Tau phosphorylation: the therapeutic challenge for neurodegenerative disease

Diane P. Hanger, Brian H. Anderton and Wendy Noble

MRC Centre for Neurodegeneration Research, King’s College London, Institute of Psychiatry, De Crespigny Park, London, SE5 8AF, UK

The microtubule-associated protein tau is integral to the pathogenesis of Alzheimer’s disease (AD), as well as several related disorders, termed tauopathies, in which tau is deposited in affected brain regions. In the tauopathies, pathological tau is in an elevated state of phosphorylation and is aberrantly cleaved. It also exhibits abnormal conformations and becomes aggregated, resulting in neurofibrillary tau pathology. Recent evidence suggests that relatively early disease-associated changes in soluble tau proteins, including phosphorylation, are involved in the induction of neuronal death. Here, we summarize recent developments that suggest new therapeutic strategies to prevent or reduce the progression of pathology in the tauopathies. A list of tau phosphorylation sites identified in the tauopathies and in controls accompanies this review.

The increasing need for new drugs targeting the tauopathies

Alzheimer’s disease (AD) is the most common form of dementia and the most prevalent of the tauopathies. Increasing age is the most significant risk factor for dementia, with ~5% of the population being affected at 65 years of age, increasing to more than 20% from the age of 80 (http://www.alz.co.uk/alzheimers). Currently, in excess of 30 million people worldwide are suffering from dementia and thus therapies for AD are urgently needed. If no new treatments for AD become available, the number of affected individuals is predicted to reach 100 million by 2050 (http://www.alz.co.uk/alzheimers). Existing treatments for AD do not effectively halt or slow disease progression, and this is due in part to a lack of knowledge of the mechanisms involved in disease pathogenesis.

In addition to AD, the tauopathies encompass a range of neurodegenerative disorders, including progressive supranuclear palsy (PSP), Pick’s disease, corticobasal degeneration and fronto-temporal dementia linked to chromosome 17 with parkinsonism (FTDP-17T), in which mutations in the tau gene (microtubule-associated protein tau [MAPT]) are causal. The precise pathological signatures and clinical characteristics of the tauopathies are disease-dependent but all exhibit distinctive intracellular inclusions, such as flame-shaped or globular neurofibrillary tangles and/or neurophil threads (fine filamentous structures found primarily in dendrites). These various inclusions are comprised of insoluble, highly phosphorylated forms of tau and are likely to play a part in the neuronal loss evident in tauopathies [1].

Recent evidence suggests that targeting tau phosphorylation through inhibition of protein kinases could represent a valid therapeutic approach to reduce tau aggregation and associated neuronal death [2]. However, given the large number of phosphorylation sites and the many protein kinases implicated in tau phosphorylation, the potential therapeutic targets are numerous [3,4]. In this review, we summarize current knowledge of tau phosphorylation in disease and consider how this could be targeted in AD and other tauopathies.

Tau exists as multiple isoforms of a highly soluble phosphoprotein

In the adult human central nervous system (CNS), alternative splicing generates six tau isoforms that differ by the regulated inclusion of two inserts near the N terminus and either three or four imperfect repeat sequences, corresponding to the highly conserved, microtubule-binding domain, in the C-terminal half of tau (Figure 1a). Splicing generates two sets of tau isoforms, each containing either three (3R) or four (4R) microtubule-binding repeats with differential affinity for microtubules. The 4R:3R tau ratios for both mRNA and protein are approximately equal in normal brain, but disturbances, usually increases, in these ratios occur in most of the neurodegenerative tauopathies.

Glossary

Alternative splicing: a process by which different forms of mRNA are produced from a single gene. In the case of tau, exons 2, 3 and 10 are variably included in the mature transcript for the translation of six CNS isoforms.

Amyloid: in the context of neurodegenerative disease, amyloid is mis-folded protein that forms an extracellular aggregate with β-pleated sheet structure. In AD, extracellular deposits of Aβ are formed from amyloid precursor protein.

Neurodegeneration: the process of progressive loss of neurons in diseased brain.

Neurofibrillary tangles: filaments of proteins deposited within neurons; a characteristic pathological feature of AD brain.

Protein kinases: enzymes that catalyse the transfer of a phosphate group from a donor, such as ATP, to a protein substrate (e.g. tau).

Protein phosphatases: enzymes that catalyse the hydrolysis of esters of phosphoric acid, resulting in removal of phosphate groups from proteins.

Tauopathy: neurodegenerative disease in which intracellular pathological aggregates of tau protein are present in brain.

Transgenic mouse: a mouse that has been genetically engineered to express a particular gene of interest.
Within neurons, tau is found predominantly in axons, where it exists as a highly soluble, phosphorylated protein that stabilizes and promotes the polymerization of microtubules principally through the microtubule-binding domain. The processes of phosphorylation and splicing of tau are both developmentally regulated, with increased phosphorylation occurring at embryonic and early developmental stages, a time at which only one tau isoform is expressed, this being the smallest of the 3R variants lacking the N-terminal inserts (Figure 1a) [5,6]. By contrast, all six tau isoforms are present in mature brain, and phosphorylation of these tau species is relatively reduced compared to that in foetal brain. Such developmentally regulated changes in tau are likely to be related to an increased requirement for neuronal plasticity during embryogenesis and early development. Increased tau phosphorylation reduces the amount of microtubule-bound tau, and 3R tau isoforms also bind less tightly than 4R tau to microtubules. These factors thus support the notion that the requirement for increased plasticity can be met by elevated phosphorylation of 3R tau during periods of prolific synaptogenesis. Furthermore, reduced plasticity is apparent in fully differentiated adult neurons in which 4R tau isoforms are also expressed and tau phosphorylation is decreased [7].

Phosphorylation in the microtubule-binding domain (residues 244–368) of tau is believed to be crucial in regulating microtubule stabilization. In particular, phosphorylation of the orthologous residues in adjacent microtubule-binding repeats at S262 and S356 has been suggested to detach tau from microtubules [8]. Phosphorylation of tau at sites distinct from the microtubule-binding domain might also be involved in regulation of cytoskeletal stability because S214 and T231 in tau also reduce its ability to bind to microtubules [9]. The putative binding site of the peptidyl-prolyl isomerase Pin1 (peptidyl-prolyl cis/trans isomerase, NIMA-interacting 1) is phosphorylated serine or threonine followed by proline, of which there are 15 such motifs in tau, including T231-P232 [10]. However, Pin1 interacts only with phosphorylated T231, with its binding resulting in a conformational change that restores the ability of tau to bind to microtubules [9]. The concentration of phosphorylated T231 in cerebrospinal fluid is associated with increased neurofibrillary tangle load in AD, and a deficit in Pin1 results in neurodegeneration, together indicating that phosphorylation at this residue might be significant in AD pathogenesis [12]. However, the relative importance of specific individual phosphorylation sites (e.g. S214, T231, S262, S356) or subsets of phosphorylation sites (e.g. in the microtubule-binding

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**Figure 1.** Six isoforms of human CNS tau and phosphorylation sites detected on tau from Alzheimer brain. (a) Illustration of the six isoforms of human CNS tau, exons 2, 3, and 10 are alternatively-spliced. Exons 2 and 3 (E2 and E3) encode two different inserts of 28 amino acids near the N-terminus of tau. Absence of E2 and E3 gives rise to 0N tau isoforms, whereas inclusion of E2 produces 1N and inclusion of both E2 and E3 results in 2N tau isoforms. M1–M4 represent the four imperfect-repeat microtubule-binding domains, M2 being encoded by exon 10. Lack of M2 produces 3R tau and inclusion results in 4R tau isoforms. The proline-rich domain (PRD) in the centre of the tau polypeptide is indicated. Alternative-splicing produces tau isoforms ranging in size from 352–441 amino acids. (b) Positioning of phosphorylation sites on tau from Alzheimer brain. Approximately 45 sites have been identified, and they seem to cluster in the PRD and in the C-terminal region, with few sites evident within the microtubule-binding domain of tau. Six of the phosphorylation sites have been identified only by phospho-specific antibody labelling (indicated in orange); the remaining phosphorylation sites have been identified by direct means (mass spectrometry and/or Edman degradation).
domain) for tau function and/or pathogenesis remains to be firmly established.

**Pathological tau phosphorylation**

In AD, extracellular deposition of neurotoxic amyloid β-peptide (Aβ) derived from amyloid precursor protein (APP) is a characteristic neuropathological feature of the disease. However, tau increasingly seems obligatory to the processes leading to neurodegeneration in the tauopathies and lack, or a reduced amount, of tau is associated with neuroprotection and resistance to Aβ toxicity [13–15]. Conversely, elevated tau expression enhances susceptibility to toxic stimuli and/or neurodegeneration and also results in increased production of neurotoxic amyloidogenic peptides [16].

Based on reactivity to phospho-specific tau antibodies, the pattern of tau phosphorylation in the various tauopathies seems to be similar irrespective of the underlying cause of the pathology. Tau abnormalities, whether they are due to mutations in the tau gene causing amino acid changes or an altered 4R:3R tau ratio, cause deposition in the brain of highly phosphorylated tau in an aberrant conformation. However, as mentioned above, although intracellular accumulations of phosphorylated tau have been assumed to be harmful to cells, an alternative role is one of protection against toxic events. An increase in the steady-state level of tau might result in excess cytoplasmic tau becoming deposited in insoluble intracellular inclusions in an effort to restore to physiological levels the amount of tau available for microtubule binding in neurons. A recent study has shown that tangles continue to form in tau-expressing transgenic mice even after tau expression is switched off, suggesting that soluble, rather than aggregated, tau species are neurotoxic, a view supported by the apparent dissociation between the number of tangles and the extent of neuronal death in this study and in comparable models of disease [14,17].

**Tau as a therapeutic target**

The principal existing strategies targeting tau in neurodegenerative disease include (i) reducing tau phosphorylation through inhibition of specific protein kinases, (ii) disaggregating tau inclusions and (iii) tau immunotherapy. The issue of whether phosphorylation of tau precedes or follows tau aggregation remains a subject of debate, but reducing tau phosphorylation is regarded by many as the preferred target, and some transgenic animal studies have shown this to be a valid strategy [18].

Inhibition of proline-directed protein kinases, such as glycogen synthase kinase-3 (GSK-3) and cyclin-dependent kinase-5 (cdk5) has recently been pursued in transgenic models of tauopathies. For example, inhibiting GSK-3 in tangle-forming transgenic mice reduces tau phosphorylation, insoluble tau load and neurodegeneration [19,20]. Lithium, an inhibitor of GSK-3, has also been shown to reduce the total amount of neuronal tau, a factor that, as mentioned above, might have additional neuroprotective benefit [21]. Similar beneficial effects have been observed using kinase inhibitors of relatively broad specificity to target cdk5, extracellular signal-regulated kinase-2 (ERK2) and cyclic AMP-dependent protein kinase (PKA), amongst others [22]. Furthermore, recent studies in double transgenic mice overexpressing a combination of mutant P301L tau with either APP or GSK-3 have suggested that tauopathy in these animals is mediated by activation of GSK-3, indicating that, at least in these models, GSK-3 has a crucial role in pathogenesis [23]. Cdk5 has also been implicated in the development of neurofibrillary pathology because its overexpression in other double transgenic mice has been shown to elevate or induce tau hyperphosphorylation and tangle formation [24,14]. Non-receptor tyrosine kinases are also associated with AD pathology. Fyn, Syk and c-Abl phosphorylate tau at Y18, Y197 and Y394, respectively [25], and neurons from Fyn-knockout mice are resistant to Aβ toxicity [26,27]. The tyrosine phosphorylation state of tau also seems to correlate with its propensity to aggregate [28]. The form of tau present in detergent-resistant membranes, which are likely to harbour lipid rafts and might be important in the initiation of the neurotoxic response to Aβ, is also tyrosine phosphorylated [27]. Tau contains five Src homology 3 (SH3) domains within PXXP motifs that are responsible for binding of tyrosine kinases [29], suggesting a possible role for tyrosine phosphorylation of tau in signal transduction pathways. Thus, targeting kinases is a rational strategy that is currently undergoing clinical investigation by pharmaceutical companies, with some GSK-3 inhibitors having entered clinical trials for AD [30].

Aβ-disaggregating agents in AD have not yet proved to be a viable therapy, and recent trials have been halted owing to lack of efficacy, possibly caused by release of toxic oligomers of Aβ [31,32]. However, a recent meeting report has pointed to the potential therapeutic use of agents that disaggregate tangles, and a compound is currently in phase II trials (http://www.alzforum.org/new/detail.asp?id=1892). Indeed, pre-clinical studies of tau aggregation inhibitors have shown promise [33–35]. Evidence suggests that oligomeric forms of tau might also have a role in disease pathogenesis, and dissolution of tangles using drugs targeting tau aggregation could conceivably result in increased amounts of available tau oligomers [36]. Thus, it remains to be seen whether disaggregating tangles is beneficial or whether release of tau oligomers might instead enhance pathogenicity and disease progression in the tauopathies.

Protocols for tau immunotherapy have been largely overlooked because, unlike Aβ, pathological tau deposits are intraneuronal, and it was assumed that antibodies directed at tau would not be internalized into neurons and would therefore fail to come into contact with their target. However, a recent report has shown that immunization with a phosphorylated tau epitope does reduce the development of tauopathy and also slows progression of the tangle-related behavioural phenotype in tau transgenic mice [37]. However, tau immunotherapy strategies, although providing promise for the future, remain under-developed. Targeting kinases therefore remains the most rational strategy for finding new drugs to ameliorate tau-induced neurodegeneration.

**Identifying relevant tau kinases and phosphatases**

Many kinases have been shown to phosphorylate tau in vitro and in cells [4,38–41]. It is important therefore to
identify which tau kinases to target, and this process requires knowledge of all of the phosphorylation sites in tau in both physiological and pathological states. There have been a large number of studies aimed at identifying phosphorylation sites generated by the actions of individual kinases on recombinant tau protein in vitro (Figure 1b and Table 1). These reports have produced a vast amount of information regarding the activities of specific kinases on tau, but the identities of the true physiological and pathological kinases in vivo remain unknown. The pattern of tau phosphorylation sites has led to speculation that individual kinases could modulate the functional properties of tau, but there exists considerable overlap of the residues phosphorylated by different kinases. The most promising candidate kinases for tau phosphorylation include GSK-3, cdk5, casein kinase 1 (CK1) and PKA (see Table 1), as well as microtubule-affinity-regulating kinase and the stress-activated protein kinases, including the p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) families, all of which have been the subject of much research [2,4,18,39-46]. Other tau kinases, including p42/p44 MAPKs (ERKs 1 and 2), protein kinase C, brain-specific kinases 1 and 2, and tau-tubulin kinases 1 and 2 also have the potential to be involved in tau phosphorylation and should not be overlooked. Table 1 provides a summary of the directly identified phosphorylation sites on Alzheimer tau, along with details of which of these sites are generated on recombinant human tau in vitro by the actions of specific kinases. A complete up-to-date listing of tau phosphorylation sites, including in vivo sites identified in AD, PSP, control human brain and rat foetal brain, in addition to in vitro kinase activity, can be found at http://cnr.iop.kcl.ac.uk/hangerlab/tautable. This fully comprehensive listing illustrates the multitude of coincident residues phosphorylated by multiple kinases, suggesting that tau is a somewhat promiscuous substrate. The data raise the possibility that the overall level of tau phosphorylation might be more important than modification of individual residues. There might also be a hierarchical process of phosphorylation that involves priming of tau by one kinase for another, as suggested previously for the action of GSK-3 on tau [47,48].

**Identification of phosphorylation sites on human brain tau**

An approach to identify sites of tau phosphorylation in vivo, using either direct sequencing methods (mass spectrometric analysis or Edman degradation of phospho-peptides) or indirect methods such as phospho-specific tau antibodies, has resulted in the identification of many phosphorylation sites on tau extracted from brain in either physiological or pathological states [4,49]. Mapping antibody phosphoepitopes on tau has also generated useful reagents, but this approach has the disadvantage that it requires prior knowledge of antibody epitopes [50-52]. Phosphopeptide sequencing of tau using mass spectrometry and/or Edman degradation has identified all or most of the phosphorylation sites on tau-enriched preparations from human and rat brain. The number of tau phosphorylation sites on Alzheimer tau is significantly greater than that of soluble tau from post-mortem control human brain from which ~10 phosphorylation sites have been identified [4]. Recent data, including more than 90% coverage of the tau sequence by mass spectrometric analysis, combined with six additional sites indicated by specific antibody immunoreactivity, has brought the total number of identified phosphorylation sites on insoluble tau from AD brain to 45 (Figure 1b and see http://cnr.iop.kcl.ac.uk/hangerlab/tautable), which represents more than half of the total of 85 phosphorylatable residues (45 serines, 35 threonines and 5 tyrosines) in tau [4,49]. The biochemical and morphological

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**Table 1. Phosphorylation sites directly identified in Alzheimer tau and by candidate pathological protein kinases on human tau in vitro**

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*Single letter amino acid abbreviations indicate the sites of all of the phosphorylatable residues in tau (S, serine; T, threonine; Y, tyrosine). Numbering is based on the sequence of the largest isoform of human CNS tau.

*An asterisk (*) indicates phosphorylation sites directly identified in tau extracted from Alzheimer brain or after incubation of recombinant human tau with selected candidate protein kinases with pathological involvement in Alzheimer’s disease. A fully comprehensive listing of tau phosphorylation, including Alzheimer tau, PSP-tau, tau from control adult human and foetal rat brain and phosphorylation of recombinant human tau by these and other serine/threonine and tyrosine kinases, is available at http://cnr.iop.kcl.ac.uk/hangerlab/tautable.

*Grey boxes indicate sites where phosphorylation occurs at one of two or four closely-spaced residues on tau.
differences observed between the varying tau inclusions in the different tauopathies, together with the apparent reduced stability of tau phosphorylation in PSP compared with AD brain [53], suggests that the precise molecular mechanisms and/or kinases involved in tau-mediated neurodegeneration might not be identical for all tauopathies.

It is likely that there are many more tau phosphorylation sites in physiologically normal human brain than have been identified in post-mortem material because tau extracted from biopsy tissue becomes rapidly dephosphorylated after excision [54]. Tau phosphorylation sites seem to be turned over rapidly in normal brain in a cyclical process catalysed by the concerted actions of protein kinases and phosphatases. In the tauopathies, it seems that this cycle becomes ‘frozen’, or at least the balance is tipped in favour of phosphorylated tau, resulting in the appearance of abnormal tau in an enhanced and more stable state of phosphorylation. This highly phosphorylated material seems to build up gradually into characteristic intracellular aggregates of tau. The number of phosphorylated residues present in tau from AD brain exceeds that detected during development, estimated at ~18 sites in tau extracted from foetal rat brain [55] (also see http://cmr.iop.kcl.ac.uk/hangerlab/tautable), although the overall stoichiometry of phosphorylation is reported to be similar in AD and foetal tau [56]. This direct identification of multiple phosphorylation sites in tau from embryonic brain is in keeping with reports of increased tau phosphorylation identified using phospho-specific tau antibodies during periods of increased synaptic plasticity [57]. It might be, therefore, that all of the phosphorylated residues found in Alzheimer tau are also phosphorylated in foetal and normal adult brain but that the stoichiometry and/or stability of tau phosphorylation could be greater in AD. However, it seems that no individual molecule of tau is phosphorylated at all possible sites, even in the tauopathies where tau phosphorylation is markedly increased, because of the apparent heterogeneity of phosphorylated species in these disorders [58].

**Multiple kinases are likely to be involved in generating phosphorylated tau**

A comparison of the patterns of phosphorylation sites on tau extracted from normal and AD brain with sites identified on recombinant human tau by candidate tau kinases indicates the likelihood of more than one kinase being involved in tau phosphorylation. This is important because current therapeutic strategies are aimed at specific and complete inhibition of individual tau kinases and off-target kinase inhibition is regarded as disadvantageous to drug discovery. One possibility is that tau might be primed by one kinase before subsequent phosphorylation by a second kinase that recognizes a nearby phosphorylated residue. For example, PKA has been shown to prime for CK1 on the hedgehog signalling effector Cubitus interruptus, and CK1 phosphorylates other substrates, including β-catenin, before substrate recognition by GSK-3 [59]. In the case of tau, dual-specificity tyrosine-phosphorylation regulated kinase 1A (DYRK1A) and cdk5 have been suggested to act as priming kinases for subsequent GSK-3 phosphorylation [47,48]. However, formal proof for in vivo priming of tau by these kinases is currently lacking. Furthermore, activation of one kinase might induce increased activity of related tau kinases, resulting in a kinase cascade. For example, CK1 and c-Abl activate cdk5 by phosphorylating residues S159 and Y15, respectively [60,61]. Interactions between cdk5 and kinases that phosphorylate neurofilaments have also been reported, with resulting changes in phosphorylation at specific sites, highlighting the need to investigate the potential for indirect effects of kinase inhibition on target substrates [62]. An alternative therapeutic strategy to specific and potent inhibition of an individual kinase therefore might be to target multiple tau kinases with the aim of reducing the overall level of tau phosphorylation (Figure 2).

[Figure 2. Involvement of multiple interacting candidate tau kinases and phosphatases in Aβ-induced neurodegeneration. Extracellular Aβ activates candidate protein kinases through several different mechanisms, including those represented in this summary. Numerous interactions between protein serine/threonine (pink) and tyrosine (Abl and Fyn, pale blue) kinases as well as phosphatases (PP1 and PP2A, yellow) have been reported. These should be considered in the design of therapeutic strategies involving kinase inhibition. As indicated by *, inhibition or knockout of cdk5 activates GSK-3. Dashed and solid lines indicate indirect and direct interactions, respectively, and red lines indicate inhibitory relationships between enzymes. Abbreviations: CaMKII, calcium-calmodulin kinase II; MARK, microtubule affinity-regulating kinase; MEK, mitogen-activated protein kinase kinase; SAPK, stress-activated protein kinase.]
and Box 1). Such a strategy has shown promising preclinical results in models of AD [22], has recently been suggested for kinases implicated in cancer [63], and might be a suitable approach, particularly when targeting diseases, such as the tauopathies, that primarily affect elderly patients.

- It is conceivable that inactivation of specific phosphatases might be responsible for tau pathology. However, phosphatase activation is unlikely to become a viable therapeutic target for pharmacological intervention in the near term because the mechanisms involved and the role of phosphatases in tau pathology are only poorly understood.

**Concluding remarks**

The involvement of tau in neurodegenerative processes that lead to AD and related tauopathies is now well-established; however, the role of tau phosphorylation in such disorders is much less certain. It has been presumed that tau phosphorylation is a pre-requisite for its aggregation, although this has yet to be proven. An alternative possibility is that tau aggregates before becoming phosphorylated, leaving it in a conformationally altered state that could protect the deposited tau from the action of protein phosphatases. The existence of potentially toxic, phosphorylated forms of soluble oligomeric tau has been demonstrated in models of AD, indicating that this approach will need careful validation [64,65].

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