REVIEW ARTICLE

Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration

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This review attempts to summarize what is known about tau phosphorylation in the context of both normal cellular function and dysfunction. However, conceptions of tau function continue to evolve, and it is likely that the regulation of tau distribution and metabolism is complex. The roles of microtubule-associated kinases and phosphatases have yet to be fully described, but may afford insight into how tau phosphorylation at the distal end of the axon regulates cytoskeletal–membrane interactions. Finally, lipid and glycosaminoglycan modification of tau structure affords yet more complexity for regulation and aggregation. Continued work will help to determine what is causal and what is co-incidental in Alzheimer's disease, and may lead to identification of therapeutic targets for halting the progression of paired helical filament formation.

INTRODUCTION

The regulation of the neuronal cytoskeleton by phosphorylation has been recognized as a potential site for formation of neuropathology caused by both intrinsic and extrinsic factors. Tubulin, neurofilaments, microtubule-associated proteins (MAPs), kinesins, actin and actin-binding proteins form key structural components of the axonal and dendritic cytoskeleton [1]. The discovery that highly phosphorylated tau protein is the major component of the paired helical filaments (PHFs) of Alzheimer's disease (AD) and other neurodegenerative diseases has focused attention on the nature and enzymology of the post-translational modification of this particular MAP [2]. Clues to the pathogenesis of PHF formation, however, have taken unexpected turns, resulting in a tangled web of potentially causal and correlative data. AD pathology consists of intracellular tau-based neurofibrillary tangles (NFTs) formed of PHFs and extracellular β-amyloid plaques. Generally, the degree of dementia correlates with the sites and severity of tau-based NFTs [3,4]. Genotype plays a role in predisposition to AD, with early-onset cases ($<$ 50 years old) linked to mutations in the amyloid precursor protein (APP) or novel transmembrane proteins termed presenilins [5]. The gene dosage of APP is also a potential factor, since Down's syndrome patients (trisomy 21) have an increased level of APP and show Alzheimer-like neuropathology upon autopsy [6]. Genotype affects predisposition to late-onset AD, with the apoE4 allele associated with a 2–5-fold increased risk [7]. Thus one dominant question has been whether tau-based pathologies are primary independent lesions of AD, are secondary to amyloid plaque formation, or are formed via a cellular defect common to APP and tau protein processing. The reader is referred to several recent reviews concerning the molecular biology of AD for more complete information concerning the roles of APP and related presenilin genes [5,8–13].

Intense scrutiny using biochemical, immunochemical, transgenic and functional approaches has been focused on the protein kinases and phosphatases that modify tau protein; however, the phosphorylation state of tau alone may not be sufficient for PHF formation. Recent data suggest that formation of PHFs *in vitro* can be accomplished in a phosphorylation-independent manner via interaction of sulphated glycosamioglycans (such as heparin and heparan sulphate) with microtubule-binding domains of adjacent tau three- and four-repeat monomers [14]. However, phosphorylation can produce functional changes in tau–microtubule interactions, and may serve as a point for therapeutic intervention in neurodegenerative disease. This review will focus on the complexities of tau phosphorylation, and will provide a comprehensive overview of the protein kinases, phosphatases and signal pathways that regulate tau. The consequences of tau modification will be analysed with respect to altered turnover, cellular trafficking, and microtubule binding and stability. Other paradigms have been described for signal transduction through the cytoskeleton, suggesting a hierarchical series of control points [15]. Additional post-translational modifications which alter tau will be described, with a goal of defining several novel sites for potential therapeutic intervention to prevent or slow PHF formation.

TAU IS A MICROTUBULE-ASSOCIATED PHOSPHOPROTEIN

Tau was originally isolated as a protein which co-purified with tubulin and promoted tubulin assembly into microtubules [16,17]. Subsequent molecular characterizations have shown tau to be a family of microtubule-binding proteins which derive from a single gene [18–21]. Tau is found predominantly in central-nervous-system neurons, although reports have suggested that tau can be expressed in other cell types. Tau proteins promote microtubule assembly, and interact with microtubules via specific microtubule-binding domains. A model showing domain struc-

Abbreviations used: AD, Alzheimer's disease; AGE, advanced glycation end-product; apo, apolipoprotein; APP, amyloid precursor protein; β-AP, β-amyloid protein; bFGF, basic fibroblast growth factor; CaM, calmodulin; CaM kinase II, Ca^{2+}/CaM-dependent kinase II; cdk, cyclin-dependent kinase; GSK, glycogen synthase kinase; MAP, microtubule-associated protein; MAPK, mitogen-activated protein kinase; NFT, neurofibrillary tangle; NGF, nerve growth factor; PHF, paired helical filament; PKA, protein kinase A; PKC, protein kinase C; PP1 (etc.), protein phosphatase 1 (etc.).

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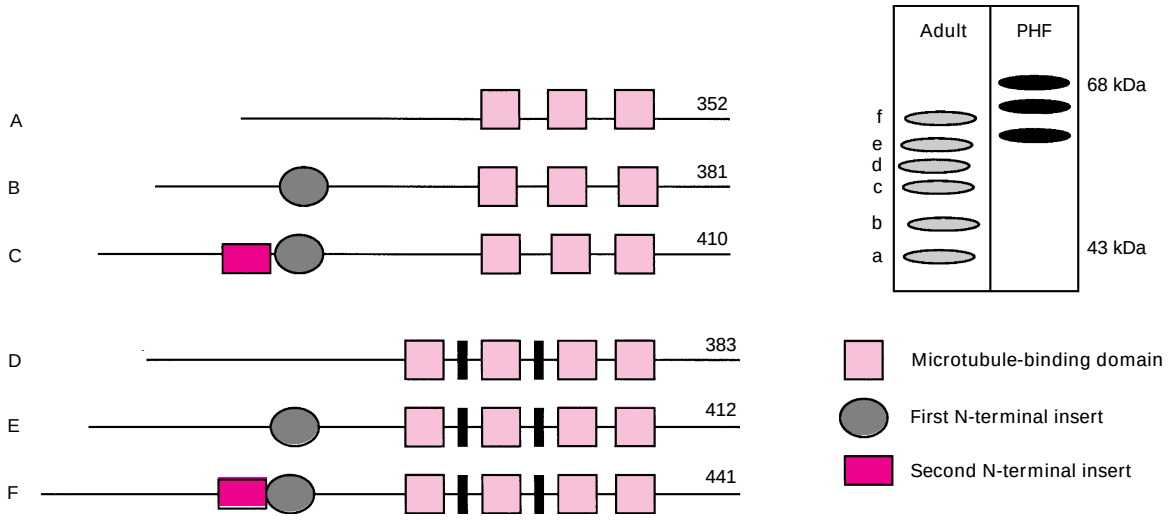

Figure 1 Alternative splicing and domain structure of tau

The adult human brain expresses six isoforms of tau which are derived from a single gene via alternative splicing. Microtubule-binding repeats are located in the C-terminal half of tau. Two N-terminal inserts and inserts surrounding microtubule-binding domain 2 are characteristic of the larger isoforms. Fetal tau corresponds to isoform A. The pattern of isoforms seen in the adult brain following electrophoresis are shown in the box. PHF tau consists of a characteristic triplet of peptides ranging between 60 and 68 kDa.

tures is shown in Figure 1. Alternative splicing of the mRNA occurs, producing six separate isoforms in adults, with variation in the number of microtubule-binding repeat domains (three and four) and in the number and size of N-terminal inserts [22]. A form of tau, termed 'big tau' is localized in the peripheral nervous system, and consists of a four-repeat form of tau with a 254-amino-acid insert in the N-terminus [23].

The isoform composition of tau undergoes developmental regulation, with fetal tissue showing expression of the smallest form of tau protein, containing three microtubule repeats and no inserts (Figure 1). Interestingly, fetal tau is highly phosphorylated, and shows immunoreactivity with several phosphorylation-sensitive antisera which react with PHFs [24,25]. This similar pattern of phosphorylation at AD-related sites on tau led to the idea that fetal tau phosphorylation was recapitulated in AD [26–28]. However, recent data suggest that the patterns and sites of phosphorylation are somewhat similar between forms of tau, and differ with regard to the extent and persistence of phosphorylation; these differences may be related to the balance of protein kinase versus phosphatase activities. Tau expression shifts during the first weeks of development, with expression of all six isoforms in adults. Thus part of the complexity of PHF formation involves determination of multiple potential sites of phosphorylation on six different isoforms. Subsequent analysis showed that all six isoforms can be highly phosphorylated and isolated from PHFs [29,30].

The hyperphosphorylation hypothesis of AD derived from the sequential discoveries that the PHF-related proteins from AD brain are, in fact, persistently phosphorylated forms of tau protein, some of which are ubiquinated [31–33]. PHFs were characterized by their insolubility, and by their ability to be stained with specific monoclonal antibodies such as Alz-50 [34]. The mobility of these proteins as a triplet of around 64–68 kDa led to the name A68 protein(s). Subsequent analysis of Sarkosyl-extracted PHF preparations from AD brain demonstrated that A68 proteins consist of derivatized isoforms of tau which are highly phosphorylated; initial characterizations suggested that phosphorylation occurred on unusual sites, as determined by tau-directed antisera [35–39]. The slowed electrophoretic mobility of A68 proteins was restored by treatment with alkaline phosphatase at elevated temperatures. Thus the focus shifted to determination of sites of phosphorylation, protein kinases which could account for A68-directed sites and, later, protein phosphatases which might dephosphorylate specific sites on tau [40]. In addition, the finding that phosphorylated tau fails to bind microtubules led to the hypothesis that highly phosphorylated PHF tau was microtubule-assembly-incompetent, leading to destabilization of the neuronal cytoskeleton and cellular demise [41].

Characterization of tau via phosphorylation-sensitive antisera has been instrumental in the determination of sites of tau phosphorylation [42,43]. Figure 2 depicts the largest human isoform of tau, showing sites recognized by phosphorylation-sensitive antisera and sites of action of putative protein phosphatases. Several antisera have been instrumental in developing the scheme of PHF tau phosphorylation. Tau-1 monoclonal antibody recognizes a dephosphorylated site between residues 189 and 207; conditions that favour dephosphorylation increase the staining intensity of this epitope on blots and in cells. In contrast, the 5E2 monoclonal antibody recognizes a phosphorylation-independent site at residues 156–175. Epitope mapping studies identified sites of the AT series of phosphorylation-dependent monoclonal antibodies: Ser-202 for AT8; N-terminal site for AT10; Thr-231 for AT180; Thr-181 for AT270. The PHF-1 phosphorylation-sensitive monoclonal antibody recognizes Ser-396/-404, while the Alz-50 antibody maps in the N-terminal region. Several groups of workers have developed polyclonal tau-directed antisera, focused on the N-and C-termini and on specific regions involved in microtubule binding and regulation. Thus Figure 2 is only a partial map of sites and antibodies. Of note, however, is the fact that most of these epitopes are outside the microtubule-binding domain. Two aspects stemming from these analyses were that: (a) tau is 'hyper'-phosphorylated in AD, potentially on sites unique to the disease; and (b) tau–microtubule interactions are compromised by the hyperphosphorylation.

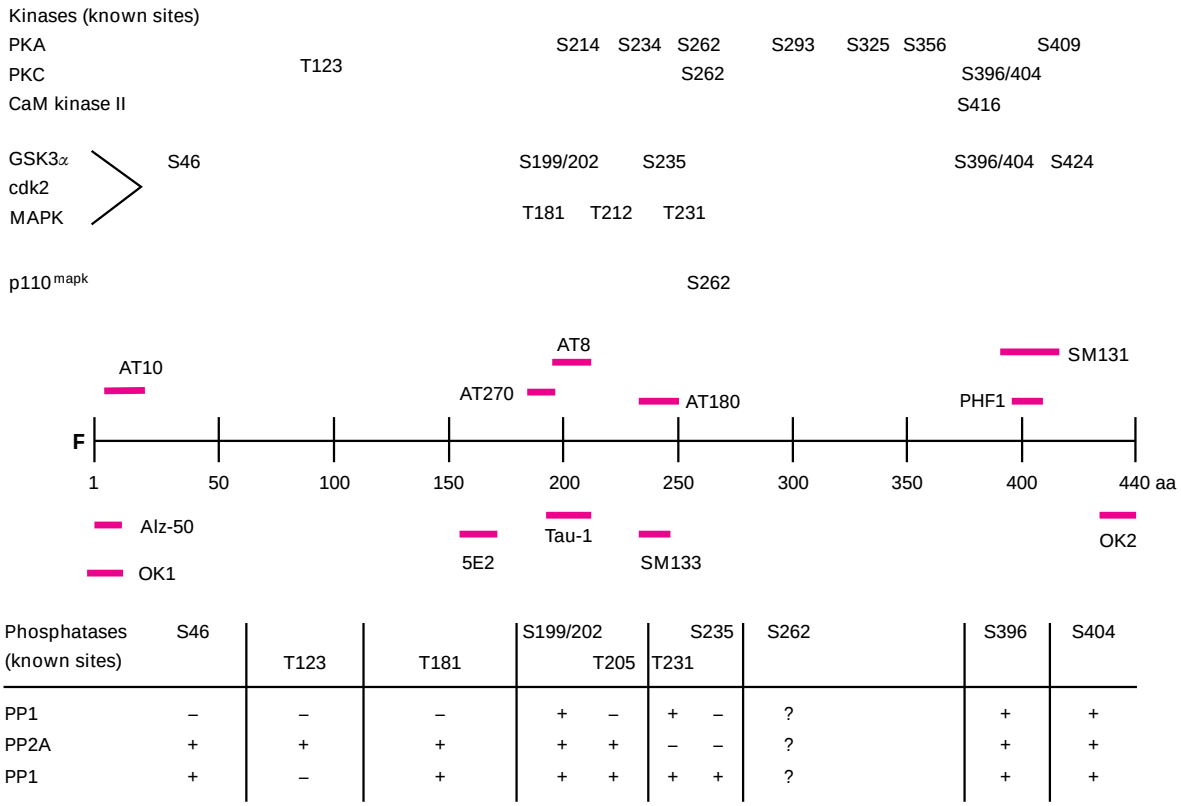

Figure 2 Phosphorylation sites on human tau-(1–441) (F)

Tau is recognized by a series of monoclonal and polyclonal antibodies, many of which are phosphorylation-dependent. Monoclonal antibodies Alz-50, AT10, AT270, AT8, AT180, PHF-1, SMI-31 and SMI-33 all recognize tau in a phosphorylation-dependent manner. Tau-1 monoclonal antibody recognizes dephosphorylated tau preferentially. Monoclonal antibody 5E2 and polyclonal antisera OK1 and OK2 recognize all isoforms of tau in a phosphorylation-independent manner. Numerous other polyclonal antisera have been developed to regions of tau, but are not shown here. The top panel attempts to show the major known sites of protein kinase action, while the bottom panel depicts putative sites of phosphatase action. Many of these sites were determined *in vitro*, using both immunological and biochemical approaches.

Several issues have altered the prospects for the hyperphosphorylation hypothesis of PHF formation. First, MS and sequence analysis of fetal, adult and PHF tau suggested considerable overlap between AD and adult patterns of phosphorylation, although there were quantitative differences in levels [44]. Secondly, studies of tau from intact human, primate and rat brains suggested that most, if not all, of the AD-specific sites on tau are seen in living neurons [45–48]. *In vitro* analysis of neuronal cultures suggested that specific sites (i.e. AT8) could be reversibly phosphorylated in response to various signals such as basic fibroblast growth factor (bFGF) [49]. Finally, biopsies from apparently normal human and primate brains showed that there is rapid dephosphorylation of most sites on tau, an effect which could be blocked via incubation with specific inhibitors of Ser/Thr phosphatases such as okadaic acid, calyculin A and FK-506/-520 [45,46].

The focus on tau phosphorylation and PHF formation is a clear extension of a large body of evidence suggesting that phosphorylation of tau plays a role in microtubule binding and function. Interestingly, targeted deletions of tau did not lead to widespread alterations in the cytoskeleton, suggesting that there is redundancy of function within the neuronal cytoskeleton [50]. In spite of the intensive study of tau and its modifications, critical data are lacking showing a direct causal link between excessive or persistent tau phosphorylation and the ultimate formation of NFTs. We pose the following strategic questions to act as a guide for interpreting the relationships between phosphorylated tau, PHFs and the development of therapeutics.

What drives PHF assembly?

Figure 3 shows a hypothetical model of tau phosphorylation, proteolysis, compartmentation, dimerization and PHF formation. Several questions are posed by the biology of tau phosphorylation. For instance, even though fetal tau is highly phosphorylated, there is no evidence that it spontaneously begins to self-aggregate [51]. Although there are developmental factors which may account for this finding, it also suggests that something more than stoichiometry and site of phosphorylation plays a role in PHF formation. Trafficking and compartmentalization may be of prime importance. Initial data suggest that overexpression of tau in transgenic mice or lamprey giant neurons leads to somatodendritic accumulation in a phosphorylation-dependent manner [20,52]. Thus phosphorylation may help to govern both the microtubule binding and the cellular fate of tau.

Persistent phosphorylation at many sites leads to a quantitative increase in phosphorylated tau

Phosphorylated tau then self-associates *in vivo* faster than it can be degraded, leading to conditions favouring dimer formation. These may ultimately cross-link, possibly under conditions of

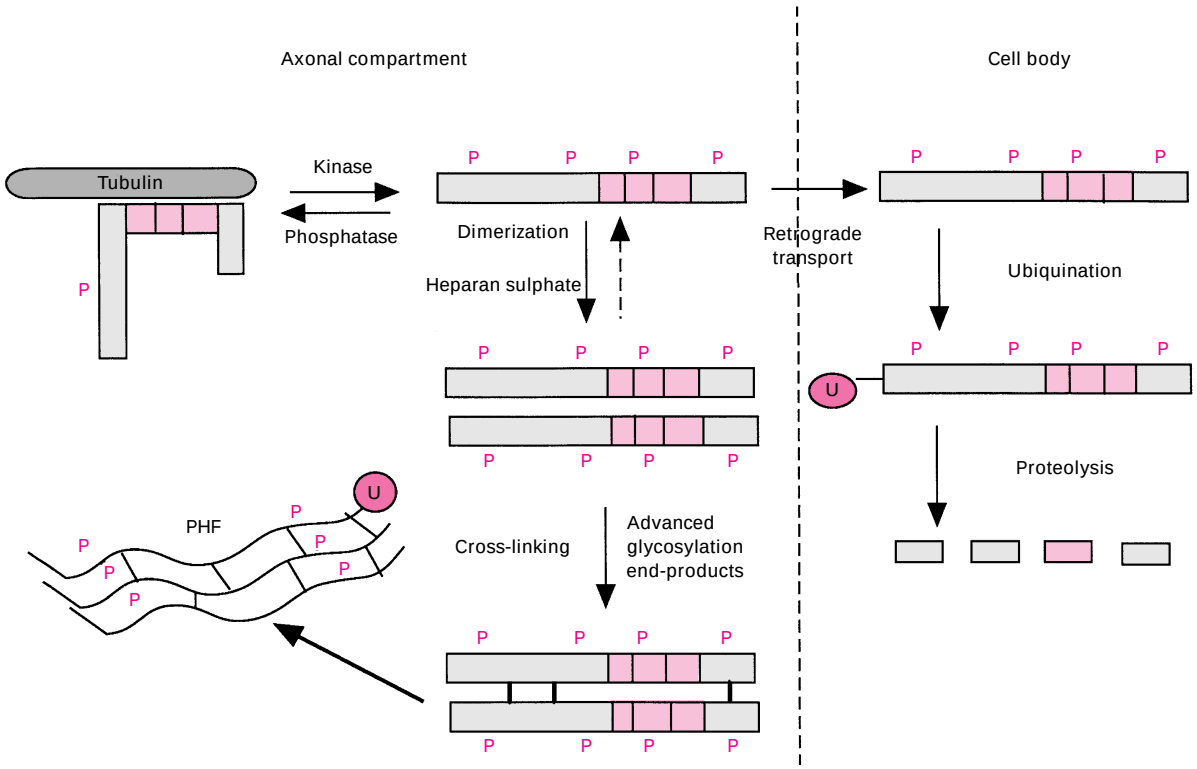

Figure 3 Scheme showing the compartmental distribution of three-repeat tau in a model neuron

In this model, kinases and phosphatases in the cytoskeleton regulate tau–microtubule interactions. Normally, tau is transported back to the cell body, ubiquinated (U) and proteolysed. In AD, other factors (heparan; dimer formation) form a nucleus of insoluble phosphorylated tau. Additional factors (glycation; covalent cross-links) lead to the formation of insoluble PHF, which builds up intracellularly.

oxidative stress or in the presence of sulphated glycosaminoglycans, leading to a stable, insoluble core of phosphorylated tau to initiate PHF and NFT formation. Drugs which activate phosphatases should decrease PHF formation, since the quantity of phospho-tau is critical to the formation of PHFs. However, studies using recombinant tau phosphorylated by numerous extracts and purified protein kinases failed to demonstrate phosphorylation-dependent aggregation into PHFs [53,54].

Excess phosphorylation on specific sites triggers reduced microtubule binding and ultimate aggregation

These specific sites (e.g. Thr-231 or Ser-396 adjacent to the microtubule-binding domain; Ser-262 in the microtubule-binding domain) must be dephosphorylated to prevent dimerization and subsequent PHF formation [55]. The prediction is that drugs that increase the activity of the specific phosphatases directed at the trigger site will decrease PHF formation. Conversely, treatments that reduce phosphatase activity should lead to PHF formation *in vivo*. To date, inhibition of phosphatase activity *in vitro* and *in vivo* has only led to accretion of phosphorylated tau epitopes, with no clear evidence of PHF formation.

Regulated phosphorylation is important for compartmentalization of tau pools

In this model, one function of the phosphorylation of tau is to regulate microtubule stability. When tau becomes heavily phosphorylated in one compartment (e.g. the axon), it is targeted for segregation to the soma for proteolytic degradation. In this case, increasing the levels of phosphorylation would augment tau degradation, and phosphatase inhibitors could increase tau turnover. This would point to a trafficking defect as a possible problem in AD. This concept is supported somewhat by findings that tau turnover and proteolytic processing are altered in cells treated with phosphatase inhibitors [56].

Conditions that favour cross-linking of tau lead to trapping of phosphate groups on an insoluble form of tau

The cross-linking of tau possibly occurs via glycation, transglutamination or heparin-related molecules. In this case, tau cannot be dephosphorylated by protein phosphatases, due to steric hindrance caused by dimer formation. Phospho-tau is predominant in a given compartment and thus gets trapped. Agents that alter phosphatase activity should have little effect on the accretion of PHFs if cross-linking is the key first step. Drugs that interfere with the binding of sulphated glycans (e.g. polycationic molecules) may thus arrest PHF progression [14].

Trafficking of tau is independent of phosphorylation, but dependent on other factors such as Rab proteins

In this case, phosphatase inhibitors are predicted to have little effect on PHF formation. However, correct trafficking is needed to target excess tau for ubiquination and degradation.

Agents that alter the pattern of tau-directed kinase activity would alter PHF formation

Since tau phosphorylation is maintained in a dynamic balance by multiple protein kinases and phosphatases, agents that alter the activity of one class of kinase could shift the pattern of tau phosphorylation, leading to altered trafficking and/or degradation. For example, agents that increase cAMP levels, such as forskolin, may alter the overall balance of tau phosphorylation. Treatment with phosphodiesterase inhibitors and forskolin activates protein kinase A (PKA). This can directly phosphorylate tau at PKA sites, but will also indirectly block (via PKA inhibition of Raf-1) mitogen-activated protein kinase (MAPK) activity directed towards the 17 Ser/Pro motifs in tau [57]. Altered phosphorylation may lead to shifts in compartmentation or degradation.

The presence of extracellular β-AP fibrils triggers excessive phosphorylation of tau

The result of this is that tau-directed protein kinase activity greatly exceeds phosphatase actions. In this case, PHFs would be expected to be formed in transgenic mice that overexpress APP or following direct injections of β-amyloid-(1–42) fibrils (β-AP). Analyses in primary neuronal cultures suggest that β-AP may increase tau phosphorylation; however, PHFs have not yet been described in animals that overexpress APP [58].

Alterations in intracellular glycosaminoglycan production may trigger formation of both PHF and plaques

In vitro analysis, immunochemistry and several biochemical systems have been used to show that heparin-like molecules can stimulate the formation of PHFs *in vitro* from three-repeat tau in a phosphorylation-independent manner, may activate tau-directed protein kinases, and may augment the formation of amyloid plaques. Thus a common defect may be in the enhanced neuronal formation of intracellular glycans, leading to PHF formation.

The primary premise is that tau protein is a substrate for numerous protein kinases *in vivo* and *in vitro*, and that tau biology can be regulated by such modifications. The extent to which such modifications play a role in PHF formation must be critically evaluated in the light of data from multiple systems.

REGULATION BY PROTEIN KINASES AND PHOSPHATASES

Antibody studies of post-mortem and biopsy-derived brain

Analysis of phosphorylated tau was initially performed in post-mortem samples from AD and age-matched non-AD brain tissues. The development of monoclonal antisera to PHFs from AD brain gave strong support to the notion that specific sites were selectively phosphorylated on AD tau [59,60]. However, by definition, most of these studies were performed retrospectively using tissue exposed to varying post-mortem intervals.

In 1992, we performed a prospective biochemical analysis of tau phosphorylation in brain slices prepared from human temporal lobes [61]. Tissues of normal appearance removed incidental to elective surgery for temporal-lobe epilepsy were incubated under conditions that perturbed protein phosphatases and kinases. Initially we observed that treatment with micromolar concentrations of okadaic acid produced mobility shifts in tau protein that led to its co-migration with authentic A68 preparations from AD brains and its recognition with Alz-50 monoclonal antibody. *In vitro* incubations with calcineurin reversed these changes, and suggested that the actions of protein phosphatase could be manipulated to produce AD-like tau. However, subsequent analysis of biopsy brain tissue immediately following neocortical resection led to the unexpected finding that tau protein is highly phosphorylated in living tissues. This surprising finding was confirmed in separate, blinded studies, and indicated that most of the sites on tau previously thought to be AD-specific were present in normal-appearing brain [46,62]. Further investigation revealed that these sites were rapidly dephosphorylated during an initial 1–2 h post-mortem period, and led us to conclude that many of the differences seen in tau phosphorylation resulted from the activity of post-mortem phosphatases in control compared with AD brains. This could result from low levels of phosphatase(s) in AD, from the build-up of inhibitors of phosphatases or via steric hindrance caused by PHF formation.

MS analysis

Analysis of adult and fetal rat tau, and tau from AD brains, has been carried out using protein sequencing and MS analysis [25,44,63]. The analysis of AD tau revealed the presence of persistent sites of phosphorylation on sites commonly recognized by tau-directed monoclonal antibodies, including Thr-231, Ser-235, Tau-1 peptides, Ser-396/-404 and Ser-262. Analysis of fetal and adult rat tau revealed that fetal tau was modified at up to 10 sites, whereas adult tau was much less phosphorylated. Phosphorylated Ser-262 has been detected in fetal, adult and PHF tau preparations, and is rapidly dephosphorylated *in vitro*; however, the extent to which this modification alters microtubule–tau interactions is still somewhat controversial [47]. There is reasonable convergence between immunological and MS approaches with regard to sites that are phosphorylated on tau. Thus the site-specific interactions of protein kinases and phosphatases with tau has been the subject of considerable interest.

Tau phosphorylation in cultured neuronal cells

The paucity of biochemical systems for the prospective analysis of human tau phosphorylation has led to the use of human- and rat-derived neuronal cell lines and primary cultures. In general, these systems do not show the adult pattern of tau isoforms, and variably express fetal isoforms and forms of high-molecular-mass tau. Primary rat neocortical and hippocampal cultures were used to show that glutamate increased Alz-50 and 5E2 staining in a calcium-dependent manner [64,65]. These increases were also mimicked by the calcium ionophore A23187, and were associated with increased ubiquination of tau. This suggested a link between excitotoxicity and tau phosphorylation. Additional studies using primary rat neocortical cultures showed increased Alz-50 staining following microtubule destabilization with colchicine, and decreased Alz-50 staining upon microtubule stabilization with taxol [66]. One interpretation is that tau bound to microtubules is somewhat sequestered from protein kinase activity. Immortalized PC-12 cells have also been used to characterize tau phosphorylation. Recently, M1 cholinergic agonists have been reported to decrease tau phosphorylation at PHF-1 and AT8 sites in a dose-related manner, suggesting a link between cholinergic innervation and tau [67].

Human neuroblastoma cells are more homogeneous than rat brain primary cultures and express human isoforms of tau. The LAN cell line expressed a 55 kDa fetal-like isoform of tau which was stained with PHF-1, Alz-50 and Tau-1. Heat shock at 45 °C led to decreased PHF-1 staining, while treatment with okadaic acid led to loss of Tau-1 staining, suggesting that tau-directed kinases and phosphatases are expressed [68]. Considerably more

Table 1 Tau-directed protein kinase sites

Abbreviations: TKR, tyrosine kinase receptor; ds, double-stranded.

	Signal	Sites on human tau-(1–441)	Microtubule-associated?
(a) Second-messenger kinases			
PKA	cAMP	Ser-214, Ser-262, Ser-324, Ser-356, Ser-409, Ser-416	No
PKC	Ca^{2+}/lipid	Ser-324	No
CaM kinase II	Ca^{2+}/CaM	Ser-409, Ser-416	Yes
(b) Proline-directed kinases			
MAPK (p42, p44, p40, p49)	TKRs	Ser-46, Ser-199, Ser-202, Ser-235, Ser-396, Ser-404, Ser-422	Yes
cdk5 (tau kinase II)	?	Ser-195, Ser-202, Thr-205, Thr-231, Ser-235, Ser-396, Ser-404	Yes
CDC2	?	Ser-195, Ser-202, Thr-205, Thr-231, Ser-235, Ser-396, Ser-404	No
GSK3α	?	Ser-199, Ser-202, Thr-212, Thr-231, Ser-235, Ser-262, Ser-324, Ser-356, Ser-396, Ser-404	Yes
GSK3β (tau kinase I)	?	Ser-199, Thr-231, Ser-396, Ser-413	Yes
(c) Other kinases			
$p110^{mapk}$	?	Ser-262	Yes
Casein kinase I	?	Ser-396, Ser-404, Alz-50, SMI-34	Yes
Casein kinase II	?	Ser-396, Ser-404, SMI-34	?
DNA-dependent kinase	ds DNA	Not determined	?

work has been done using the SH-SY5Y neuroblastoma line [69–72]. Tau phosphorylation in this line has been correlated with mitosis, suggesting that cyclin-dependent kinases (cdks) can modify tau. These cells show phosphorylation at AD-related sites, including AT8, PHF-1 and AT180 sites. Okadaic acid increased staining at phosphorylation-sensitive epitopes such as Alz-50, while Tau-1 staining decreased along with tau–microtubule binding. Two interesting findings from these studies focused on phosphorylation-induced shifts in tau compartmentation, with dephosphorylated tau located in nuclei and phosphorylated tau located in different cytoplasmic compartments and processes. One report has shown that lamin and fibronectin alter PHF-1 staining in retinoic-acid-differentiated SH-SY5Y cells, suggesting that the extracellular matrix can influence neurite outgrowth and tau phosphorylation [73]. The NT2N cell line has the most representative patterns of tau-directed phosphorylation, and responds to phosphatase inhibitors [74]. Recent analysis revealed that microtubule disruption in this cell line led to increased turnover of tau phosphorylation sites and recovery of dephosphorylated tau. Phosphorylation at Ser-202 and Thr-205 was reversed exclusively by protein phosphatase 2A (PP2A), since inhibitors of this enzyme, but not of PP2B, led to accretion of phosphate groups on tau. This cell system has many advantages with respect to determining prospective biochemical systems that alter tau.

Overall, the relative lack of adult tau isoforms and the lack of formation of visible PHFs following perturbation of phosphorylation systems limits the utility of *in vitro* systems. In spite of this, cells in culture remain a valuable tool for the determination of tau trafficking and its regulation by phosphorylation.

Tau-directed kinases

We have arbitrarily divided tau-directed protein kinases into three categories: (a) second-messenger-activated kinases [protein kinase C (PKC), PKA and Ca^{2+}/calmodulin-dependent kinase (CaM kinase) II]; (b) Ser/Pro-directed kinases [MAPK family; glycogen synthase kinase 3 (GSK3); cdk2 and cdk5]; and (c) other tau-directed kinases (Ser-262 kinase/$p100^{mapk}$; casein kinases; DNA-dependent protein kinase). This is summarized in Table 1.

Tau can undergo two modes of phosphorylation, one which changes the electrophoretic mobilities of tau and another which does not. Phosphorylation by CaM kinase II decreases the electrophoretic mobility of all tau isoforms [75,76]. A phosphorylation site located at Ser-416 in the C-terminal tail of the protein outside the region of the internal repeats may be responsible for this shift [77]. Phosphorylation at this site not only decreases the electrophoretic mobility of tau but also makes the protein long and stiff [78]. Recent data suggest that CaM kinase II may also phosphorylate Ser-262 and Ser-356 *in vitro* [79].

Phosphorylation of tau by PKA can slow its migration on SDS/polyacrylamide gels and inhibit its degradation by calpain [80]. This could result in a protease-resistant tau population which may contribute to the formation of PHFs. Recent analysis of the sites phosphorylated by PKA suggests that Ser-214, -262, -324, -356, -409 and -416 are preferentially modified. However, *in situ* analysis of phosphorylation indicated that Ser-262 and Ser-356 are maintained in a regulated state of phosphorylation by PKA [81]. Ser-262 has been the focus of considerable attention, since it resides in the first microtubule-binding repeat, and phosphorylation of this site has been detected in PHFs. PKA and CaM kinase II phosphorylation of this site suggests that agents that activate cAMP and/or Ca^{2+} can lead to the directed phosphorylation of tau, causing a decrease in microtubule binding. Interestingly, a protein kinase termed $p110^{mapk}$ has been isolated which also phosphorylates Ser-262 on tau [82]. However, as discussed below, increased levels of cAMP have divergent effects, and PKA can act to modulate the activity of other tau-directed protein kinases ($p42^{mapk}$).

PKC can also phosphorylate tau *in vitro*, but, unlike CaM kinase II, this does not change its electrophoretic mobility [83,84]. However, *in vitro* phosphorylation by PKC reduced tau–microtubule interactions. Direct injection of a constitutively active form of PKC into murine neuroblastoma cells increased PHF-1 and Alz-50 immunoreactivity, with little change in AT8 or Tau-1 staining [85]. Cells injected with the active kinase failed to elaborate filipodia, whereas injection with intact PKC did not cause changes in neurite outgrowth in these cells. Thus there is evidence that, under certain conditions, tau may be modified by active PKC without causing AD-like shifts in electrophoretic mobility.

Proline-directed Ser/Thr protein kinases from brain can induce AD-like changes in tau [86,87]. Three major proline-directed protein kinase families have been characterized: MAPKs, GSK3 and CDC2-like kinases (cdk2 and cdk5). Several members of the MAPK family can phosphorylate tau, such as $p42^{mapk}$ [88], $p44^{mapk}$ [89], a phosphatase-resistant form of extracellular-signal-regulated kinase 2 (ERK2) termed $PK40^{erk2}$ [90,91], and a novel MAPK termed $p49^{3F12}$ [92]. Many of the PHF-related antibodies recognize Ser/Thr-Pro motifs, and modification of tau by MAPKs leads to enhanced staining with these antibodies. MAPKs have been co-localized in PHF-positive neurons. Interestingly, the novel $p49^{3F12}$ MAPK co-localizes in Alz-50-positive human neurons, and resides on human chromosome 21. Thus overexpression of this protein kinase may have a role in the formation of PHFs in Down's syndrome. MAPKs can phosphorylate tau in cultured neurons and in brain slices, primarily via activation of tyrosine kinase receptors and protein kinase cascades. In brain slices, activation of $p42^{mapk}$ via nerve growth factor (NGF) or blockade of PP2A led to enhanced tau phosphorylation [57]. In addition, a small pool of MAPK associates with the cytoskeleton, and is constitutively active. Thus MAPKs in the brain are prime candidates for regulation of the neuronal cytoskeleton.

Proline-directed protein kinase activity is associated with tau–cytoskeletal preparations from brain [93,94]. Subsequent analysis focused on GSK3 (tau protein kinase I) [95]. GSK3 comprises closely related α and β forms [96], both of which associate with microtubules and phosphorylate tau [97–99]. GSK3α phosphorylated tau at Ser-199, -202, -235, -396 and -404; heparin stimulated phosphorylation by GSK3α at Thr-212 Thr-231, Ser-262, Ser-324 and Ser-356 [100,101]. Heparin stimulation of Ser-262 phosphorylation is provocative, since this site is in the microtubule-binding domain. The actions of GSK are synergistic with those of other kinases [102], and one report suggests that this activity is necessary for β-AP toxicity *in vitro* [103]. Co-transfection of non-neuronal cells with human tau and GSKs indicated that GSKs could induce an AD-like phosphorylation, leading to sequestration of phosphorylated tau in the cytosol and loss of microtubule binding [104]. The signals that control GSK activation in neurons have not been fully characterized, however.

Several lines of evidence suggest that a CDC2-like protein kinase (tau protein kinase II) can also modify tau [105–109]. CDC2 and related cyclins (e.g. cyclin B) are not enriched in post-mitotic neurons, whereas cdk5 is highly enriched in neurons [110,111]. Recent analysis suggests that cdk5 is complexed with a neuron-specific 23 kDa regulatory protein [112]. This cdc2-like kinase may itself be regulated by phosphorylation in a manner analogous to cyclin-dependent kinases; however, more work is needed to establish these details. Unlike other cdks, cdk5 does not contain a PSTAIR motif, but does express a PSSALRE motif [113]. cdc2-like kinases co-localize with the cytoskeleton, and can also modify neurofilaments. Sites phosphorylated on tau by this kinase include Ser-195, -202, -235, -396 and -404, and Thr-205 and -231. Interestingly, phosphorylation of tau by the CDC2 homologue $p34^{cdc28}$ from yeast was enhanced by charged species such as heparin, sphingosine and related compounds [105]. Thus the Ser/Pro kinases co-localize with microtubule networks, modify sites associated with PHFs and, in some circumstances, are stimulated by heparin and related compounds.

The other tau-directed kinases include the $p110^{mapk}$ protein kinase, which shows specificity for Ser-262 and is associated with microtubules [82]. Other substrates for this protein kinase have not been fully elucidated. Both casein kinases I and II phosphorylate tau *in vitro*; casein kinase I has been reported to phosphorylate tau at PHF-1 and SMI-34 sites [114,115]. Finally, DNA-dependent protein kinase II can phosphorylate recombinant tau protein, as determined by measuring incorporation of ^{32}P into tau protein [116].

The question remains as to which protein kinase(s) phosphorylate tau in the AD brain, and which sites are normal, regulatory sites of phosphorylation. It is still unclear if tau is the substrate for all these kinases *in vivo*, or whether specific subsets of kinases co-localize with tau to provide regulated phosphorylation. The putative functions of phosphorylation include regulation of microtubule binding, alterations in subcellular compartmentation and altered proteolytic processing.

Tau-directed phosphatases

All sites of tau phosphorylation are at Ser/Thr residues; hence the candidate phosphatases include PP1, PP2A, calcineurin (PP2B) and PP2C. All have been reported to dephosphorylate tau *in vitro*, with some degree of overlapping site specificity [117]. However, the preponderance of data suggest that PP2A and calcineurin are crucial for the *in vivo* regulation at many of the best-characterized sites of tau phosphorylation (Figure 2). Ser/Thr phosphatases are differentiated on the basis of their primary structure, subunit composition and regulation by protein and toxin inhibitors [118]. The inhibitors of PP1 include heat-stable inhibitor-1, okadaic acid (K_i 100 nM) and calyculin A (K_i 50 nM); inhibitors of PP2A include two protein inhibitors, I-1 and I-2, okaidaic acid (K_i 1–10 nM) and calyculin A (K_i 1–5 nM). Calcineurin is activated by Ca^{2+}/CaM, and can be inhibited by immunosuppressive compounds such as cyclosporin, FK-506 and FK-520, and by high concentrations of okadaic acid (K_i 5 μM) [119]. Cell-permeable phosphatase inhibitors have been useful for determining both the sites and enzymology of dephosphorylation. Finally, both phosphatase and kinase activities can be further regulated by spatial–temporal subcellular distributions of enzyme activity [120]. Calcineurin has been localized to the cytoskeleton, as have several of the protein kinases [121].

The actions of PP1 on tau have been determined primarily from *in vitro* analysis of PHF and recombinant tau. *In vitro* analysis of tau has been useful, since dephosphorylation can often restore microtubule binding to phosphorylated or PHF tau. PP1 can dephosphorylate Ser-199, Ser-202, Thr-231 and Ser-393/-404, as determined using purified enzyme and alterations in tau immunoreactivity [122]. Inhibition of phosphatases with okadaic acid and calyculin A in brain slices, primary brain cultures and neuronal cell lines has generated data suggesting PP1 involvement in tau dephosphorylation [123]. However, there are problems with the interpretation of such results. *In vitro*, there is a reasonable difference in the K_i values for okadaic acid between PP1 (100 nM), PP2A (1–10 nM) and calcineurin (5 μM); *in vivo*, levels of phosphatases vary considerably between brain regions and developmental stages. Coupled with the variable penetration of inhibitors, such factors confound simple conclusions about which phosphatase acts at a specific site *in vivo*. Intraventricular infusions of okadaic acid for up to 8 weeks also led to accretion of phosphorylated tau, as judged by increased AT8 (Ser-202) and SMI-31 (Ser-396/-404) immunoreactivity in rats [124]. Treated rats also showed impairment of memory, and formation of plaque-like structures in the brain. Correspondingly, there was a decrease in PP1 + PP2A activity. Although this dramatic effect was correlated with AD-like changes, it is impossible to ascribe this to PP1 or PP2A alone. Analysis of PP1 activity in post-mortem AD brains suggested a small but statistically significant loss of activity

[125,126]. However, since PP1 is a broad-spectrum phosphatase, it is unlikely that changes in levels would cause the unique and specific pathologies of AD without causing changes in other phosphopeptides.

PP2A is likely to be one of the more important tau-directed phosphatases; it is also associated with microtubules, and is regulated in a complex manner by carboxymethylation, by association with regulatory subunits and by several recently characterized inhibitors [127]. Site-specific dephosphorylation of tau by PP2A *in vitro* has been observed at several of the key PHF sites, including Ser-46, Ser-199, Ser-202, Thr-205, Ser-396 and Ser-404 [128–132]. The suggestion has been made that PP2A counteracts Pro-directed kinases, although there is no restrictive motif which would suggest that this is the case. Dephosphorylation of recombinant tau, PHF tau and human tau phosphorylated *in situ* with PP2A leads to restoration of microtubule binding, although this effect has not been linked with a specific site(s) of dephosphorylation. PP2A activity also shows a small, but significant, decrease in activity in AD brains [125,126]. Site-specific dephosphorylation of tau at Ser-202 and Thr-205 has been suggested from studies in NT2N cells [74]. Microtubule depolymerization by colchicine or nocodazole led to dephosphorylation of these two sites; this dephosphorylation was blocked by okadaic acid and calyculin A (500 and 100 nM respectively), but not by FK-506 or FK-520. This type of site-specificity and the co-localization of PP2A in the cytoskeleton strongly suggests that this phosphatase plays a critical role in tau dephosphorylation. Although it is unlikely that loss of PP2A enzyme leads to PHF accumulation, it is intriguing to speculate that a build-up of heat-stable protein inhibitors of PP2A could lead to persistent tau phosphorylation.

Calcineurin (PP2B) has also been identified as a likely tau-directed phosphatase *in vivo* [133–137]. This phosphatase has a narrow substrate specificity, and may preferentially dephosphorylate substrates with the motif Arg-Xaa-Xaa-Ser/Thr-(Pro) [138]. However, this motif is not invariant. Calcineurin is regulated by Ca^{2+} and CaM, and consists of a 61 kDa catalytic subunit and an 18 kDa calcium-binding regulatory subunit [139,140]. There are two isoforms of calcineurin in brain, Aα and Aβ; each catalytic subunit is transcribed from a separate gene, with Aα being more prominent in brain [141,142]. Putative sites on tau that are selectively dephosphorylated by calcineurin include Thr-231 and Ser-235. A list of the sites and motifs in tau that are potentially dephosphorylated by calcineurin is shown in Table 2. Note that the site that best resembles the calcineurin consensus sequence is Thr-212, and that several of the sites shown to be substrates by antibody binding analysis loosely fit the Arg-Xaa-Xaa-Ser/Thr-(Pro) motif. Even though numerous studies have demonstrated that calcineurin can dephosphorylate tau *in vitro*, studies using inhibitors of calcineurin in brain slices and primary cultures have suggested that inhibition of calcineurin alone is not sufficient to produce complete, PHF-like, changes in tau. We recently infused antisense phosphorothioate oligonucleotides directed against the Aα, Aβ and B subunits of calcineurin into the lateral ventricles of adult rats, and achieved a 50–60% reduction in protein levels and activity of this phosphatase [143]. We found that phosphorylation of tau persisted at Thr-231 and Thr-181 in animals with reduced levels of calcineurin. The Tau-1 site was not affected. This suggests that, either directly or indirectly, a decrease in calcineurin activity leads to persistent phosphorylation of tau on sites associated with PHFs. However, there was no evidence for PHF formation or plaque formation in treated rats. Studies in which both PP2A and PP2B activities are lowered may be needed to determine whether persistent phosphorylation of tau leads to PHF formation. In humans, AD brains do not show profound alterations in calcineurin levels, although this phosphatase was co-localized with PHFs [134].

Table 2 Calcineurin (PP2B) dephosphorylation sites

	Motif
Known PP2B sequence sites	
RII peptide	Arg-Arg-Val-Ser-(Pro)
Inhibitor 1	Arg-Arg-Pro-Thr-(Pro)
DARPP-32	Arg-Arg-Pro-Thr-(Pro)
HSP-25	Arg-Ser-Pro-Ser-(Pro)
GAP-43 (Ser-96)	Pro-Ala-Thr-Ser-(Pro)
GAP-43 (Thr-172)	Ala-Ala-Thr-Thr-(Pro)
Consensus:	Arg-Xaa-Xaa-Ser/Thr-(Pro)
Putative PP2B sites on tau	
Ser-46	Leu-Lys-Glu-Ser-(Pro)*
Ser-129	Arg-Met-Val-Ser-(Lys)
Thr-181	Ala-Pro-Lys-Thr-(Pro)*
Ser-199	Gly-Tyr-Ser-Ser (Pro)*
Ser-202	Ser-Pro-Gly-Ser-(Pro)*
Thr-212	Arg-Ser-Arg-Thr-(Pro)
Ser-214	Arg-Thr-Pro-Ser-(Leu)
Thr-231	Val-Val-Arg-Thr-(Pro)
Ser-235	Pro-Pro-Lys-Ser-(Pro)*
Thr-245	Arg-Lys-Gln-Thr-(Ala)
Ser-262	Lys-Ile-Gly-Ser-(Thr)
Ser-320	Lys-Val-Thr-Ser-(Lys)
Ser-352	Arg-Val-Gln-Ser-(Lys)
Ser-396	Val-Tyr-Lys-Ser-(Pro)*
Ser-404	Gly-Asp-Thr-Ser-(Pro)*
Ser-409	Arg-His-Leu-Ser-(Asn)

* Denotes sites determined from *in vitro* antibody studies.

PP2C has been reported to dephosphorylate tau phosphorylated by PKA *in vitro*, but did not act on PHF tau [122]. In addition, PP2C activity is not altered in AD brains [125,126]. Thus this phosphatase is not likely to play a role in tau dephosphorylation. Other changes in acid phosphatase activity have been noted in AD brains, but these are not likely to be affiliated with tau regulation [144]. From the available evidence, PP2A and calcineurin remain the best candidates for additional study. Targeted gene disruptions for calcineurin have been carried out, and initial analyses indicate persistent phosphorylation of tau. Additional studies using protein-based, antisense oligonucleotide and toxin phosphatase inhibitors may help to determine if loss of phosphatase activity can cause persistent phosphorylation of tau and subsequent phosphorylation-dependent formation of PHF structures.

Signal transduction and tau phosphorylation

One issue that has not received full attention is the signal transduction pathways that normally regulate the state of tau phosphorylation. In one sense, progress has proceeded 'backwards', in that considerable attention has been directed towards the sites and enzymology of tau phosphorylation, with little emphasis on the reverse paradigm, i.e. investigating which receptor or extracellular-matrix pathways modify tau. It is clear that tau phosphorylation and dephosphorylation can be modified by the second messengers calcium and cAMP via PKA, PKC, CaM kinase II and calcineurin. In a similar vein, brain slice models and primary cultures have been used to document that NGF and bFGF, likely activators of MAPK cascades, can lead to phosphorylation of tau at proline-directed sites. Interestingly, bFGF has been reported to increase β-AP production. Glutamate

activates calcineurin via *N*-methyl-D-aspartate receptors, but this neurotransmitter has bifunctional effects. Initial reports suggested that glutamate could increase tau phosphorylation, as determined using Alz-50, Tau-1 and AT8 in cultured neurons; however, glutamate also activates calcineurin [145,146]. This latter effect is temporally delayed but sustained, and explains how one signal could have bidirectional control of phosphorylation [147]. This mechanism has been shown for MAP-2, another cytoskeletal protein for which binding to microtubules is regulated by phosphorylation.

Activation of tau phosphorylation by β-AP-(1–42) fibrils *in vitro* has been reported [59]. Although β-AP pathology and molecular biology are well characterized, the mechanisms of direct neurotoxicity remain somewhat controversial [148]. Free-radical damage, backbone rearrangements, glycation products and, importantly, fibril formation have been linked with increased toxicity of β-AP. In one report, secreted APP increased MAPK activity, ^{32}P incorporation into tau and AT8 reactivity; this effect was blocked in PC-12 cells transfected with a dominant-negative inhibitory form of Ras [149]. Subsequent analysis showed that β-AP fibrils, but not soluble β-AP, increased tau phosphorylation at Ser-202, Ser-396 and Ser-404, with no change in Tau-1 inmmunoreactivity in cultured rat hippocampal neurons. There was a decrease in cell viability which correlated with increased PHF-1 staining. Furthermore, β-AP fibrils led to loss of tau–microtubule interactions and increased somatodendritic compartmentation. Several lines of transgenic mice have been developed in which APP is overexpressed; to date, none of these lines have shown PHF formation, although other pathological features of AD were present. Thus other factors may need to be present in addition to APP in order that tau-based PHFs develop. If β-AP fibrils do trigger PHF formation *in vivo*, then drugs that block fibril formation (e.g. hexadecyl-*N*-methylpiperidinium bromide) would be predicted to block PHF formation, possibly via blockade of MAPK activation [150]. Additional work is needed to determine both the normal and abnormal pathways and factors that regulate tau phosphorylation.

CONSEQUENCES OF TAU PHOSPHORYLATION

Altered proteolysis

One key structural change that has been linked with the regulated phosphorylation of tau is altered turnover and proteolysis. The best characterized effect has been the reduction in tau cleavage by the calcium-activated protease calpain following PKA-induced phosphorylation [78]. PKA phosphorylates tau in its C-terminus, and analysis of calpain-induced tau cleavage indicates that most of the proteolysis occurs in this domain [151–153]. This has been observed in LAN-5 neuroblastoma cells as well; treatment with okadaic acid, forskolin and rolipram all decreased calpain-induced hydrolysis of tau [152]. In SH-SY5Y neuroblastoma cells, induction of calcium influx via A23187 led to complex changes in tau [154]. Alz-50 immunoreactivity was increased, and this effect was mimicked by phorbol esters and blocked by inhibitors of PKC. At the same time, calcium influx led to a marked loss of total tau via direct activation of calpain. *In vitro* analysis of Alz-50 tau indicated that it was resistant to proteolysis by calpain. Thus two of the second-messenger protein kinases may lead to increased resistance of tau to proteases. Recent data have suggested that tau is proteolysed by cathepsins D, B and L in the hippocampus [155]. Issues raised in that study suggest that the extra-lysosomal build-up of cathepsin D led to formation of a 29 kDa tau proteolytic product which was subject to modification by protein phosphorylation. Thus alterations in lysosomal trafficking of tau and/or loss of lysosomal function may lead to aberrant processing of tau.

Suggestions have been made that phosphorylation of tau favours dimerization rather than monomer formation [156]. However, many of these studies were performed *in vitro*, and may not reflect what happens when phosphorylated tau is exposed to microtubules and cellular proteases. Conformational studies have yet to show that phosphorylation produces consistent changes in the structure of tau, leading to a restricted conformation favouring PHF formation. Of note are the numerous studies in which phosphorylation-dependent assembly of tau filaments was not detected. Thus phosphorylation analysis must be taken in context with the subcellular milieu with respect to tau and PHF formation.

Altered microtubule binding

One basis for the hyperphosphorylation hypothesis was that site-specific or extensive phosphorylation of tau protein leads to a loss of microtubule binding, thus weakening the neuronal cytoskeleton [157]. As a result, sites of phosphorylation in or near the microtubule-binding domains (Ser-262; Ser-393/-404) have been the focus of such investigations [158,159]. However, the dynamics of microtubules and the neuronal cytoskeleton are much more complex and are modulated by more that tau phosphorylation [160]. Phosphorylation of tau affects both microtubule assembly kinetics and microtubule binding *in vitro* [161–164]. In general, tau with four microtubule repeats promotes assembly at a faster rate, and highly phosphorylated tau is less able to bind and assemble microtubules [165–168]. The binding of tau *in vitro* consists of many weak, but flexible, binding arrays, allowing the molecule to pivot and bind in a range of conformations. Highly phosphorylated PHF tau is assembly-incompetent, but can show some restored activity after dephosphorylation. Similarly, tau isolated from fresh human biopsies shows assembly competence which is intermediate between that of fully dephosphorylated and PHF tau [169]. However, it is still not clear exactly how the phosphorylation of sites outside the binding domain can regulate tau–microtubule interactions.

Careful analysis of the axonal distribution and transport of tau has found the highest concentration of this protein in the distal end nearest the growth cone [170,171]. Since this region is most sensitive to the destabilizing effects of colchicine or nocodazole, the conclusion has been reached that tau has effects other than on microtubule stability, and may be involved in axonal growth itself. Axoplasmic transport analysis using pulse-labelling has suggested that two pools of soluble and Triton X-100-insoluble tau are differentially transported, including species that are phosphorylated [172]. Thus PHF tau may lead to a failure of axonal growth and plasticity at the distal end, rather than causing a massive destabilization and collapse of the microtubular network.

Altered intracellular trafficking/polarity

Transfection studies with tau in heterologous systems are consistent with the concept that tau organizes microtubules and promotes process outgrowth. Initial studies in Sf9 cells transformed with a baculovirus vector containing tau showed marked changes in cell morphology; these cells elaborated long processes with bundles of microtubules [173]. Stable transfection of CHO cells with tau has been used to characterize changes in tau phosphorylation and microtubule bundling. CHO cells trans-

fected with tau showed an increased bundling of microtubules, with no alteration in the levels of tubulin [174]. Tau can be phosphorylated and accumulate in CHO cells, with some evidence of process formation [175]. Tau phosphorylation in CHO cells was cell-cycle-dependent; during interphase, tau was primarily bound to microtubules [176]. During mitosis, substantial phosphorylation occurred on Ser-202, Thr-205, Thr-231, Ser-235, Ser-396 and Ser-404, and this was accompanied by a loss of microtubule-bound tau. Phosphorylated tau was then concentrated in cytoplasmic pools. These sites are regulated by proline-directed kinases, implying that mitotic signals can activate MAPKs, cdks or GSK3, leading to phosphorylation of tau and collapse of the microtubule network in preparation for cell division. Thus transfection analysis of heterologous cells supports the idea that tau bundles microtubules in a phosphorylation-dependent manner.

Tau, however, is primarily expressed in neuronal cells, and has been implicated in neurite outgrowth and the formation of cell polarity. Antisense oligonucleotides directed against tau block neurite outgrowth in cultured neurons, and the timing of tau mRNA expression *in vivo* coincides with neurite outgrowth [177–179]. Fetal tau is expressed abundantly during neuro- and synapto-genesis, and expression switches to a lower level of adult isoforms after the first few postnatal weeks in the rat. This suggests that highly phosphorylated fetal tau, which lacks several N-terminal inserts and has only three microtubule repeats, has a specialized role during axonal growth and synaptogenesis [180]. However, the mechanisms by which tau establishes polarity, is concentrated in distal axons and interacts with membranes remain to be established. Studies using chimaeric constructs of tau and MAP-2 identified domains and mechanisms that act to sort these two proteins to axon and dendrite respectively [181]. First, the N-terminal domain of MAP-2 blocked its entry into axons. Secondly, phosphorylation of tau in the cell body at Tau-1 sites may reduce its binding to somatic microtubules and allow its loading into the axon. This implies a role for proline-directed kinases in tau sorting. Tau mRNA may also play a role in the determination of polarity, since it is localized on microtubules in the proximal section of the axon [182,183]. This implies that local translation of tau at a site determined by microtubular organization sites can lead to locally high levels of tau, which can theoretically cause bundling and forward movement of neurites.

Epitope-tagged tau and constructs containing the N-terminus of tau have been used to determine the interaction of the protein with neuronal membranes in PC-12 cells [184]. Using FLAG-tagged tau constructs, Brandt et al. [185] determined that the N-terminus of tau interacts with membranes, and that over-expression of a truncated N-terminus of tau can block NGF-induced neurite outgrowth. Tau was shown to associate with the plasma membrane, using both subcellular fractionation and immuno-electron microscopy. Tau was also concentrated at growth cones of hippoocampal and PC-12 cells. These data indicate a second, and perhaps more important, function for tau protein, namely in isoform-specific interactions between the distal axonal cytoskeleton and the growth-cone cell membrane. It will be important to determine whether the N-terminus of tau interacts with internal membrane proteins at or near the growth cone. Two possible candidates include GAP-43, a growth-cone-associated membrane protein, and SNAP-25, a SNARE protein involved in synaptogenesis and synaptic vesicle docking [186]. Interestingly, phosphorylation of GAP-43 is altered in relation to PHFs in the AD brain, suggesting a possible link [187].

In the light of discussions above suggesting the importance of tau in neuronal development and neurodegeneration, it was somewhat surprising to find that targeted deletions of tau protein led to only minor changes in the axonal calibre of small-fibre axons in restricted brain regions [50]. One explanation for this result is that there is considerable redundancy in the nervous system, and that other related MAPs subserve a function similar to that of tau in its absence. Transgenic and microinjection strategies have been used to approach the issue of whether the overexpression of tau protein can lead to neuropathology. Plasmids containing human tau driven by the strong cytomegalovirus promoter have been injected into lamprey giant neurons, resulting in phosphorylation at Tau-1 and PHF-1 sites [52]. PHF-1-reactive tau was excluded from the axon, and in some cases heavy somatodendritic accumulation was reported. Similarly, in murine models made transgenic with human tau-(1–441), some neurons were shown which accumulated phosphorylated tau in the somatodendritic compartment [20]. These approaches, which have only been described in preliminary form, are supportive of the idea that the intracellular trafficking of tau is modulated by a combination of protein phosphorylation, developmental expression, and selective transport and membrane interactions.

INTEGRATED ROLE OF TAU PHOSPHORYLATION IN THE BRAIN

Is persistently phosphorylated tau causal or reflective of PHFs?

To date, in spite of considerable research, this question remains open. There is considerable evidence that persistently phosphorylated tau protein is a hallmark pathology of AD and related diseases, and that tau may serve as a potential cerebrospinal fluid marker for AD. The neuropathology of PHFs correlates well with the severity of AD, and suggests a progressive process that is initiated by one or more factors. To date, there is no compelling evidence to suggest that PHF tau is caused by an overactive kinase. Similarly, *in vitro* analysis suggests that phosphorylation alone is not sufficient to induce PHF formation. PP2A and PP2B are likely to regulate many of the PHF-like sites of tau, although absolute site-specific dephosphorylation may not predominate. Rather, there may be promiscuity between sites, such that the protein phosphatase in proximity to tau in a given compartment may act preferentially on it. There is still a possibility that the accumulation of heat-stable inhibitors of PP2A may lower activity in selected neurons, leading to PHF accumulation. However, it is possible that the failure to dephosphorylate tau in AD results from steric hindrance, and that PHF formation occurs in a phosphorylation-independent manner. A possible model showing the roles of kinases, phosphatases and tau trafficking is shown in Figure 4.

Numerous cell-biology studies have shown that domains other than the microtubule-binding region are of importance, with the C-terminus playing possible roles in axonal targeting and the N-terminus involved in membrane–tau interactions. It is possible that phosphorylation events in the N- and C-terminal domains alter axonal transport and/or membrane interactions. Transgenic mice in which specific domains or phosphorylation sites are deleted may give additional clues as to how such regions function *in vivo*.

Finally, species differences in the primary structure of tau may suggest how and why tau accretes into PHFs in the primate brain [188]. Life-span arguments have been raised to explain the lack of formation of PHFs in rodent species, implying that PHFs form as a function of age. If this is the case, then long-lived mammalian species should also show PHFs. Hence structural differences in tau or in the regulatory cascade may play a role in primate PHF formation.

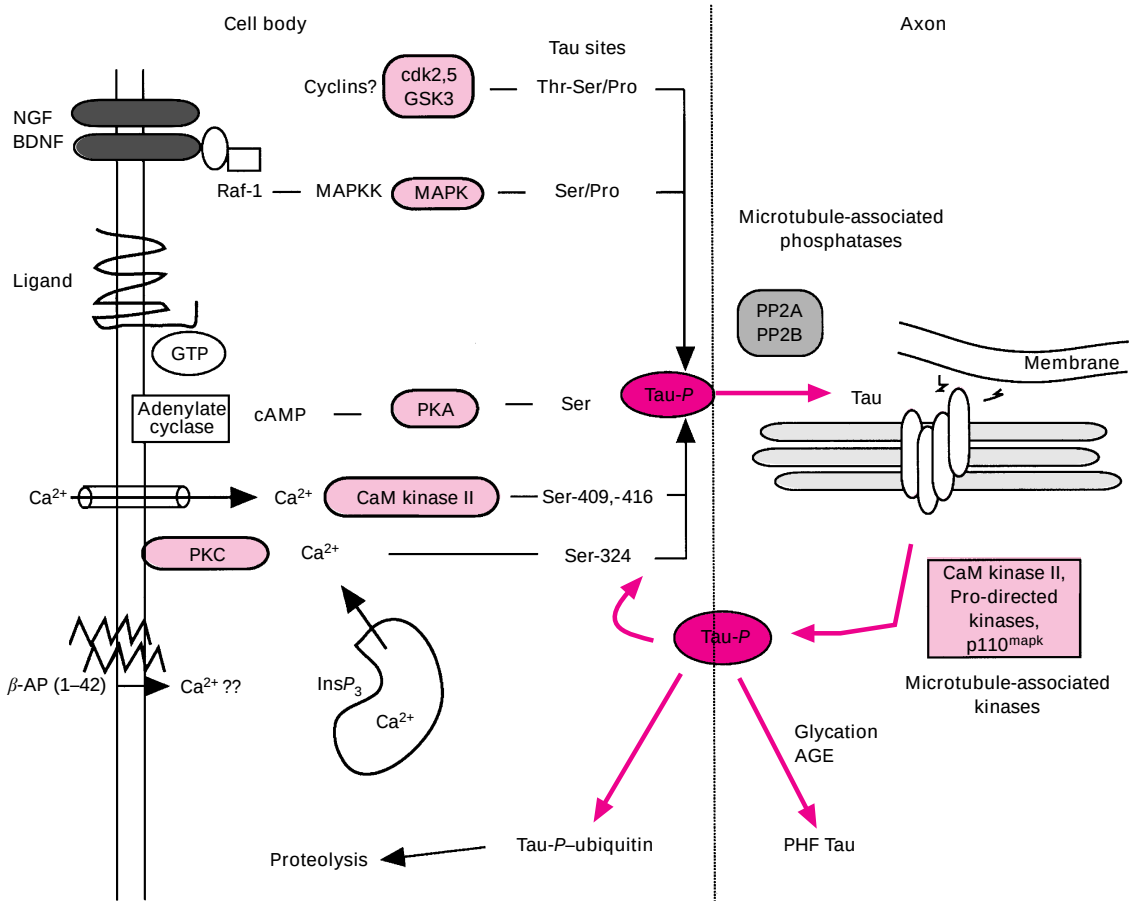

Figure 4 Regulation of tau phosphorylation and trafficking in a model neuron

Tau phosphorylation may regulate the entry of tau into the axon and binding of tau at the distal end of the microtubular system in axons, and may ultimately target tau for retrograde transport and lysosomal degradation. Ligands which may affect the activation of tau-directed protein kinases are also shown, indicating the many signals which may increase tau phosphorylation. β-AP fibrils have been reported to increase tau phosphorylation and to alter calcium homoeostasis in some model systems. AGEs may help to augment PHF formation. Abbreviations: BDNF, brain-derived neurotrophic factor; MAPKK, MAPK kinase.

Other post-translational modifications of tau

Glycation of tau can occur via the non-enzymic formation of 'advanced glycation end-products' or AGEs [189]. These moieties form between glucose and protein moieties over time, particularly under conditions of oxidative stress. AGE formation can promote β-AP fibril formation *in vitro*. Tau can be glycated *in vitro*, resulting in a loss of microtubule binding [190,191]. Monospecific antibodies against AGEs were used to co-localize such products with authentic PHFs; when introduced into SH-SY5Y cells, glycated tau stimulated oxidative stress [192]. Lysine residues in the microtubule domain are potential sites for such modifications. The receptor for AGE products ('RAGE') has recently been cloned and characterized [193]. It is located on microglial cells and subsets of neurons. Importantly, it may modulate many of the toxic reactions caused by AGE-modified β-AP. Interestingly, neurons from fetal Down's-syndrome brains show increased oxidative stress and undergo rapid apoptosis *in vitro*, suggesting that a defect in reactive oxygen generation or response may also occur in trisomy 21 [194]. AGE–tau conjugates may also more likely to form covalent cross-links between each other.

PHF tau is also modified by glycolipids. A recent MS and NMR analysis of acid-resistant PHF tau revealed the presence of several glycolipids consisting of glucose pentamers, hexamers and tridecamers variably associated with esterified fatty acids (C_{14}–C_{20}) [195]. This intriguing analysis is consonant with the AGE hypothesis and, further, demonstrates the presence of hydrophobic, potentially self-associating, fatty acids. Recent studies have shown that heparin and heparan can induce PHF-like assembly *in vitro* [14,196]. Heparan, a sulphated glycosaminoglycan, is associated with PHFs. Recombinant tau was able to bind heparin–Sepharose whether or not it was phosphorylated. Furthermore, heparin catalysed the formation of PHF structures *in vitro* from three-repeat tau, suggesting that excess sulphated glycosaminoglycan can facilitate PHF formation in cells. This interesting twist suggests that aberrant lipid metabolism may be a culprit in PHF formation. The interplay between phosphorylation as a targeting event, tau glycation, oxidation and heparan-mediated PHF formation remains to be elucidated.

Other factors and modifications have been reported to influence tau. Aluminium, long an element of controversy in AD research, can cause non-enzymic phosphate incorporation into recombinant tau *in vitro* [197]. *In vivo*, aluminium salts have been reported to bind PHF tau, and to induce co-deposits of other tangle-related proteins such as β-AP, ubiquitin and α_1-anti-

chymotrypsin [198]. Aluminium salts administered intracisternally to rabbits cause temporal increases in AT8, Alz-50 and PHF-1 staining [199]. However, the relationship between aluminium and PHF remains primarily experimental at this stage.

Transglutaminase may also form cross-links between tau monomers, primarily in the C-terminal portion [200]. Electron microscopy suggested that transglutamate links formed a filamentous structure [201]. Interestingly, such cross-links were Alz-50-positive [202]. Ubiquitin is also conjugated to PHF tau. Structural studies suggest that tau protein is N-terminally processed and may be phosphorylated prior to ubiquitin conjugation [203]. The persistence of ubiquitin in PHF tangles suggests a failure of normal pathways leading to proteolysis [204]. Thus work on how tau forms filaments under physiological conditions, when covalently modified by phosphate, glucose, lipids and cross-links, remains a key area of research [205].

Apolipoprotein E4 (apoE4) has been hypothesized to play a direct role in binding tau and altering its susceptibility to kinases and phosphatases [206,207]. In a similar vein, apoE4 has been reported to interact with β-AP [208]. In neuroblastoma lines, apoE3 promotes, while apoE4 inhibits, neurite outgrowth in a microtubule-dependent manner. Several studies have reported direct interactions between apoE4 and tau *in vitro*. Although intriguing, several major issues argue against a simple role for tau–apoE4 interactions [209]. First, apoE4 must cross neuronal membranes to bind tau. Secondly, tau–apoE4 interactions must be altered over time to account for the late-onset disease. Finally, apoE4 is a risk factor, not a causal link. However, it is possible that apoE4 somehow alters neuronal lipid or glycolipid metabolism, leading to increased intracellular glycation of tau. Mice deficient in apoE show neuronal degeneration characterized by a loss of synapses, dendritic and cytoskeletal changes and a decrease in tubulin [210]. This suggests that apoE may play a role in the maintenance and/or repair in the central nervous system.

Potential sites for therapeutic intervention

One goal of understanding alterations in PHF tau is to identify possible therapeutic avenues that might prevent or slow PHF formation [211]. Cytoskeletal stabilization via drugs was initially suggested as a possible mode of therapy, based on the presumption that PHF tau destabilizes the cytoskeleton [212]. However, due to the dynamic nature of the cytoskeleton, excess stability is as damaging as destabilization. Hence drugs that mimic nocodazole would be predicted to induce serious neurotoxicity.

Tau-directed protein kinases are potential targets for modification in AD. However, most of the protein kinases that affect tau also affect other substrates. Inhibitors of protein kinases have not found much clinical utility as a result. Agents that alter second-messenger generation are useful therapeutic agents, and thus of potential utility. One intriguing prospect would be to determine whether increases in cAMP levels can alter tau disposition in AD or a related model. One prediction is an altered turnover and/or trafficking of tau protein. Agents such as rolipram, forskolin and other phosphodiesterase inhibitors may be used to determine whether PKA-induced tau phosphorylation alters tau dynamics. Agents that activate MAPK and related protein kinases may also be useful in redirecting tau traffic. Such factors include NGF and related growth-promoting ligands for tyrosine kinase receptors. However, recent studies with specific inhibitors (P098059) of MAPK kinase in primary cultures indicated that okadaic acid could still induce tau phosphorylation, suggesting that other protein kinases and/or phosphatases play important roles in the regulation of tau [213].

Phosphatases are important targets for immunosuppressive drug action [214]. Calcineurin inhibition by FK-506/FK-520 and cyclosporin leads to altered patterns of tau phosphorylation, but these agents alone cannot induce PHF-like tau in human brain slices. One speculation is that the inhibition of calcineurin might lead to altered turnover of selected sites on tau (Thr-181, Thr-231, Ser-235), altering the dynamics of the N-terminus of tau. This perturbation could have a paradoxical effect, leading to increased tau turnover. Since FK-506 is in clinical use and can penetrate the brain to some extent, this hypothesis could be tested. Alternatively, agents that activate intracellular calcineurin may also be predicted to alter tau turnover and trafficking. Agents such as thapsigargin and calcium-channel agonists could have such an effect, but would suffer from pleotropic activation of other calcium-sensitive enzyme systems. Similarly, since tau phosphorylation can also be regulated by microtubule-associated PP2A in neurons, agents that activate PP2A (or prevent its inhibition by endogenous inhibitory proteins) may also be of utility in preventing excess tau phosphorylation [215].

Finally, drugs that alter tau–tau interactions may play a role in the prevention or slowing of PHF accumulation. Non-neuroleptic phenothiazine derivatives have been reported to block tau–tau interactions at the level of the microtubule repeat domain in protease-resistant PHF core proteins [216]. This report demonstrated an assay for screening compounds for anti-aggregator properties. It is now feasible to set up *in vitro* assays, using both recombinant human and PHF-derived tau, to screen for agents that block heparan-induced aggregation. The identification of agents that have selective profiles for tau aggregation with minimal side effects will be crucial.

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