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Tau-Based Treatment Strategies in Neurodegenerative Diseases

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Summary: Neurofibrillary tangles are a characteristic hallmark of Alzheimer's and other neurodegenerative diseases, such as Pick's disease (PiD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). These diseases are summarized as tauopathies, because neurofibrillary tangles are composed of intracellular aggregates of the microtubule-associated protein tau. The molecular mechanisms of tau-mediated neurotoxicity are not well understood; however, pathologic hyperphosphorylation and aggregation of tau play a central role in neurodegeneration and neuronal dysfunction. The present review, therefore, focuses on therapeutic approaches that aim to inhibit tau phosphorylation and aggregation or to dissolve preexisting tau aggregates. Further experimental therapy strategies include the enhancement of tau clearance by activation of proteolytic, proteasomal, or autophagosomal degradation pathways or anti-tau directed immunotherapy. Hyperphosphorylated tau does not bind microtubules, leading to microtubule instability and transport impairment. Pharmacological stabilization of microtubule networks might counteract this effect. In several tauopathies there is a shift toward four-repeat tau isoforms, and interference with the splicing machinery to decrease four-repeat splicing might be another therapeutic option. **Key Words:** Tau, Alzheimer's disease, phosphorylation, aggregation, neurodegeneration, therapy.

INTRODUCTION

Cell biology and function of tau

The microtubule-associated protein (MAP) tau is expressed mainly in neurons, where it regulates microtubule assembly and stability.[1,2] The synthesis of tau is upregulated along with tubulin during neuronal differentiation; in mature neurons, tau occurs mostly in the axon and is largely excluded from the somatodendritic compartment, whereas other MAPs (e.g., MAP2) predominate in dendrites.[3,4] Microtubules must be able to dynamically grow and shrink, to support cellular shape changes. This is a prerequisite to establishing neuronal polarity and axonal outgrowth.[5,6] In addition, microtubules serve as tracks for the transport of cellular cargoes (vesicles, organelles) along axons and dendrites.[7] Both the dynamics and transport properties of microtubules are regulated by MAPs, such as tau. Their interactions are in turn regulated by post-translational modifications, such as phosphorylation.[8,9]

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Due to its content of hydrophilic residues, tau is a highly soluble protein.[10] It represents a prototype of the class of natively unfolded (or intrinsically unstructured) proteins.[11] Six isoforms of tau are expressed in the adult human CNS by alternative splicing of exons 2, 3, and 10 of the single *MAPT* gene (alias *TAU*) on chromosome 17q21.3; *MAPT* comprises 16 exons (FIG. 1B).[12,13] Tau isoforms differ by the absence or presence of one or two N-terminal inserts (N1, N2), as well as exclusion or inclusion of the second of four pseudo-repeats (~31 residues each) in the microtubule-binding domain (3R, 4R) (FIG. 1A).[10,13] Tau-microtubule binding is mediated by the repeat domain and requires the presence of both N- and C-terminally flanking proline-rich domains which target tau to the microtubule surface.[14] The number of repeats can modulate tau–microtubule affinity.[15–17] Consistent with the weaker microtubule binding of three-repeat (3R) tau isoforms, only 3R tau is expressed during neurogenesis, when higher microtubule dynamics are required.[18] Phosphorylation of the repeat region and, to a lesser extent, also of the flanking regions impairs the tau–microtubule interaction and leads to tau detachment from microtubules.[19,20]

Whereas the microtubule interaction domain of tau resides in the C-terminal half (the assembly fragment),

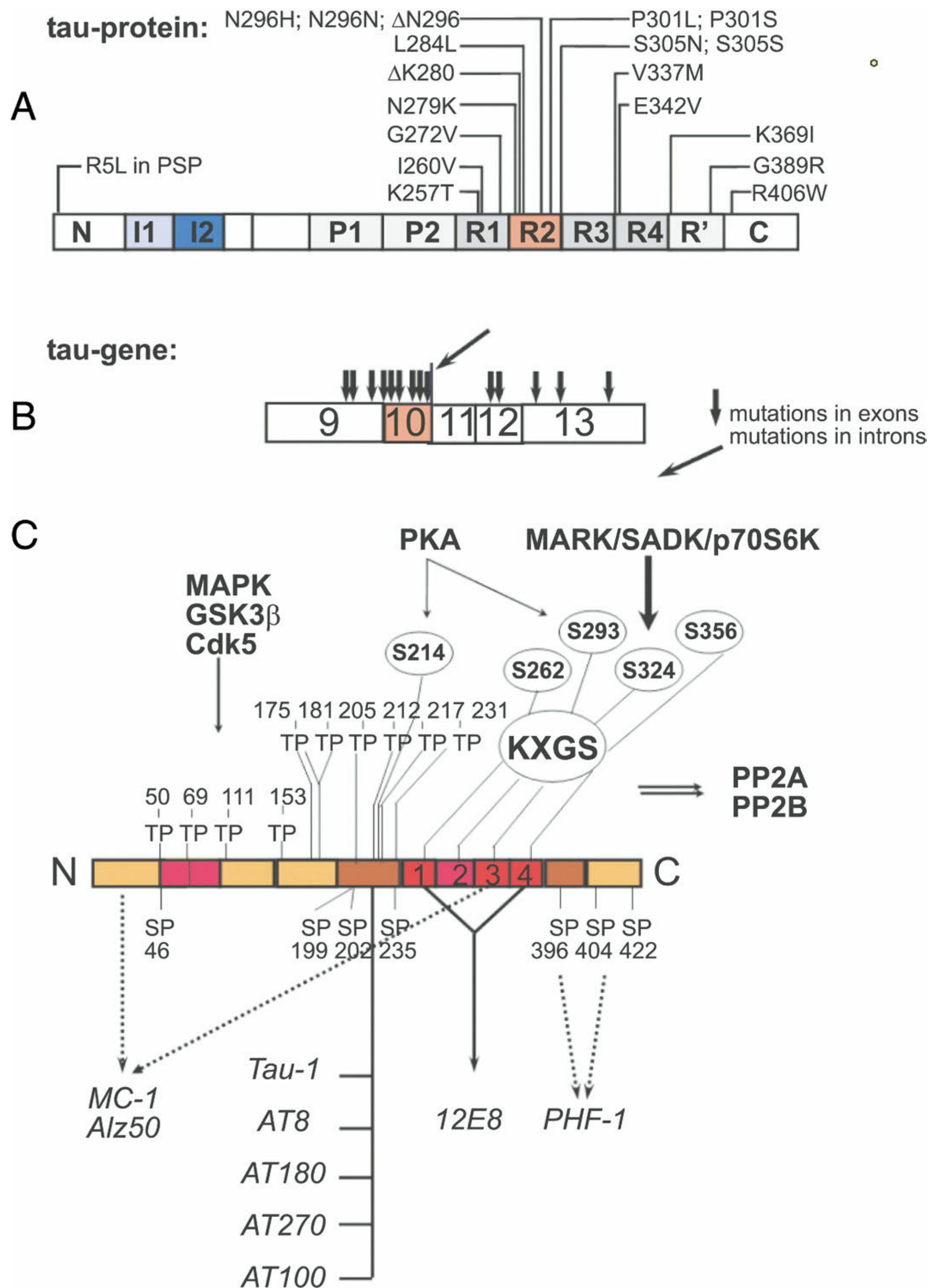

FIG. 1. Diagram of tau domains, mutations, phosphorylation sites, and antibody epitopes. (A) Human tau contains between 352 and 441 residues, depending on the splicing isoform. The isoforms are generated by the presence or absence of inserts I1, I2, and R2. The N-terminal half constitutes the projection domain, which protrudes away from the microtubule surface. The C-terminal half constitutes the assembly domain, which binds and stabilizes microtubules. The core of this domain is formed by the three or four repeats of ~31 residues each (R1–R4; R2 may be absent in the three-repeat isoforms of tau). The repeat domain is also responsible for the aggregation of tau into PHFs. The diagram shows many of the mutations observed in cases of FTDP-17. Most of them map to the repeat domain and may influence microtubule binding or PHF aggregation. ΔK280 and P301L are two FTDP-17 mutations which are prone to aggregation. (B) Diagram of the tau gene in the region covering the repeat domain (exons 9–13). The MAPT gene consists of 16 exons. Exons 1 and 14 are not translated. The repeat domain is encoded by exons 9–12. Exclusion or inclusion of exon 10 gives rise to three-repeat or four-repeat tau isoforms. Most intronic tau mutations localize to the 5′ region of intron 10, they increase the incorporation of exon 10 (increased expression of 4R-isoforms), but they are silent on the protein level. Exon 10 mutations can also shift splicing toward four-repeat tau (for details, see http://www.alzforum.org).[227] (C) Diagram of the longest tau isoform, tau441, with phosphorylation sites, kinases, and antibody epitopes. Tau isoforms differ by the presence or absence of 2 N-terminal inserts N1, N2) and a second repetitive amino acid sequence, R2. Phosphorylation of S214 by PKA and S262/S356 by MARK, PKA, or other kinases leads to tau detachment from the microtubule. Phosphorylation of SP/TP sites in the flanking regions have a modulating effect on tau-microtubule affinity. Epitopes of different diagnostic phospho-epitopes are indicated.

the N-terminal half projects away from the microtubule surface.[21] Its functions are not well defined. It could act as a spacer, or interact with other proteins—for example, signaling molecules such as kinases, phosphatases, heat shock proteins (HSPs) and other cytoskeletal elements.[22–25] Because MAPs and microtubule motors both bind to the surface of microtubules, they can potentially interfere with each other. Thus, the overexpression of tau can retard motor-dependent transport, particularly in the anterograde direction.[26–29] This can lead to oxidative damage and synaptic degeneration.[30,31]

Tauopathies

Filamentous or amorphous aggregates of hyperphosphorylated tau have been described in the context of various neurodegenerative diseases. These tauopathies cover a wide spectrum of neurodegenerative diseases with prominent tau pathology, including dementia syndromes (Alzheimer's disease [AD] and Pick's disease), which can be accompanied by parkinsonism (progressive supranuclear palsy [PSP], corticobasal degeneration [CBD], frontotemporal dementia and parkinsonism linked to chromosome 17 [FTDP-17]), and motor neuron diseases (amyotrophic lateral sclerosis–Parkinson–dementia complex, or ALS-PDC).

The neurofibrillary tangles (NFTs) of Alzheimer's disease consist of intracellular filamentous aggregates of hyperphosphorylated tau protein (FIG. 2).[32,33] Tau can assemble into filamentous strands, which are termed either straight filaments or paired helical filaments (PHFs), depending on whether ultrastructure exhibits periodic crossovers of the two strands (FIG. 3). Twisted or nontwisted filaments of tau are found in AD, PSP, FTD, and CBD. Additionally, amorphous tau aggregates have been described in astrocytes of CBD brains. Hirano bodies,

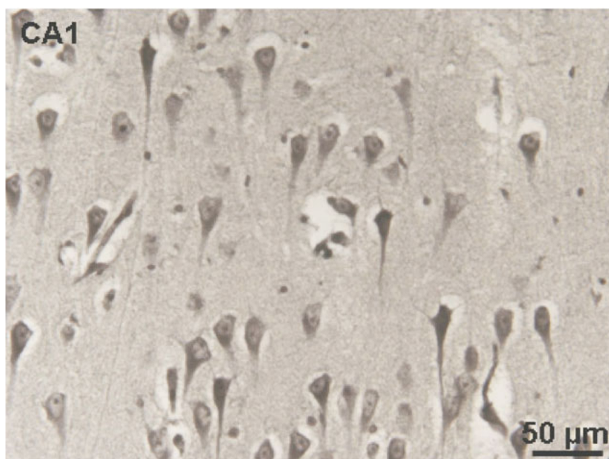

FIG. 2. Section from Alzheimer's disease (AD) brain, showing pyramidal cells of the hippocampal CA1 region with typical flame-shaped neurofibrillary tangles consisting of tau protein. Gallyas silver staining. (Courtesy of A. Nissen.)

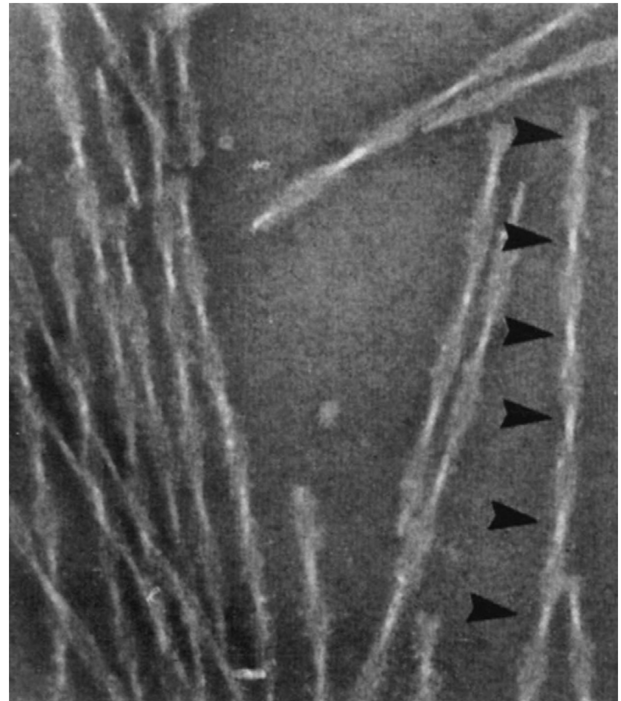

FIG. 3. Electron micrograph of negatively stained paired helical filaments isolated from AD brain. Note the two twisted strands with the typical crossover periodicity of ~80 nm (arrowheads). (Courtesy of E.M. Mandelkow.)

which are actin-rich, paracrystalline structures that contain nonfibrillary aggregates of tau, have been identified in AD brain and in other tauopathies.[34]

Tau aggregates are not restricted to neurons but are also seen in glial cells, such as the coiled bodies and argyrophilic threads in oligodendrocytes of CBD and PSP, as well as the tufted astrocytes found in PSP. Tau pathology is predominant in oligodendrocytes in multisystem atrophy, and the various mutations of FTDP-17 differ by the proportion of neuronal or glial tau pathology.[35,36] In AD patients, the number of NFTs correlates strongly with the degree of cognitive decline and neurodegeneration, and tau is commonly used as a diagnostic marker of AD.[37] The identification of patients in early disease stages is important, in light of recent findings of a positive correlation between early treatment with disease-modifying drugs and a prolonged maintenance of cognitive function.[38]

Total tau levels in CSF are elevated under circumstances of extensive neuronal degeneration, such as AD, Creutzfeldt–Jakob syndrome, frontotemporal dementia (FTD), and stroke, but not in Parkinson's disease and progressive supranuclear palsy (PSP).[39–43] The CSF levels of total and phosphorylated tau are therefore useful biomarkers for the diagnosis of AD, in which phospho-tau concentrations in CSF are increased with an average sensitivity of 78% and specificity of 92%.[44,45] Especially high CSF concentrations of tau

phosphorylated at T181, T231, S199, and S396/404 (FIG. 1B) have been suggested to be reliable predictors for the conversion of mild cognitive impairment into AD.[46–48] Noninvasive diagnostic methods for tauopathies include positron emission tomography imaging with plaque-binding and especially tangle-binding tracers such as FDDNP, similar to the amyloid-binding radiotracer Pittsburgh compound B.[49,50]

PATHOPHYSIOLOGY

In AD, tau pathology is accompanied by extracellular amyloid plaques and intracellular NFTs that are distributed mostly in the entorhinal region, hippocampus, and cortex. This is in contrast to other tauopathies, in which amyloid plaques are absent and NFTs are preferentially localized in different brain regions, such as the brain stem and basal ganglia (in PSP) or in the frontotemporal cortex (in FTDP-17). The identification of tau mutations in FTDP-17 families and in sporadic FTD has provided compelling evidence for a causative role for tau in neurodegeneration, rather than tau pathology being a mere side effect observed in neurodegenerative diseases.[51–53]

Tau mutations in FTDP-17 are either intronic, localized close to the splice-donor site following exon 10 and resulting in overproduction of four-repeat tau isoforms, or comprise missense, deletion, or silent mutations in the coding regions (FIG. 1B).[52,53] The mutations lie mostly in or near the repeat domain, which constitutes the core of the microtubule binding domain and also the PHF assembly domain; the mutations result in reduced microtubule binding ability or in enhanced tau aggregation into PHFs (R5L, K257T, G272V, ΔK280, P301L, P301S, V337M, and R406W).[54–60] Mutations in exon 10 can affect both protein properties and exon 10 splicing (e.g., ΔK280, ΔN296 and N296H), the latter shifting the isoform ratio of three- to four-repeat tau from approximately 1:1 in healthy individuals toward the four-repeat isoforms.[61,62] Similar findings have been obtained in PSP and CBD, two tauopathies that share the *MAPT* H1 haplotype as a risk factor.[63,64] Filamentous tau aggregates in PSP and CBD consist of four-repeat isoforms.[65]

Why tau, a highly soluble protein, aggregates into PHFs is still a matter of debate. In AD brains, tau is redistributed from the axonal to the somatodendritic compartment and is hyperphosphorylated at 25 different sites at least, which are known to interfere with the affinity of tau–microtubule binding (FIG. 1C).[66] Pathologic hyperphosphorylation might thus lead to tau-microtubule detachment, subsequent instability of microtubule networks, impaired axonal transport and synaptic degeneration (FIG. 4). In addition to this loss-of-function effect, caused by microtubule instability, a toxic gain-of-function was assigned to aggregated PHF-tau (FIG. 4).[67–69] Moreover, hyperphosphorylated, nonaggregated

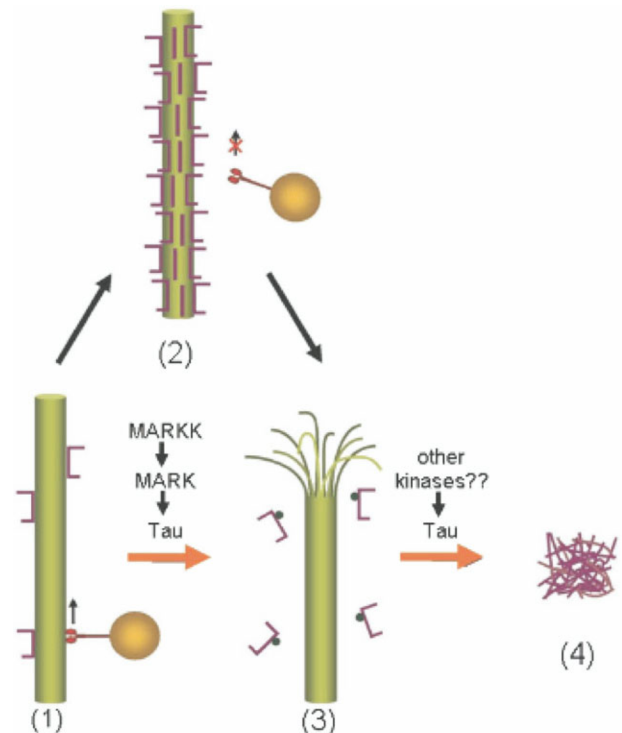

FIG. 4. Possible mechanisms of tau toxicity. (1) Microtubules stabilized by tau protein support axonal transport by motor proteins. (2) Excess bound tau can reduce the attachment of motor proteins and thus impair axonal traffic. (3) In AD, tau becomes hyperphosphorylated by several protein kinases (e.g., MARK) and detaches from microtubules. The microtubules break down (resulting in perturbed axonal transport) and the detached tau self-assembles into paired helical filaments. (4) The PHFs bundle together and form neurofibrillary tangles that obstruct the cell interior.[27]

tau might be toxic by itself, as suggested by recent findings in *Drosophila* fruit fly and mouse models.[70–73]

Prevention of mis-splicing, hyperphosphorylation, and aggregation of tau are therefore promising therapeutic targets to interfere with tau-based toxicity and neurodegeneration. New approaches currently under intense investigation include various kinase and aggregation inhibitors, microtubule-stabilizing agents, enhancers of tau clearance (e.g., HSP90 inhibitors, immunotherapy), and recently described techniques to shift the ratio of mis-spliced tau toward three-repeat isoforms.

TAU-BASED THERAPEUTIC APPROACHES

Antiphosphorylation strategies

The largest tau isoform contains 45 serine (S), 35 threonine (T), and 5 tyrosine (Y) residues (FIG. 1C). Among these, 17 sites are part of serine/proline (SP) or threonine/proline (TP) motifs and represent targets of proline-directed SP/TP protein kinases such as glycogen synthase kinase 3β (GSK-3β), cyclin-dependent kinases (cdk5, cdc2), mitogen-activated protein kinase (MAPK), stress-activated kinases (JNK, p38), and others.[74,75] Ad-

ditional sites are targeted by different kinases, including cAMP-dependent protein kinase (PKA), protein kinase C (PKC), calcium/calmodulin dependent protein kinase II (CaMKII), serum- and glucocorticoid-inducible kinase (SGK), protein kinase B (PKB), microtubule-affinity regulating kinase (MARK), and SAD kinase (SADK).[76] The KIGS or KCGS motifs in the repeat domain (S262, S293, S324, S356) can be phosphorylated by MARK, PKA, PKB, SADK, CaMKII, and p70S6K.[77–80] PKA additionally phosphorylates other sites including S214, a phospho-epitope that is especially upregulated during mitosis.[81] In AD brain, tau is hyperphosphorylated at nearly all phosphorylation sites, with approximately nine phosphates per molecule, in contrast to the two to three phosphorylated residues observed in healthy control brains.[82]

Various antibodies directed against these phospho-epitopes preferentially recognize AD brain-derived tau and are used as diagnostic tools. Phosphorylation of S214 or the KXGS sites within the repeat domain strongly inhibits tau–microtubule binding, thereby raising soluble tau concentrations and increasing microtubule instability. Tau-microtubule detachment seems to be an early event in the course of the disease, because pretangle tau is phosphorylated at S262/S356 (12E8 epitope) in AD brains and in mouse models of tauopathy.[83,84] Similarly, S214 phosphorylation was found to precede PHF formation in a triple transgenic mouse model of mutant APP, presenilin, and the FTDP-17 tau mutation P301L.[85] However, phosphorylation of the KXGS motifs, especially S262, has been shown to inhibit the aggregation of tau into PHFs *in vitro*.[86] Rather than promoting tau aggregation, the detachment from microtubules might render free cytosolic tau more accessible to phosphorylation by different SP/TP directed kinases than microtubule-bound tau would be.

There is a widespread assumption that hyperphosphorylation at SP or TP motifs leads to aggregation (although this is a matter of debate). Independently of aggregation, phosphorylation may be toxic to neurons (e.g., by microtubule destabilization, repeat domain phosphorylation) or by other mechanisms (e.g., activation of the mTOR pathway, SP/TP phosphorylation).[87] Most approaches to interfering with tau phosphorylation rely on inhibiting different kinases or activation of phosphatases.[88]

MARK. The KXGS motifs in the repeat region of tau are phosphorylated by MARK/Par-1. The decreased microtubule affinity of KXGS phosphorylated tau and subsequent microtubule instability[77] might be the mechanism by which MARK exerts its function in cell differentiation, neurite outgrowth, and maintenance of axonal transport.[89–91] The PHF tau is hyperphosphorylated at S262/S356, and fluorescence resonance energy transfer experiments revealed active MARK in close proximity to phosphorylated S262/S356 residues in AD brain PHFs.[92]

MARK/Par-1 mediated S262/S356 phosphorylation was shown to enhance the toxic phenotype caused by human tau expression in the *Drosophila* fruit fly retina, which was absent in a S262A/S356A expressing mutant.[72] In addition, S262A/S356A mutants were less phosphorylated at SP/TP sites that become hyperphosphorylated during disease progression.[83] This finding hints toward a priming effect of S262/S356 phosphorylation for subsequent pathological tau hyperphosphorylation.

MARK/Par-1 belongs to the calcium/calmodulin-dependent protein kinases. Its activity depends on phosphorylation by activating kinases (MARK kinase/thousand and one amino acid kinase [MARKK/TAO-1] or LKB1).[91] Inhibitory regulation of MARK can additionally be achieved by binding to PAK5 or to scaffolding proteins or by subcellular localization.[91,93] The activating MARKK is a member of the Ste20 family of kinases and phosphorylates MARK at a threonine residue in the activation loop. Other functions of MARKK/TAO-1 involve mitosis progression and MAPK/p38 signaling upon stress induced activation.[94,95] By these mechanisms, apart from the toxic effects of S262/S356 phosphorylated tau, MARKK might contribute to neurodegeneration and cell death.

Binding of testis-specific protein kinase 1 (TESK1) inhibits MARKK activity by protein–protein interaction.[96,97] TESK1 is similar to LIM kinase, in that it phosphorylates cofilin and plays a role in the organization of the actin cytoskeleton.[97–99] Note that active LIM kinase (LIMK) is also a downstream effector of Aβ-mediated toxicity.[100] Pharmacological inhibitors of MARK/MARKK include staurosporine (unselectively) and hymenialdisine, which also inhibits GSK3β.[90,101] Given the important and early role of MARK phosphorylation in the pathogenesis of tauopathies, the application of MARK inhibitors would be a worthwhile approach.

PKA. cAMP-dependent protein kinase A (PKA) has been shown to phosphorylate tau at S262/S356 and S214 *in vitro* and *in vivo*.[102,103] Phosphorylation by PKA results in reduced microtubule-binding affinity and promotes the subsequent phosphorylation of different diagnostic phospho-epitopes. PKA phosphorylation at S214 is required to prime tau for its sequential phosphorylation by GSK-3β, thereby giving rise to the AD-specific AT100 phospho-epitope.[102] Activation of PKA by forskolin resulted in spatial memory deficits in rats.[103] Isoquinoline sulfonamide derivatives and staurosporines are known to inhibit PKA with low selectivity and also affect PKG, PKC, and the myosin light chain kinase (MLCK). PKA inhibitors with higher selectivity are KT-5720 and PKI. PKI binds to the catalytic subunit of PKA and promotes its nuclear export, thereby preventing

cAMP response element regulated gene expression.[104] However, none of these inhibitors have been tested in AD model systems to date.

Cdk5. Cyclin dependent kinase 5 (Cdk5) accumulates in tangle-bearing neurons of AD brains, and kinase activity was found to be upregulated in AD.[105] Cdk5 is regulated by the neuron-specific activator p35. The myristoylated and membrane-bound domain of p35 can be cleaved by calpain to p25. This leads to a cytosolic redistribution of p25-bound cdk5 and aberrant tau phosphorylation.[106] Overexpression of p25 in a rodent model resulted in increased tau phosphorylation and neurodegeneration and SP/TP phosphorylation by cdk5 increased tau toxicity in a *Drosophila* fruit fly model.[71,107,108] Recent evidence suggests that cdk5/p25 might be a downstream effector of Aβ mediated toxicity, leaving cdk5 as a promising therapeutic target.[109] One must be aware, however, that the inhibition of cdk5 was accompanied by activation of GSK-3β in neuronal cell culture and that p35 knock-out mice revealed increased GSK-3β activity and tau phosphorylation.[110,111]

Cdk5 inhibition can be achieved by active site-directed inhibitors, by 2,6,9-trisubstituted purines and aloisines, which are small-molecule inhibitors that interfere with the cdk5/p25 complex formation (Cdk5 inhibitory peptide [CIP]), and by calpain inhibitors, which prevent p25 generation.[101,112,113] The use of active site inhibitors is hampered by their lack of selectivity toward cdk5. Indirubins, which belong to the chemical class indolinones, also inhibit GSK-3. Likewise, purines are not highly selective for cdk5 and can inhibit ERK2. The purine roscovitine has been tested in p25 transgenic mice, and reduced tau phosphorylation and neurodegeneration was demonstrated upon roscovitine treatment. Roscovitine shows a higher selectivity toward cdk5 than olomoucine, another purine inhibitor of cyclin-dependent kinases, which has been tested successfully in Niemann Pick type C (NPC) mice, and aminopurvalanol, which efficiently inhibited cdk *in vitro*.[114] Less cdk5 specificity is achieved by flavopiridol, indirubin, 1-aza-9-oxafluorenes, and aminothiazoles, all of which inhibit cyclin-dependent kinases in general in addition to GSK3.[115] The cdk5 inhibitory peptide (CIP) interferes with cdk5/p25 binding and is more selective toward cdk5 than to other cyclin kinases. Calpain inhibitors have shown beneficial results in AD models, however their clinical application is restricted by their poor ability to cross the blood–brain barrier (BBB).[116,117] In contrast, roscovitine has been described to penetrate the BBB at a sufficient rate.[118]

GSK3. The GSK-3 isoforms α and β are encoded by two different genes.[119] Both isoforms are involved in glucose metabolism, cell proliferation, wnt signaling, and apoptosis.[120,121] GSK-3β activity is regulated by phosphorylation; S9 phosphorylation inhibits kinase activity, and Y216 phosphorylation activates GSK-3β.[121] Tau has been identified as an important substrate of GSK-3 *in vitro* and *in vivo* and can be phosphorylated at primed (e.g., T231) and unprimed epitopes (PHF-1, Tau-1, and AT8).[122–124] Priming involves the prior phosphorylation of a serine or threonine residue, four amino acids C-terminal to the target phosphorylation site.[125]

Active GSK-3 was detected in pretangle neurons and NFTs in AD brains, and coexpression of the GSK-3 homolog *shaggy* with tau led to tau hyperphosphorylation, filamentous tau aggregation, and neurotoxicity in *Drosophila* fruit fly.[126,127] In line with these findings, GSK-3β overexpressing transgenic mice showed increased tau phosphorylation and deficits in spatial memory.[128,129] Tau phosphorylation by GSK-3 can be enhanced by Aβ -treatment, which thus provides a possible link between tau and Aβ pathology.[130] GSK-3β activity is negatively regulated by phosphorylation at S9 through different kinases (e.g., AKT–protein kinase B).

Various small-molecule inhibitors such as flavopiridol, paullones, and aloisines are directed against the ATP-binding site of GSK-3 and therefore display a low selectivity toward GSK-3α or -β, and most of them also target cdk5. The recently described staurosporine-derived bisazaindolylmaleimide derivatives as well as pyrazolopyrazines and pyrazolopyridines are characterized by a higher selectivity toward GSK-3β. Oral administration of the aminothiazole AR-A014418 resulted in decreased tau aggregation in a transgenic mouse model of P301L tau.[131,132]

Lithium, a well-characterized mood-stabilizer, competes with magnesium for GSK-3 binding; it leads to reduced tau-phosphorylation, aggregation and axonal degeneration in transgenic mice.[133,134] Valproate, another mood-stabilizing drug (and an antiepileptic), also inhibits GSK-3β and is currently being tested in clinical AD trials.

Phosphatases. Tau is dephosphorylated by protein phosphatase-2A (PP-2A) and to a minor extent by PP-1 and PP-2B (calcineurin).[23,135] In AD brain, the mRNA levels of PP-2A and PP-1 are reduced compared with controls.[136,137] A decrease in phosphatase activity might result in impaired tau dephosphorylation, as well as enhanced tau phosphorylation, because various tau-directed kinases are activated by phosphorylation themselves. Indeed, inhibition of PP-2A by okadaic acid resulted in AD-like tau hyperphosphorylation in rat brain slice cultures.[138] To date, no pharmacologic approaches to activate PP-2A have been reported. However, memantine, an *N*-methyl-D-aspartate (NMDA) receptor antagonist that is well established for the treatment of AD, has been described as antagonizing the okadaic acid–mediated inhibition of PP-2A in hippocampal slice cultures.[139] These findings further support the choice of phosphatases as potential pharmacological targets in AD.

Antiaggregation strategies

Tau, a natively unfolded protein, is highly soluble and does not readily aggregate into filaments. *In vitro*, polyanionic cofactors such as glycosaminoglycans, acidic peptides, RNA, or fatty acid micelles can promote PHF assembly.[140–143] Aggregation is further promoted by high tau concentrations. *In vitro* studies have shown that tau phosphorylation is not necessary to drive tau into PHFs.[140,141] On the contrary, phosphorylation of the KXGS motifs in the repeat region inhibits tau aggregation *in vitro*.[86] Tau comprises two hydrophobic hexapeptide motifs[144,145] in the repeat domains (275VQIINK280 at the beginning of R2 and 306VQIVYK311 at the beginning of R3) that form a cross β-structure and make up the PHF core. The N- and C-terminal flanking regions are thought to form the PHF fuzzy coat.[146] Proteolytic cleavage of the inhibitory N- and C-termini exposes the aggregation-prone hexapeptide motifs, thereby greatly facilitating the nucleation of filaments.[144,147]

There has been debate on what form of tau is the toxic species for neurons. Elevation of tau, phosphorylation of tau at different sites, and truncation by different proteases have been investigated.[148–150] Recent findings point to toxic functions of tau aggregates. For example, in stable tau cell lines, toxicity paralleled tau aggregation and removal of aggregates rescued the toxic phenotype.[151,152] Similarly, in inducible transgenic mice, only the proaggregation tau was toxic; antiaggregation tau was not.[68,69] Thus, preventing the buildup of aggregates by small-molecule inhibitors could be a promising strategy in the treatment of tauopathies. The non-neuroleptic phenothiazine methylene blue, which penetrates the BBB, and its desmethyl derivatives have been described to inhibit tau aggregation, with K_i values in the nanomolar range.[153] Recent high-throughput screens yielded various potential drug candidates. Members of different substance classes, such as anthraquinones, N-phenylamines, phenylthiazolhydrazides, and rhodanines, can inhibit tau aggregation and, even more importantly, disassemble existing filaments.[67,154–156] The respective lead substances were identified in a high-throughput *in vitro* screen of approximately 200,000 compounds followed by chemical modification and retesting in a tau-expressing stable neuronal cell line with marked tau aggregation (FIG. 5).[157]

PTH. The phenylthiazolyl-hydrazides (PTH) (FIG. 5) were found by in silico screening of 77 primary screen hits. From this, 49 PTH derivatives were synthesized and a PTH lead structure as well as a structure–activity relationship could be verified. The structure–activity relationship distinguishes PTHs from nonselective inhibitors without a structure–activity relationship.[155] The tau–PTH interaction was studied by saturation transfer difference NMR and appeared to be hydrophobic.[158] The exact structural interaction of tau and PTH still needs to be determined. One could envision interference with nucleation or elongation steps of tau assembly, either mediated by steric inhibition of tau–tau interaction, stabilization of a non–aggregation prone tau conformation or interaction with polyanionic cofactors of aggregation.

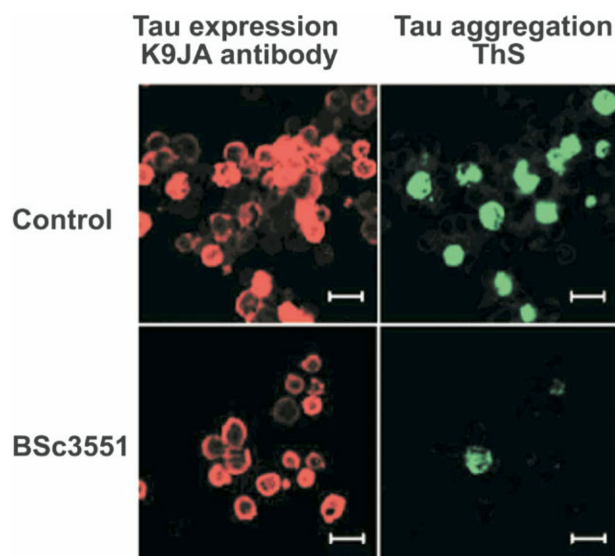

FIG. 5. Tau mutant expression, aggregation, and inhibition in a cellular model. The left panels show the expression of the tau repeat domain with the ΔK280 mutation after addition of doxycycline in an inducible N2a cell line, stained with tau antibody (red). Tau aggregates are formed and can be detected by the fluorescence of thioflavine S (right, green). Top row, control without aggregation inhibitor compound, bottom row, with inhibitor BSc3551 (a phenylthiazolyl hydrazide derivative). Note the strong reduction of aggregation by the compound. Scale bar: 20 μm. (Modified from Pickhardt et al.[155])

Rhodanines and anthraquinones. The recently published substance class of rhodanines is preferable to anthraquinones, due to reduced cytotoxicity and absence of mutagenicity. Notably, a rhodanine derivative, the aldose reductase inhibitor epalrestat, showed no toxic side effects in a long-term clinical trial.[159,160] Different rhodanine derivatives inhibit tau aggregation and promote filament disassembly in a cell culture model, with efficacies in the nanomolar range and in the absence of obvious cytotoxicity or interference with microtubule stability.[156]

N-phenylamines. N-phenylamines were proved to have a comparable activity as rhodanines, but show a lower pharmacological potency *in vitro*.[67] It will now be interesting to test these substances in mouse models for bioavailability and pharmacokinetics.

Thiacarbocyanines. Another class of small-molecule inhibitors of tau aggregation are thiacarbocyanine dyes, such as N744, which were identified by *in vitro* screens.[161,162] A multivalent cyane derivative, the cyclic bis-thiacarbocyanine, inhibits tau aggregation *in vitro* with approximately a fourfold higher potency than the monovalent cyane itself.[163] The mechanism by which multivalent ligands interfere with protein aggregation

has been speculated to involve their binding to oligomers, thereby preventing incorporation of new molecules into filaments.[164] Alternatively, these small-molecule inhibitors might decrease the concentration of aggregation-prone proteins by sequestration into protein–inhibitor complexes.[165] Developing multivalent cyanes, which are characterized by an improved potency due to an increased local concentration of ligands, might therefore be a promising strategy.

Tau clearance

Another possible approach to prevent tau toxicity could be the augmentation of its cellular clearance, as well as the degradation of tau aggregates.

Proteasome and autophagosome activation. Ubiquitin–proteasome and autophagy–lysosomal pathways are the two major means of protein degradation within the cell.[166] Oligomers or higher-order aggregates cannot enter the proteasome for steric reasons, but will be degraded by autophagy. During autophagy, a double membrane is formed around the intracellular aggregate. This autophagosome then fuses with the lysosome (autolysosome), where acidic lysosomal hydrolases degrade the contents of the vesicle.[167] Autophagy has been described for intracellular protein aggregates such as polyQ-huntingtin and α-synuclein aggregates, and also for tau.[168,169] Rapamycin inhibits mTOR (mammalian target of rapamycin), thereby promoting autophagy. In a *Drosophila* fruit fly model expressing wild-type or R406W mutant tau, treatment with rapamycin induced autophagic tau degradation and diminished tau-induced toxicity.[169] It has been speculated that the nonclassic, proteasome-independent K63 ubiquitin pathway is involved in the autophagic route of tau. This notion is supported by the finding that P301L tau-positive inclusions in a neuronal cell line were cleared after stimulation of autophagy, a mechanism that was impaired by coexpression of the ubiquitin mutant K63R.[170]

Note, however, that the beneficial effect on tau clearance by stimulating autophagy might be counteracted by increased Aβ production in the autophagosomal system, as has been proposed by Rubinsztein et al.[171,172]

The chaperones HSP27 and HSP90 are involved in phosphorylation-dependent proteasomal degradation of tau.[24,173] HSP90-interacting proteins are degraded by the proteasome upon pharmacological inhibition of HSP90. Phosphorylation of the flanking regions enhanced the proteasomal degradation of tau in transfected cell lines that were treated with HSP90 inhibitors.[173] Phosphorylation of S262/S356 protected tau against proteasomal degradation. The HSP90 inhibitor geldanamycin is not feasible for clinical studies because of hepatotoxic side effects, but its less toxic derivatives 17-AAG and 17-DMAG have been tested as anticancer treatment in a clinical setting.[174] Their pharmacological properties, however, suggest a poor ability to cross the BBB. Recently, several HSP90 inhibitors of low molecular weight have been described, which might reach sufficient therapeutic CNS concentrations.[173]

PHF-tau has been shown to be ubiquitinated at least at four different sites, and through different lysine linkages on ubiquitin.[175,176] Tau is predominantly monoubiquitinated, and only a minor part of PHF-tau is linked to polyubiquitin chains by Lys42. Conjugation sites for monoubiquitination have been identified within the repeat region. They include Lys254, Lys257, Lys311, and Lys317, with Lys311 being a major linkage site.[176] The HSP70 co-chaperone CHIP (the acronym stands for the carboxyl terminus of the Hsc70-interacting protein) functions as the primary E3-ligase in ubiquitin-dependent tau clearance.[177–180] In line with the important function of CHIP in tau clearance, increased tau accumulation was reported in CHIP knock-out mice.[178] Activation of the co-chaperone CHIP could prove to be an attractive drug target, especially because it also stabilizes full length APP against secretase cleavage. In addition, CHIP directs APP to proteasomal degradation, by promoting APP ubiquitination. It also accelerates Aβ_{42} removal and can protect against Aβ toxicity.[181]

Proteases. It has been assumed that cytosolic proteases might also play a role in tau degradation. Recently, the puromycin-sensitive aminopeptidase PSA, a 100-kDa M1 metalloprotease, was identified by a genomic approach screening for modifiers of tauopathy.[182] Besides its function in protein turnover, PSA is involved in cell cycle regulation, and a wide range of PSA substrates are known, including enkephalins, cholecystokinin, and somatostatin, among many others.[183,184] Tau has been identified as a substrate of PSA *in vitro*. PSA mediated amino-terminal tau degradation of AD brain-derived soluble or insoluble tau was impaired, relative to control tau.[185] At first sight, activation of PSA appears to be an attractive therapeutic target, but regarding the large number of physiologic processes in which PSA is involved, side effects are likely to occur.

When activating tau proteolysis, it has to be considered that proteolytic tau fragments of the repeat domain might act as a seed for tau aggregation. A stepwise proteolysis of the tau repeat domain has been described in an inducible FTDP-17Δ280 mutant tau cell line.[151] There, a PHF nucleating fragment was generated by subsequent cleavage, first between K257/S258, followed by cleavage at I360 or K353. The responsible protease has not yet been identified, and it is not known whether it is upregulated in AD. This could, however, be a promising target for pharmacological inhibition.

Two cysteine proteases, caspase-3 and calpain-1, have been described as downstream targets of Aβ-induced toxicity in cell culture models. In cultured hippocampal neurons, Aβ-induced toxicity was mediated by calpain-1

activation and generation of a 17-kD tau fragment. Both tau fragmentation and neurotoxic effects of Aβ could be diminished by calpain-1 inhibition.[186] To date, however, no direct proof for the suggested neurotoxicity of the 17-kD tau fragment has been provided.

Tau can be cleaved *in vitro* by the serine-aspartyl protease caspase-3 at Asp421, which increased and accelerated the aggregation of both truncated and full-length tau. In addition, the caspase-3 cleaved tau fragment exerted toxic effects in tissue-culture experiments.[187–189] Caspase-3 is activated in AD, and PHFs from AD-brains were recognized by antibodies directed against the caspase-3 cleavage site of tau.[150,187,188] The caspase-3 specific inhibitor AcDEVD-CHO inhibited tau cleavage and reduced Aβ-mediated toxicity in organotypic rat brain slices.[190]

Tau vaccination

Intriguing new treatment strategies, currently being investigated in multicenter clinical trials, involve passive and active immunization against Aβ. Approximately 0.1% of total circulating IgG is found within the CNS.[191] IgG is assumed to enter the CNS via BBB-deficient regions and BBB transcytosis, and CNS entry might be enhanced by an increased BBB permeability, as has been described for AD.[192–195] Similar vaccination approaches have been tested for a tau-directed immunotherapy in mice, even though tau aggregates are intracellular (in contrast to amyloid plaques).[196,197] Neuronal pinocytic uptake of antibodies has been reported, partially mediated by the neuronal Fcγ or Thy1.1 receptors.[198–204] This increases the probability of targeting intracellular tau by an antibody-mediated approach. However, no explanation has been provided as to how tau antibodies, once in endosomal compartments, interact with the cytosolic tau protein, nor how tau immunocomplexes, once formed, are cleared by Fcγ-receptors or astrocytes and microglia-mediated phagocytosis.

Despite such concerns, one could imagine immunomediated clearance of extracellular PHFs, the so-called ghost tangles, and tau-based immunotherapy was further encouraged by the reduction of intracellular α-synuclein aggregates in synuclein vaccinated transgenic mice.[205] In the case of tau, P301L transgenic mice were immunized with a phospho-tau peptide (containing the phosphorylated PHF-1 epitope).[196] These mice responded with antibody synthesis, less of histochemically detectable tau aggregates, and slower progression of behavior deficits, compared with untreated mice. The feasibility of tau-immunotherapy must be considered critically, however, given that vaccination of C57BL/6 mice with recombinant human tau protein resulted in the onset of neurofibrillary tangle pathology, axon damage, demyelination, and gliosis.[197] This is an important finding, especially in the light of the first clinical Aβ immunization trial, which was terminated after the detection of profound neuroinflammation in some immunized patients.[206]

Isoform approaches

In approximately half of the FTDP-17 mutations, a twofold to sixfold excess of four-repeat tau over three-repeat isoforms was observed, whereas both isoforms are expressed in equal proportions in normal adult brain.[53,207] The hypothesis that four-repeat tau isoforms might play a pathogenic role is further strengthened by the predominance of filamentous tau aggregates, consisting of four-repeat isoforms, in FTDP-17 mutations and in PSP and CBD brain.[208–211] In addition, sporadic tauopathies are strongly correlated with the *MAPT* H1 haplotype, from which approximately 1.4-fold more four-repeat tau isoforms are generated than from the H2 haplotype.[63,64,212] No difference in the ratio of four- and three-repeat isoforms was found in total postmortem AD brain tau, but a roughly 1.5-fold excess of four-repeat tau has been reported in the temporal cortices of AD brains.[207,213,214]

Four-repeat tau binds microtubules with a higher affinity than three-repeat tau, and an isoform imbalance in favor of four-repeat isoforms might impair kinesin-dependent anterograde traffic, because tau and kinesins bind to the microtubule surface in a competitive fashion.[28,30,146] Shifting the tau isoform ratio from four-repeat to three-repeat isoforms by alternative splicing was therefore proposed as a therapeutic option in tauopathies. Promising results have already been achieved, with antisense oligonucleotides directed against the splice junction of exon 10 in PC12 cells leading to reduced exon 10 splicing.[215]

Spliceosome-mediated RNA trans-splicing (SMarT) is another method for mRNA reprogramming, and has been tested in tau transfected cells.[216,217] Trans-splicing occurs between the 5′ splice site of a pre-mRNA and the 3′ splice site of a second mRNA at the spliceosome. With SMarT, the endogenous target pre-mRNA becomes trans-spliced to an exogenously delivered pre-trans-splicing molecule. This contains a binding domain that interacts with the 3′ end of the target pre-mRNA intron. As a proof of principle, an exogenous pre-trans-splicing RNA comprising human tau exons10 to 13 and a binding sequence complementary to the 3′ end of intron 9 were transfected into cells expressing tau exons 9, 10, and 11. The resulting trans-spliced tau chimera contained exons 9 to 13 with a correct and exact exon 9 to exon 10 junction. Shifting the ratio from four- to three-repeat tau could be achieved in a similar way, with a pre-trans-splicing construct containing exons 11 to 13 together with the intron 9 binding site.

Besides interfering with defective alternative splicing, the SMarT method has been successfully applied to express the correct transcripts in the case of dominant

mutations such as cystic fibrosis and hemophilia A.[218,219] The application of this exciting method in animal models of tauopathy has not been studied to date, and might be hindered by the difficulty of RNA entry into brain cells.

Microtubule-stabilizing drugs

The rationale behind microtubule-binding drug approaches in tauopathies is to compensate for the putative loss-of-function of abnormally hyperphosphorylated or aggregated tau that no longer binds and stabilizes microtubules. PHF tau cannot stabilize microtubules, and this leads to microtubule decay, transport impairment, and synaptic degeneration (FIG. 4).[146] Indeed, a microtubule stability marker, acetylated α-tubulin, is reduced in AD neurons. In tissue culture, phosphorylation of tau repeats resulted in transport impairment, and deficits in fast anterograde transport were described in tau transgenic mice.[27,220]

One major advantage of microtubule-stabilizing strategies is the availability of approved and well-established chemotherapeutic drugs such as taxol. Taxol and its derivative taxane analog TX67 stabilize microtubules and promote tubulin polymerization.[221] Weekly taxol treatment of tau transgenic mice over 12 weeks restored fast anterograde transport, increased the number of axonal microtubules, and diminished motor deficits.[222] Other microtubule-stabilizing approaches are reviewed by Michelis et al.[223,224]

The neuronal tubulin-preferring agent NAP was reported to display less side effects than the non–cell-type-specific taxol, which inhibits cell mitosis in general.[225] The octapeptide NAPVSIPQ (NAP) has a preference for neuronal tubulin and crosses the BBB readily when administered intranasally. Continuous NAP treatment of a triple transgenic mouse model characterized by Aβ and tau-based pathology reduced the levels of hyperphosphorylated soluble and insoluble tau and enhanced the cognitive function of these mice.[85,225]

PERSPECTIVES

Compelling evidence supports the crucial role of tau in the pathogenic cascade in AD and other tauopathies (Fig. 4). Therapeutic approaches to interfere with tau-mediated toxicity are still in the stage of scientific validation and clearly more work will be needed before any of these experimental strategies will find their way into clinical application. Interest in tau is growing, especially after discovery of several tau mutations in FTDP-17 and in light of recent reports that suggested tau as a downstream mediator of aβ-conferred toxicity.[186,226] Counteracting tau neurotoxicity will therefore be a promising strategy, and even more so when combined with anti-Aβ directed approaches.

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