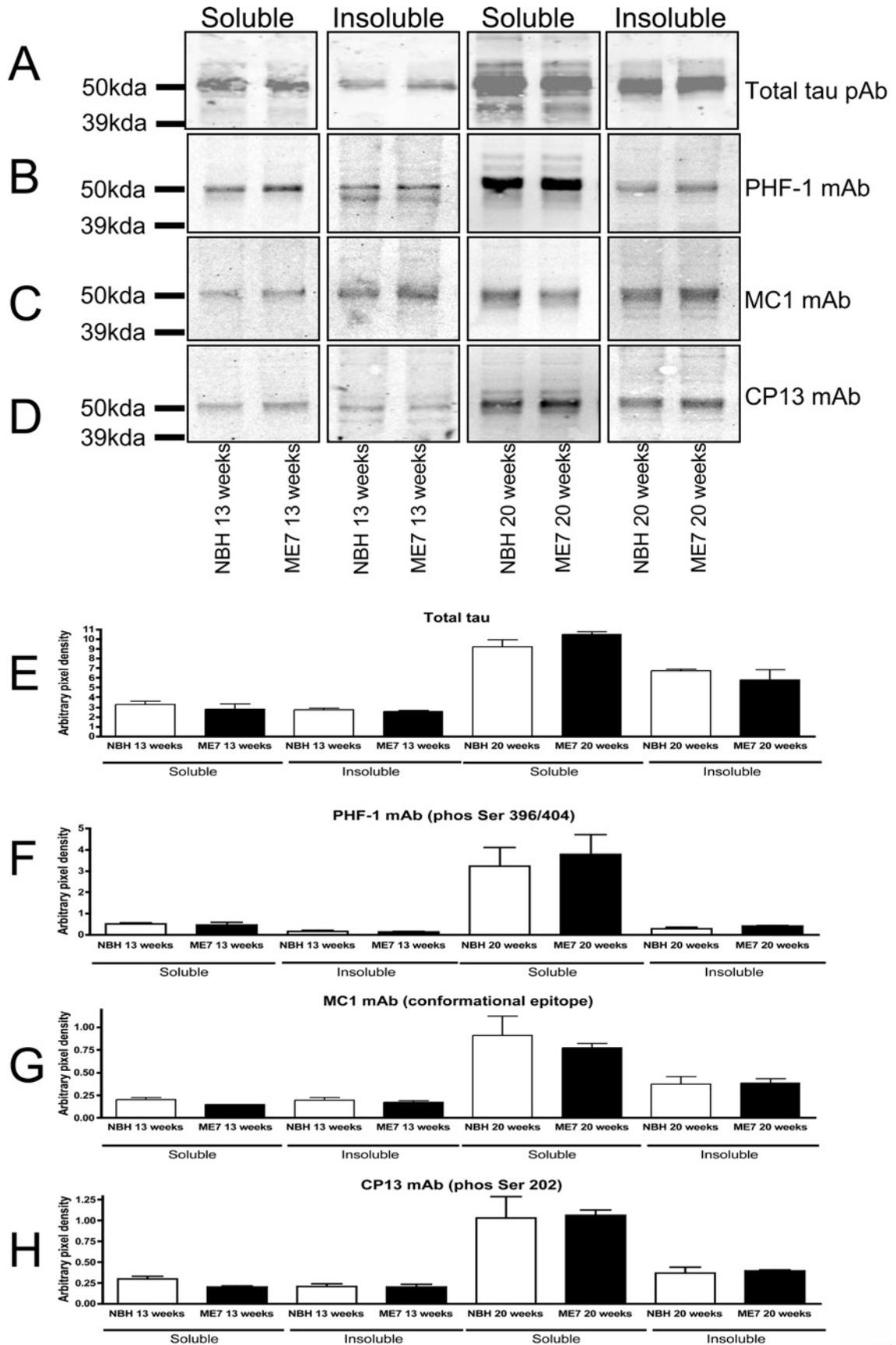
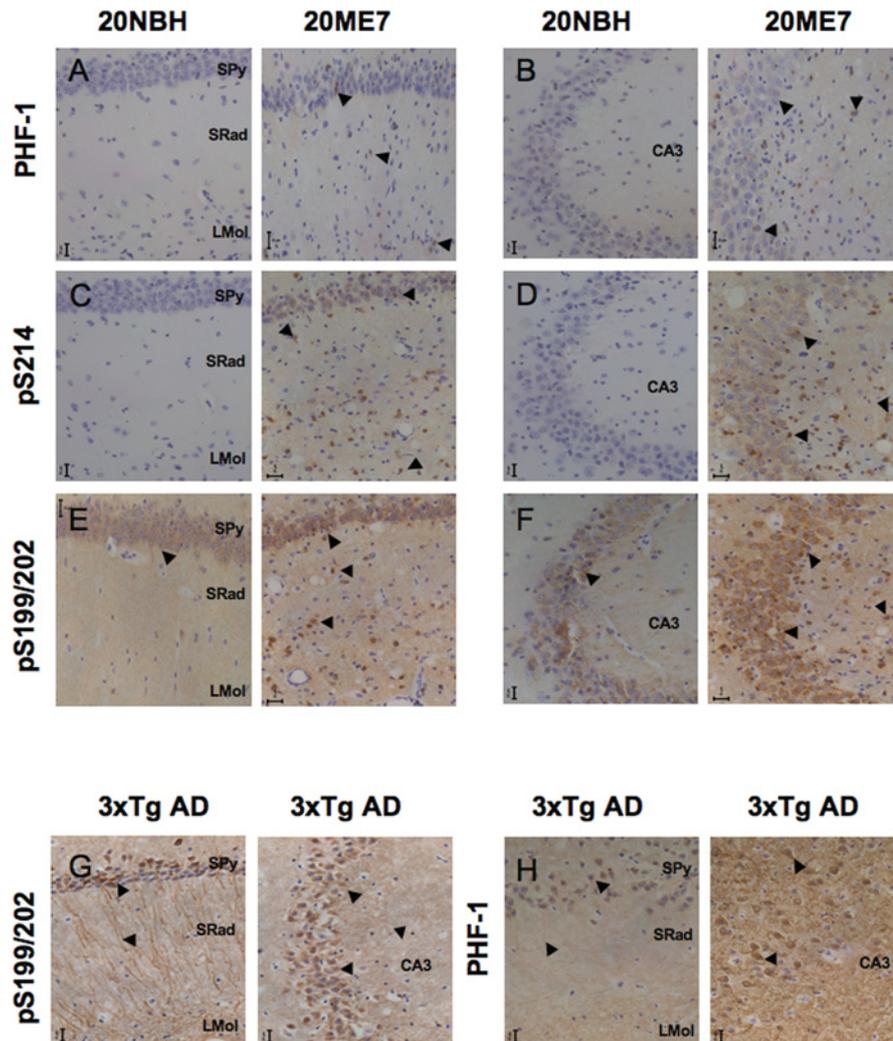


Figure 2 | Analysis of Western blots demonstrating tau phosphorylation status in detergent-extracted fractions from NBH- and ME7-treated animals

Samples of 20 μg of detergent-soluble and detergent-insoluble extracts from the hippocampus of 13 weeks and 20 weeks NBH- and ME7-treated animals were prepared, run on SDS/PAGE and immunoblotted with phosphorylation-independent and phosphorylation-dependent tau antibodies. **(A)** total tau, **(B)** MC1, **(C)** PHF-1 and **(D)** CP13. Representative blots are shown ($n=3$). Total tau detected with the phosphorylation-independent tau antibody recognized a single band migrating with an apparent molecular mass of 48–50 kDa corresponding to the band recognized by the phosphospecific antibodies. Quantitative analysis was performed by measuring protein expression relative to the fluorescence of total protein from individual samples stained with colloidal Coomassie Blue **(E–H)**. Integrated intensity data in each bar are means \pm S.E.M.

Figure 3 | Differential distribution of phospho-tau epitopes in the brains of NBH- and ME7-treated animals

Images of 10 μm adjacent coronal section from NBH- and ME7-treated animals immunostained with PHF-1, MC1, CP13, anti-pSer²¹⁴ and anti-pSer^{199/202} antibodies. **(A and B)** With the PHF-1 antibody, there was no difference in staining at 13 weeks in ME7- compared with NBH-treated animals. At 20 weeks, immunoreactive phospho-tau was localized to a distinct somal region (arrowheads), and was more pronounced in ME7- compared with NBH-treated animals. **(C and D)** There were no differences in the staining between NBH- and ME7-treated animals at 13 weeks (results not shown), but at 20 weeks, the CP13 antibody revealed increased pSer²¹⁴ staining in the ME7 tissue. Similar observations were made with the MC1 antibody (results not shown). **(E and F)** Some neuronal perikarya were detected by anti-phospho-tau antibody pSer^{199–202}, indicating pre-tangle-like structures in the hippocampus. This was restricted to 20 weeks and was more pronounced in ME7- compared with NBH-treated animals at 20 weeks. **(G, H)** Sections from 12-month old 3 \times Tg-AD mice were included as positive controls as these animals have substantial tau pathology. Note the pronounced dendritic staining (arrowhead) with the anti-pSer^{199/202} antibody. Scale bar, 20 μm . LMol, molecular layer; SPy, stratum pyramidal; SRad, stratum radiatum.



phospho-tau immunoreactivity is not associated with the neuronal compartments (axons or dendrites) that might express dysfunctional transport to drive a synaptic loss.

Nevertheless, increased phospho-tau immunoreactivity (PHF-1, pSer²¹⁴, pSer^{199/202}) in ME7- compared with NBH-treated animals is consistent with studies in BSE-infected Bo-PrP transgenic animals where increased hyperphosphorylated tau is reported [19]. It is also in accord with reports of an increase in CSF (cerebrospinal fluid) phospho-tau levels associated with sporadic CJD [13,34], studies in human vCJD (variant CJD) and experimental mouse models that showed diffuse phospho-tau-positive neurites and perikarya, associated with PrP amyloid [35]. Consistent with this, accumulations of hyperphosphorylated tau, identical with that in AD, have been described coincident with PrP^{Sc} deposits in GSS (Gerstmann-Sträussler-Scheinker) disease [36]. Dystrophic tau-immunoreactive neurites were also observed associated with PrP deposits in very old mice with advanced scrapie infection [37]. Taken together, our data suggest that modest changes in tau metabolism are associated with late-stage disease, but argue against its use as a causative pathway or indeed a useful marker in the early processes that give rise to the synaptopathy associated with disease. This still leaves unresolved the potential role of axonal transport deficits as important determinants in the early stages of prion disease.

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