Cell. Mol. Life Sci. 64 (2007) 1701 – 1714
1420-682X/07/131701-14
DOI 10.1007/s00018-007-6513-4

Cellular and Molecular Life Sciences

Biomedicine & Diseases: Review

RNA and protein-dependent mechanisms in tauopathies: consequences for therapeutic strategies

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Received 3 December 2006; received after revision 23 February 2007; accepted 20 March 2007
Online First 23 April 2007

Abstract. Tauopathies are a group of neurodegenerative diseases characterised by intracellular deposits of the microtubule-associated protein tau. The most typical example of a tauopathy is Alzheimer's disease. The importance of tau in neuronal dysfunction and degeneration has been demonstrated by the discovery of dominant mutations in the *MAPT* gene, encoding tau, in some rare dementias. Recent developments have shed light on the significance of tau phosphorylation and aggregation in pathogenesis. Furthermore, emerging evidence reveals the central role played by tau pre-mRNA processing in tauopathies. The present review focuses on the current understanding of tau-dependent pathogenic mechanisms and how realistic therapies for tauopathies can be developed.

Keywords. Tauopathies, neurodegeneration, Alzheimer's disease, tau, alternative splicing, phosphorylation, glycogen synthase kinase, aggregation.

Introduction

Several major diseases of the central nervous system are characterised neuropathologically by prominent intracellular accumulations of abnormal filaments formed by the microtubule-associated protein tau in affected neurons and are collectively referred to as tauopathies [1]. Tauopathies include dementias such as Alzheimer's disease (AD), Pick's disease (PiD), some forms of frontotemporal dementia and corticobasal degeneration (CBD) as well as movement disorders such as progressive supranuclear palsy (PSP) [2]. The most characteristic tau-containing lesions are the neurofibrillary tangles (NFTs) found in large number in AD, which are made of paired helical filaments (PHFs). Definitive evidence for the pathogenic importance of tau was provided in 1998 by the discovery of dominant mutations in the *MAPT* gene, the gene encoding tau, in the rare dementia, frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) [3–5]. Tau is mainly expressed in neurons and predominantly localised in axons and promotes microtubule polymerisation and stabilisation [1, 6]. How tau causes neuronal dysfunction and death has been the subject on intense research and reviews of the field are published regularly (for recent examples, see [1, 6–10]). The present review focuses on the current understanding of tau-dependent pathogenic mechanisms and how realistic therapies for tauopathies can be developed.

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The *MAPT* gene

The human *MAPT* gene is a large gene of about 134 kb comprising 16 exons on chromosome 17q21.3 [11] (Fig. 1). Alternative splicing of exons 2, 3 and 10 (E2, E3 and E10) in the tau pre-mRNA results in the expression of six isoforms in adult human brain. E10 encodes the second of four imperfect 31–32-amino acid microtubule-binding repeats in the C-terminal half of the tau protein. Exclusion or inclusion of E10 gives rise to tau isoforms with three (tau3R, $E10^-$) or four (tau4R, $E10^+$) microtubule-binding repeats [12]. Furthermore, inclusion or exclusion of E2 and E3 produces tau isoforms with different N termini (Fig. 1). Tau isoforms can have either no N-terminal insert, E2 or E2+E3. E3 is never present without E2. The N terminus of tau regulates its interaction with the plasma membrane [13]. The six tau isoforms resulting from alternative splicing of E2, E3 and E10 have molecular masses ranging from 48 to 56 kDa. Tau3R and tau4R are expressed in approximately equal amounts in adult human brain, but only tau3R is expressed during embryogenesis. Exon 4a encodes a 254-amino acid sequence present in a high molecular weight form of tau expressed only in the peripheral nervous system [14, 15].

Two haplotypes of the *MAPT* gene, H1 and the less common H2, have been identified [16]. *MAPT* haplotypes are defined by a region of complete linkage disequilibrium that extends over the entire *MAPT* gene and contains a series of at least 16 single nucleotide polymorphisms in different exons and introns. Noticeably, H2 also contains a 238-bp deletion in intron 9 between positions –951 and –713 [16] (Fig. 1). H2 is invariant, but several variants of H1 have been identified. The *MAPT* gene is part of an ~900-kb inversion that prevents the recombination between the H1 and H2 haplotypes [17]. Stemming from the work of Conrad *et al.* [18], association between specific *MAPT* haplotypes and a number of tauopathies has now been shown. Homozygosity of H1 is associated with PSP and CBD [16, 19, 20], while H2 shows strong negative association with both diseases [16]. Furthermore, variants of H1, such as H1c and H1b have a high degree of association with PSP and CBD [21, 22]. The H1c sub-haplotype has also been shown to be associated with AD [23, 24] and the importance of the H1c haplotype in AD has been confirmed using quantitative trait analysis [25].

By contrast, no association between *MAPT* haplotypes and PiD has been found [26]. The association of *MAPT* haplotypes and tauopathies has been reviewed recently from a genetic standpoint [24]. A heterozygous deletion of 500–650 kb within the inverted region has been detected in three individuals with learning disability [27]. However, whether the clinical phenotype is caused by the loss of one copy of the *MAPT* gene is still to be determined.

MAPT mutations in FTDP-17

To date more than 35 mutations in the *MAPT* gene have been associated with FTDP-17. A comprehensive and up-to-date database of *MAPT* mutations and polymorphisms is available on the World Wide Web at: http://www.molgen.ua.ac.be/FTDmutations/. *MAPT* mutations are either missense, deletions and silent mutations in the coding region, or intronic mutations. Missense mutations outside E10, such as V337M and R406W, affect both tau3R and tau4R and lead to the development of tau filaments containing all six human tau isoforms, whereas missense mutations in E10, such as P301L and N279K affect tau4R only. The majority of missense *MAPT* mutations in FTDP-17 reduce the affinity of tau for microtubules and its ability to promote tubulin polymerisation and microtubule stability [28–32]. This is perhaps not surprising as a large proportion of the missense mutations are found in the C terminus of tau near or inside the microtubule-binding domain. In transfected cells, mutant tau binds to microtubules, but is displaced by co-expression of wild-type tau [33]. The G272V, Δ280K, or P301L mutations, but not the R406W mutation, reduce the ability of tau to regulate the dynamic instability behaviour of microtubules [34]. However, the I260V, Δ280K, P301L, V337M and R406W mutations do not affect the rate of translocation of tau in axons in cultured neurons [35]. Transgenic mice overexpressing the missense P301L mutation, the most common one, develop NFTs and Pick-body-like lesions in various areas of the brain from about 5 months of age and display motor and behavioural deficits [36]. These animals, referred to as JNPL3 mice, are widely used in investigating mechanism downstream of tau pathology and in the development of therapeutic strategies.

Most intronic *MAPT* mutations are clustered near the 5′ end of intron 10 and increase the incorporation of E10 [3]. A mutation has also been identified at position –10 in intron 9 that also increased E10 incorporation *in vitro* [37]. Furthermore, with the exception of the P301L/S mutations, mutations within E10 also promote E10 inclusion. In turn, E10 retention results in a two- to six-fold excess of tau4R over tau3R, as opposed to both isoforms being in approximately equal proportions in normal adult brain [3, 5, 29, 38]. Fibrillar inclusions in neurons from patients carrying these mutations are mainly composed of tau4R. On the other hand, the Δ280K mutation

reduces the $E10^+/E10^-$ tau mRNA ratio (4R/3R ratio) [39].

Due to the presence of an additional repeat, tau4R has a higher affinity for microtubules than tau3R and, indeed, expression of tau4R in transfected cells displaces tau3R from microtubules [33]. Consequently, an abnormal tau isoform ratio is likely to impair microtubule properties and microtubule-dependent functions. For instance, overexpression of tau in non-neuronal cells protects microtubules from severing by katanin and tau4R has a better protective effect than tau3R [40]. Tau has been suggested to act as a regulator of kinesin-dependent, microtubule-based transport [41] and tau3R and tau4R have a differential effect on transport [42].

Mechanisms of tau E10 splicing disruption in FTDP-17

The sequence of the exon 10/intron 10 junction predicts a stem-loop structure regulating tau E10 splicing by protecting the 5′ splice site. FTDP-17 mutations +3 to +16 destabilise this stem loop, resulting in an enhancement of E10 inclusion [43, 44]. The stem loop is also destabilised by the exonic S305N mutation [44, 45] (Fig. 1).

Splicing regulatory elements within E10 have been extensively characterised. E10 contains several exonic splicing enhancers (ESEs), the best characterised being a polypurine enhancer that acts in conjunction with an A/C-rich enhancer [10] (Fig. 1). This ESE is strengthened by the N279K mutation and abolished by the Δ280K mutation, resulting in inclusion or exclusion of E10 [45, 46]. The ESE serves as a binding site for the splicing factors Tra2β and SF2/ASF [47, 48]. The affinity of Tra2β and SF2/ASF for the ESE is enhanced by the N279K mutation and decreased by the Δ280K mutation [46, 47]. Tra2β binding is antagonised by the serine-arginine-rich (SR) protein, SRp54, and this provides an additional level of regulation [49]. Inhibition of E10 inclusion requires the binding of splicing factors, such as SRp30c or SRp55, to silencer elements at the 5′ end of E10 [50, 51]. E10 regulatory elements also include an ESE near its 3′ end and an exonic splicing silencer (ESS), which is disrupted by mutations N296H/N and L284L [46]. E10 splicing can be regulated further by phosphorylation of *trans*-acting factors. For example inhibition of glycogen synthase kinase-3β activity in cultured neurons promotes E10 inclusion in tau mRNA, an effect probably mediated by phosphorylation of the SR protein SC35 [52]. On the other hand, phosphorylation of SR proteins by Cdc2-like kinases promotes E10 skipping [53].

Intronic elements controlling E10 splicing have not been as extensively characterised as exonic elements, partly due to the large size of introns flanking E10 (intron 9, 13.6 kb; intron 10, 3.8 kb). A mutation at position –10 in intron 9 promotes E10 incorporation, hence suggesting that it is located in a regulatory element [37]. The +19 mutation, 3′ of the stem loop results in a decrease in E10 incorporation, probably by disrupting a bipartite regulatory sequence [54, 55]. Few *trans*-acting factors binding to tau pre-mRNA intronic elements have been identified. The RNA binding protein, RBM4 (RNA binding motif protein 4), binds to a sequence in intron 10 and stimulates the incorporation of E10 [56]. Other factors binding to intronic elements and promoting E10 incorporation are CELF3 and CELF4 [57].

Tau E10 splicing in sporadic tauopathies

Alteration of the 4R/3R ratio in FTDP-17 highlights the importance of a tight regulation of tau isoform balance to maintain neuronal viability. A question that comes immediately to mind is whether dysregulation of tau pre-mRNA splicing may be a contributing factor to sporadic tauopathies, especially AD. Tau-containing lesions in PSP and CBD are mainly composed of tau4R [58–62], although some heterogeneity between cases has been reported [63]. Tau isoform imbalance in PSP is at least in part the result of aberrant splicing of the tau pre-mRNA leading to an excess of tau4R over tau3R [60]. In the context of AD, several studies have addressed this issue and came up with conflicting results. Studies on affected areas from post-mortem brain using various quantitative RT-PCR strategies did not reveal statistically significant differences in the 4R/3R ratio between AD and control brains [38, 64]. A quantitative RT-PCR analysis of tau isoform ratios in laser-captured neurons with or without neurofibrillary pathology revealed regional differences in the 4R/3R ratio, but failed to show an association between elevated isoform ratio and AD pathology [65]. On the other hand, another study measured a 4R/3R ratio of about 1.5 in the temporal cortex of post-mortem brain from AD patients [66].

The development of sporadic tauopathies is strongly influenced by *MAPT* haplotypes and a recent study has shown that the two *MAPT* haplotypes, H1 and H2, produce different levels of tau mRNA species containing E10. Specifically, an allele-specific expression analysis of post-mortem human brain and cultured cell lines has demonstrated that up to 1.43 more $E10^+$ mRNA is expressed from the H1 haplotype that from the H2 haplotype [67]. Also, a region-specific differ-

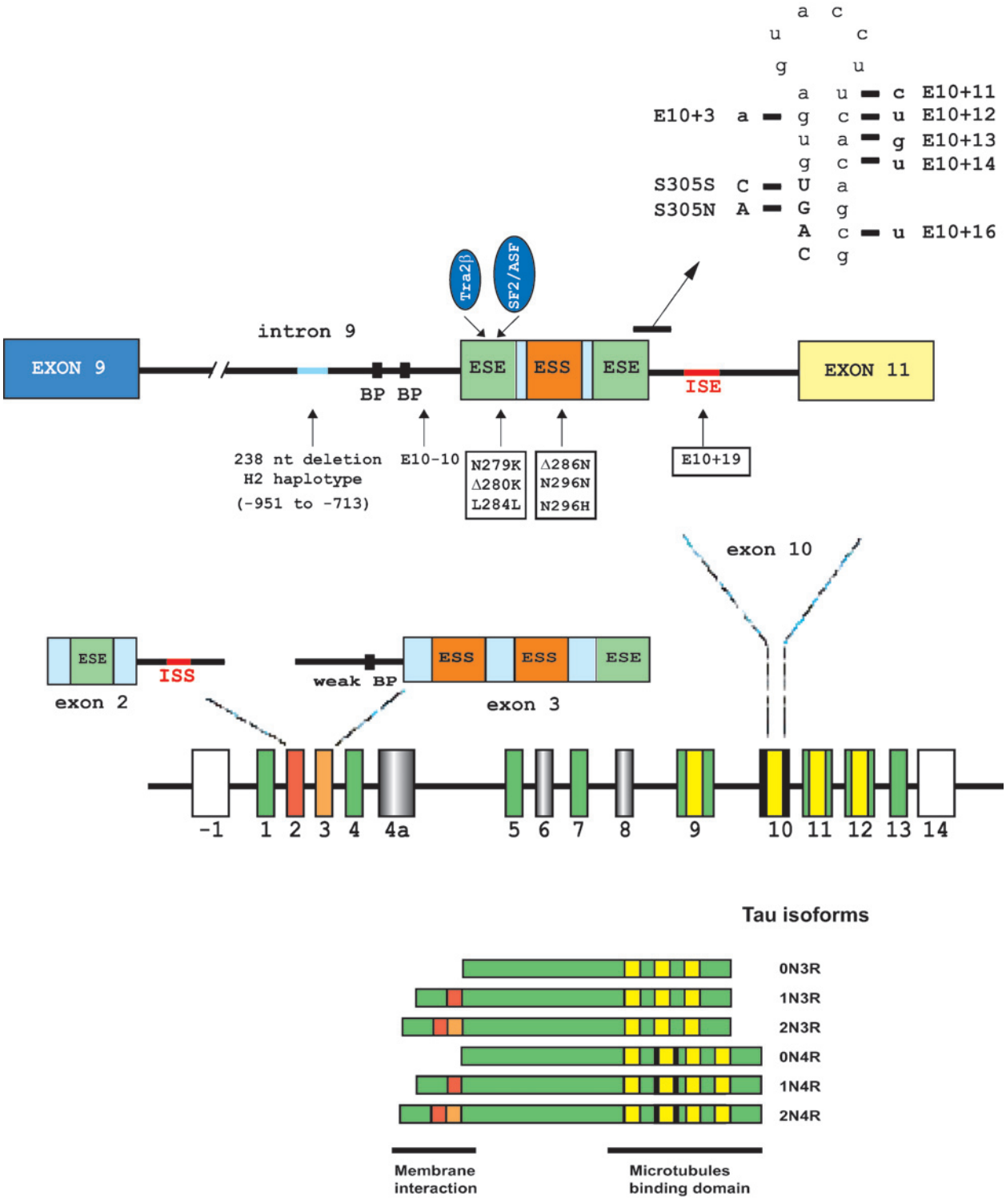

Figure 1. Structure of the human *MAPT* gene and of tau isoforms. The *MAPT* gene comprises 16 exons; exons - 1 and 14 are not translated (white boxes). Green boxes represent constitutively expressed exons. Exons 9–12 encode the microtubule-binding domain. The 238-nucleotide sequence in intron 9 deleted in the H2 haplotype is highlighted. The yellow box within each repeat encodes a 18-amino acid microtubule-binding sequence. Six tau isoforms are expressed in the central nervous system and differ by the inclusion or exclusion of exons 2, 3 and 10. E4a encodes a 254-amino acid sequence found only in a large tau isoform (110 kDa) expressed specifically in the peripheral nervous system. Exonic and intronic elements regulating the splicing of exons 2, 3 and 10 are shown, as well as some of the FTDP-17 mutations affecting E10 splicing. The exon 10/intron 10 junction sequence predicts a stem-loop structure regulating exon 10 splicing. (Partly adapted from [1, 9, 10]).

ence in the $E10^+$ transcripts produced from H1 and H2 was found, the difference being the highest in the globus pallidus, a region severely affected in PSP. Furthermore, this study showed that the total levels of tau mRNA produced from H1 or H2 were not significantly different. Similarly, an independent study found that the H1c haplotype increased the proportion of $E10^+$ transcripts, but also increased *MAPT* expression levels [68]. Thus elevated production of $E10^+$ tau mRNA may explain the susceptibility to neurodegeneration conferred by the H1 haplotype.

Tau E2 splicing in myotonic dystrophy

Several diseases, which are not primarily neurodegenerative, nevertheless display a tau pathology. One such example is myotonic dystrophy, type 1 (DM1), an inherited progressive muscle wasting disease. DM1 is caused by the expansion from 50 to over 1000 in a CTG repeat domain in the 3′ untranslated region of the DM protein kinase gene (*DMPK*) [69]. The CUG expansion in the mRNA acts through a *trans*-dominant effect resulting in decreased activity of the RNA-binding protein Muscleblind and increased activity of another RNA-binding protein, CUG-BP, a member of the CELF family of RNA-binding protein [70]. DM1 is associated with degeneration of cortical neurons with abnormal deposits of tau [71, 72]. Degeneration of cortical neurons in DM1 patients is correlated with reduced levels of E2-containing tau transcripts [71, 72]. Tau E10 splicing is also dysregulated in DM1, but its connection with the phenotype is uncertain [73]. Interestingly, the splicing of tau E2 is regulated by a CELF protein, ETR-3 [74].

Splicing in non-tau dependent dementias

FTDP-17 is part of a larger group of dementias, referred to as frontotemporal lobar degeneration (FTLD) characterised by similar clinical phenotype and pattern of neuronal degeneration. FTLD cases not linked to *MAPT* mutations do not show any tau pathology in the form of tau deposits nor show biochemical abnormalities of the tau protein or abnormal tau isoform composition [75, 76]. The genes causing some familial cases of FTLD have been identified. These include *CHMP2B*, encoding charged multivesicular body protein 2B, a potential regulator of endosomal trafficking [77] and *PRGN*, encoding the growth factor progranulin. Intriguingly, the *PRGN* gene is in the same locus as the *MAPT* gene on chromosome 17 [78, 79]. A number of tau-negative FDLD cases, with or without a family history, contain ubiquitinated inclusions in affected neurons that are similar to the typical skein ubiquitin inclusions characteristic of motor neurons in amyotrophic lateral sclerosis (ALS) [80]. These cases are referred to as tau-negative FTLD with ubiquitin-positive inclusions (FTLD-U). The major ubiquitinated component of ubiquitin-positive inclusions in FTLD-U and ALS has been identified as the protein TDP-43 [81, 82]. TDP-43 (TAR-DNA-binding protein 43) in FTLD-U is hyperphosphorylated, proteolysed and ubiquitinated. TDP-43 is a DNA- and RNA-binding protein that has been shown to promote exon 9 skipping in cystic fibrosis transmembrane conductance regulator (CFTR) transcripts [83, 84]. Thus, by accumulating in the cytoplasm TDP-43 would not be able to perform its function as a splicing regulator and its natural targets might be misspliced.

Interestingly, a number of transcripts other than tau appear to be misspliced in AD, including Tra2β, clk2 and presenilin 2 [66]. Missplicing of multiple transcripts would be consistent with the novel concept whereby a single splicing factor can regulate the activity of functional protein networks in neurons by co-ordinating exon usage [85]. Thus, it is tempting to speculate that dysregulation of co-regulated alternative splicing might be a contributing factor to dementias. Different co-regulated networks may be disrupted in different dementias, but they may share some common targets.

Alternative splicing as a therapeutic target

From a therapeutic perspective, correcting defective alternative splicing, be it tau or a co-regulated target, would ideally require direct intervention at the RNA level. A main advantage of modifying gene expression at the RNA level over traditional gene therapy strategies is that the modified transcript is expressed under endogenous transcriptional control. Hence, the overall level of expression of the targeted transcript is not changed and the modified transcript has the same expression pattern as the endogenous target. Reduction of E10 inclusion has been achieved using oligonucleotides binding to E10 splice junctions in rat pheochromocytoma PC12 cells that express predominantly $E10^+$ tau [86]. These authors designed modified antisense oligoribonucleotides targeting the 5′ or 3′ splice junction of E10 to block the access of the splicing machinery to tau pre-mRNA. Other strategies to interfere with splicing events are based on the design of bifunctional oligoribonucleotides binding to both an exonic regulatory element and a *trans*-acting factor or hybrid molecules comprising an antisense domain and a peptide domain mimicking a *trans*-

acting factor [87–89]. However, there is no report to date on the application of these methods to the modulation of tau splicing.

Another strategy is to reprogram tau mRNA using spliceosome-mediated RNA *trans*-splicing, or SMaRT™ [90]. SMaRT creates a chimaeric mRNA through a *trans*-splicing reaction mediated by the spliceosome between the 5′ splice site of an endogenous target pre-mRNA and the 3′ splice site of an exogenously delivered pre-*trans*-splicing RNA molecule. Tau pre-mRNA has been shown to be a suitable target for SMaRT at the level of E10 splicing [91]. Furthermore, *cis*-splicing exclusion of E10 can be bypassed by *trans*-splicing and incorporation of E10 into tau mRNA can be achieved with an efficiency of approximately 30 % [91]. Thus, *trans*-splicing could be used to correct aberrant tau E10 splicing and, due to its versatility and selectivity, SMaRT would offer a distinctive advantage over other methods. In addition to correcting defective alternative splicing, SMaRT can be used to repair dominant mutations at the RNA level; in this case, a corrected transcript is expressed, while, simultaneously, the mutant form is down-regulated.

Correction of defective alternative splicing by direct intervention at the RNA level will necessarily require delivery of therapeutic sequences through viral vectors. An advantage of gene transfer methods, as compared to drug-based therapies, is that they provide long-term benefit and alleviate side effects and blood-brain barrier crossing problems. Gene therapy for brain diseases is still at an early stage of development, but several classes of viral vectors have been developed for the transduction of neurons and are of proven efficiency. Such vectors include lentivirus vectors that can transduce neurons *in vivo* and integrate the transgene into the host cell genome so maintaining long-term expression [92, 93].

Tau hyperphosphorylation

Disease-associated tau is post-translationally modified by phosphorylation, N- and C-terminal proteolytic cleavage, altered conformation, nitration, glycosylation, glycation or ubiquitination [7, 94]. The form of tau that assembles into PHFs in AD is referred to as PHF-tau. Tau hyperphosphorylation is the most prominent tau modification in all tauopathies including AD, FTDP-17, PSP, CBD and PiD [95]. Tau has 79 potential serine or threonine phosphorylation sites and four tyrosine phosphorylation sites. Biochemical and mass spectrometry analyses have shown an increase in the stoichiometry of phosphorylation in PHF tau [96, 97]. Hyperphosphorylation of cytosolic tau in AD brain induces self-aggregation, with dephosphorylation preventing its assembly into PHF [98]. Tau phosphorylation at specific sites correlates with neurodegeneration in individual neurons in the brains of transgenic mice overexpressing an FTDP-17 tau mutant [99]. Phosphorylation sites characteristic of disease-associated tau are mainly sites phosphorylated by proline-directed kinases (*e.g.* GSK-3 and cdk5). Tau is also phosphorylated by non proline-directed kinases and tyrosine kinases [100–102].

Glycogen synthase kinase-3 (GSK-3) exists as two isoforms, GSK-3α and GSK-3β, and phosphorylates tau at sites known to be abnormally phosphorylated in tauopathies [103, 104]. GSK3 also colocalises with NFTs in AD brain [105]. Overexpression of GSK-3β, or of the constitutively active S9A mutant, in transgenic mice has demonstrated that GSK-3β promotes neurodegeneration *in vivo* [106–109], with a significant reduction in overall brain volume [106]. Conditional overexpression of GSK-3β results in tau hyperphosphorylation and redistribution of tau from axons to somatodendritic regions [107]. Pathological signs in these animals include lower level of nuclear β-catenin, apoptosis (presence of TUNEL- and active caspase-reactive neurons), reactive gliosis and decreased thickness of the dentate gyrus [107, 109]. Furthermore, deficits in spatial learning in the Morris water maze were found in the hippocampus of these mice [108]. Co-expression of human tau and *shaggy*, the fly homologue of GSK-3, in *Drosophila* results in neurodegeneration that is associated both with tau hyperphosphorylation and with the presence of filamentous tau aggregates [110]. However, neurodegeneration was also observed in fly lines overexpressing human tau only.

Cyclin-dependent kinase 5 (cdk5) phosphorylates tau at sites hyperphosphorylated in tauopathies. The activity of cdk5 is regulated by its binding to the neuronal activator protein p35 and its more potent proteolytic product, p25. In cultured cells, tau phosphorylation by the cdk5/p25/p35 pathway is a key mediator of neuronal toxicity in response to Aβ treatment [111–113]. Overexpression of p25 in mice leads to the accumulation of phosphorylated tau and neurofilaments in spheroids and to the development of axonopathy [114–116]. This correlates with neuronal and axonal degeneration, with the presence of neurons with condensed nuclei and cytoplasm and few remaining axonal or dendritic processes. Interestingly, cdk5-induced neurodegeneration in these models is found in the absence of any neurofibrillary pathology. Mice inducibly expressing p25 postnatally in the forebrain develop hyperphosphorylated, aggregated, tau that precedes the deposition of NFTs in the cortex and hippocampus [117]. Extensive brain atrophy and

neuronal loss were found in areas known to be affected in AD and aged mice develop severe cognitive deficits [118]. In these animals hyperphosphorylated and aggregated tau coincides with the onset of neuronal loss, supporting the hypothesis that neurotoxicity occurs prior to the formation of mature NFTs. In addition to phosphorylating tau directly, cdk5 phosphorylates GSK-3 and regulates its activity, thus signalling between cdk5 and GSK-3 may have a cumulative effect on tau phosphorylation [119, 120].
Microtubule-affinity regulating kinase (MARK) is a non-proline-directed protein kinase that regulates microtubule dynamics and axonal transport [121, 122]. Overexpression of Par-1, the fly homolog of MARK kinase, in *Drosophila* induces tau hyperphosphorylation at sites characteristic of PHF tau [123]. Par-1 overexpression causes neuronal cell death *in vivo*; elevated levels of TUNEL reactive cells indicate the involvement of an apoptotic mechanism [123]. Par-1 overexpression also results in the phosphorylation of tau at putative cdk5 and GSK-3 sites. This could be explained by two possible mechanisms: (i) MARK phosphorylation of tau may reduce significantly the affinity of tau for microtubules leading to an increased pool of unbound tau which is more susceptible to phosphorylation by cdk5 and GSK-3, (ii) MARK/Par-1 may also alter the conformation of tau to make docking sites more accessible for additional kinases [123]. The importance of MARK/Par-1 in tau-induced neuronal degeneration was strengthened recently as it was identified as a suppressor of tau toxicity in *Drosophila* through a screen for genetic modifiers [124]. In this screen, tau-associated protein kinases and protein phosphatases accounted for the vast majority of the modifiers identified, hence supporting phosphorylation as a key pathogenic mechanism in tauopathies. However, GSK-3 and cdk5 were found not to modify toxicity.
Mutagenesis of serine and threonine residues to glutamate to mimic phosphorylation at these sites has allowed insight to be gained into the functional consequences of sustained disease-like tau hyperphosphorylation. Mutation of ten key phosphorylation sites of tau induces apoptotic cell death in the absence of tau aggregation in both PC12 cells and terminally differentiated human CNS neurons [125]. Treatment of cells expressing pseudohyperphosphorylated tau with the microtubule stabilising agent, taxol, was not sufficient to reverse the effects of mutated tau, indicating that the reduced ability of tau to stabilise microtubules is not the primary toxic effect of phosphorylation. Furthermore, pseudohyperphosphorylated tau induces neurodegeneration in specific regions of cultured hippocampal slices, suggesting that the toxic effects of tau phosphorylation are both region and neuron type specific [126].
Phosphorylation might contribute to tau toxicity by modulating its proteolytic cleavage. Tau is cleaved *in vivo* by at least two groups of proteases, caspases and calpains. Tau fragments generated by different proteases may differ in their ability to assume disease-associated conformational states, assembly into filaments and neurotoxicity [127–129]. Tau is cleaved at Asp421 by caspase-3 in response to Aβ treatment of neurons, generating a fragment initiating tau polymerisation [130]. Tau cleavage by caspase-3 at Asp412 can be prevented by mutation of Ser422 to glutamate [128].

Tau hyperphosphorylation as a therapeutic target

Tau hyperphosphorylation is a relatively early event in the development of tauopathies and as such represents a good therapeutic target. The availability of new transgenic mouse models, which recapitulate many of the features of human tauopathies, has allowed *in vivo* testing of kinase inhibitors. The use of protein kinase inhibitors in the treatment of AD has elicited a considerable interest in the pharmaceutical industry. GSK-3 is generally accepted to be a key tau kinase in tau-induced pathology and the GSK-3 inhibitor lithium is widely used to reduce tau phosphorylation *in vitro*. Perez *et al.* [131] were the first to show that chronic lithium treatment of transgenic mice not only resulted in a reduction in tau phosphorylation, but also in a reduction in the number of tau filaments. Further *in vivo* testing revealed that GSK-3 inhibition with lithium was sufficient to reduce abnormal tau phosphorylation and aggregation, concomitant with a significant improvement in axonal degeneration in JNPL3 mice, that overexpress mutant human P301L tau [132]. Similar results were obtained with the selective GSK-3 inhibitor, AR-A014418, developed by AstraZeneca [132]. Furthermore, AR-014418 reduces tau phosphorylation *in vitro* as well as protects neurons against Aβ-induced neurotoxicity in hippocampal slice cultures [133]. GSK-3 inhibition has been reported to promote tau E10 inclusion by inhibiting the phosphorylation of the SR protein, SC35 [108], suggesting that GSK-3 may be involved in the development of tauopathies not only by phosphorylating tau directly, but also by affecting E10 splicing.
The synthetic inhibitor, SRN-003–556, an orally bioavailable CNS penetrating analogue of the naturally occurring K252a, has a relatively broad specificity, inhibiting ERK2, cdc2, GSK-3β, PKA and PKC with roughly approximately equal efficacies [134]. Treatment of JNPL3 mice with SRN-003–556 results

in lower levels of phosphorylated tau and of aggregated tau species. Importantly, the development of the severe motor deficits typical of these animals was improved by the treatment, suggesting that kinase inhibition is sufficient to either prevent or reverse cell death associated with motor phenotypes. In addition to reducing tau pathology and the associated neurodegeneration, kinase inhibitors also reduce the development of amyloid pathology in transgenic mice expressing pathogenic mutants of the amyloid precursor protein [135], making them a particularly attractive therapeutic approach for AD.

The wide range of substrates modulated by protein kinases, together with kinase-kinase interactions [119, 120], may be a cause for concern; however, kinase inhibitors are increasingly used as therapeutic agents in a number of diseases, including the tyrosine kinase inhibitors gleevec and herceptin in leukaemia and breast cancer, respectively, and lithium in bipolar disorders. Both the safety record and the good tolerance reported with most kinase inhibitors in current clinical use suggest that such compounds may be very successful in developing new therapies for tauopathies.

Pathological conformations of tau

Specific conformational changes of tau unique to AD brain may take place at an early stage of the disease, preceding NFT formation, and may represent a more disease-specific alteration than phosphorylation [136, 137]. One such conformational change creates the epitope recognised by the MC1 antibody [138]. The notion of toxic tau conformation is supported by experimental evidence. For instance, cell death correlates with structural changes in tau rather than tau affinity for microtubules in cells expressing pseudophosphorylated tau [125]. Perhaps the most significant argument in favour of pathological tau conformations comes from the comparison of the phenotypes of transgenic mice overexpressing wild-type human tau or the P301L mutant on a mouse tau knockout background [139]. Mice expressing wild-type tau develop axonopathy and motor deficits from a young age in the absence of any tau aggregates, which contrasts with the excessive neurofibrillary pathology found in P301L mice in later life. Tau is hyperphosphorylated in both models, and in addition tau from P301L mice is conformationally altered, aggregated and has a somatodendritic localisation. This is consistent with the altered physical and structural characteristics exhibited by recombinant FTDP-17 tau proteins [136]. Terwel *et al.* [139] suggest that tau hyperphosphorylation alone is not sufficient to induce aggregation and suggest that the abnormal conformation of tau found in cell bodies of P301L mice is the pathological species that results in neuronal dysfunction and neurodegeneration. Of interest is the observation that specific conformational changes may also render tau more susceptible to phosphorylation rather than phosphorylation inducing changes in tau conformation [140]. Thus, conformationally altered tau may also be a precursor for subsequent pathological tau phosphorylation. Altered conformation of FTDP-17 mutant tau could make it more susceptible to hyperphosphorylation, with subsequent phosphorylation and aggregation occurring at much lower phosphorylation levels than required by wild-type tau [141].

The significance of tau aggregation in toxicity

A characteristic feature of most neurodegenerative diseases is the accumulation of misfolded proteins in typical inclusion bodies [142]. In AD, NFT distribution spreads in a defined pattern in association with disease severity [143]. A long-established view is that NFT load correlates with the loss of hippocampal neurons and synaptic degeneration in AD brain, with NFT density more strongly associated with cognitive decline than amyloid plaques. However, recent results obtained from transgenic mouse models provide strong evidence to suggest that the formation of NFTs can be dissociated from tau-induced neuronal loss [144, 145] and memory decline [145], suggesting that neurodegeneration is more likely associated with an early, soluble, form of tau than with tau aggregated in PHFs. In one model, transgenic mice inducibly overexpressing a pathogenic FTDP-17 tau mutant progressively accumulate hyperphosphorylated tau with cortical pretangles followed by NFTs and extensive brain atrophy [145]. Deficits in cognitive functions appear at an early stage and precede the development of NFTs. Suppression of mutant tau expression improved cognitive ability and rescue of neuronal loss, while NFT development continued. Further evidence for a distinction between the presence of NFTs and neurodegeneration has been shown in transgenic mice overexpressing wild-type human tau [146]. These mice progressively develop accumulations of hyperphosphorylated tau, NFTs, and severe neuronal loss with aging, but the presence of intraneuronal tau filaments did not correlate directly with neuronal death [144]. Treatment of JNPL3 mice with GSK-3 inhibitors reduces tau phosphorylation as well as axonal degeneration but, significantly, the reduced neurodegeneration observed occurred in the absence of any apparent effect on NFT number [132]. Trans-

genic mice expressing the P301S tau mutant display synaptic loss in the hippocampus before fibrillar aggregates of tau are detected [147]. These studies highlight an important division between the presence of NFTs and the onset of neurodegeneration and subsequent memory impairment. Interestingly, impairment of microtubule polymerisation and stabilisation is induced by non-fibrilised, hyperphosphorylated cytosolic tau from AD brain, while filamentous aggregated tau loses the ability to inhibit the assembly of microtubules [148]. Whereas filamentous aggregates of tau, such as PHFs, may not be pathogenic, other evidence suggests that small oligomeric forms of tau in a specific conformation may play a role. Furthermore, hyperphosphorylation alters the structure of tau, with aggregated tau showing significant secondary β-sheet or α-helix structure [149, 150].

On the other hand, aggregated tau repeat domains have been shown to be toxic to N2a neuroblastoma cells, whereas soluble mutants of the repeat domains are not. In this model, the degree of aggregation and the extent of neurotoxicity found were directly related to the propensity of tau to adopt a secondary β-sheet structure [151]. Furthermore, although aggregates were unaffected by treatment with proteasome inhibitors, the use of *N*-phenylamine small molecule inhibitors with anti-aggregation properties were able to disassemble pre-existing aggregates. Of most significance was the finding that the reduction of tau aggregates brought about by either suppression of tau expression or chemical intervention, is beneficial. Taken together these results may suggest the possible existence of a separate pool of aggregated tau that is not in an abnormal conformation. Such a possibility is supported by the observation that aggregated tau appears prior to detectable MC1 immunoreactivity in JNPL3 mice [36].

Another view on the significance of tau aggregation is that aggregates are formed as the result of overburdening of the ubiquitin-proteasome pathway attempting to degrade pathological tau [152]. Polyubiquitination of tau at Lys-6 has been demonstrated in PHF tau extracts [153]. The E3 ubiquitin ligase, CHIP (carboxyl terminus of the Hsc70-interacting protein), has recently been shown to act as the primary ubiquitin ligase for tau, with tau lesions in AD showing CHIP immunoreactivity [152, 154, 155]. Non-neuronal cells overexpressing CHIP, GSK-3β and tau develop insoluble ubiquitinated tau aggregates, whereas cells lacking exogenous CHIP produce soluble phosphorylated tau in the absence of ubiquitinated tau aggregates [152]. Significantly, the presence of soluble phosphorylated tau was correlated with increased cell death, suggesting that soluble phosphorylated tau is toxic, whereas insoluble, aggregated tau is not and that ubiquitination of phosphorylated tau is in fact a protective mechanism, preventing the accumulation of toxic tau species. This is in agreement with earlier work reporting elevated levels of tau aggregates in CHIP-knockout mice [156].

Concluding remarks

Although tauopathies are different in their clinical manifestation and pathology, they share similarities in regard to tau metabolism, especially phosphorylation and RNA processing. Our current understanding of the mechanisms of tauopathies at the molecular level now offers the prospect of realistic therapies. Of particular significance is the role played by impaired alternative splicing of tau pre-mRNA in tauopathies as an increasing number of neurological disorders are being linked to impairment of RNA processing [157–160].

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