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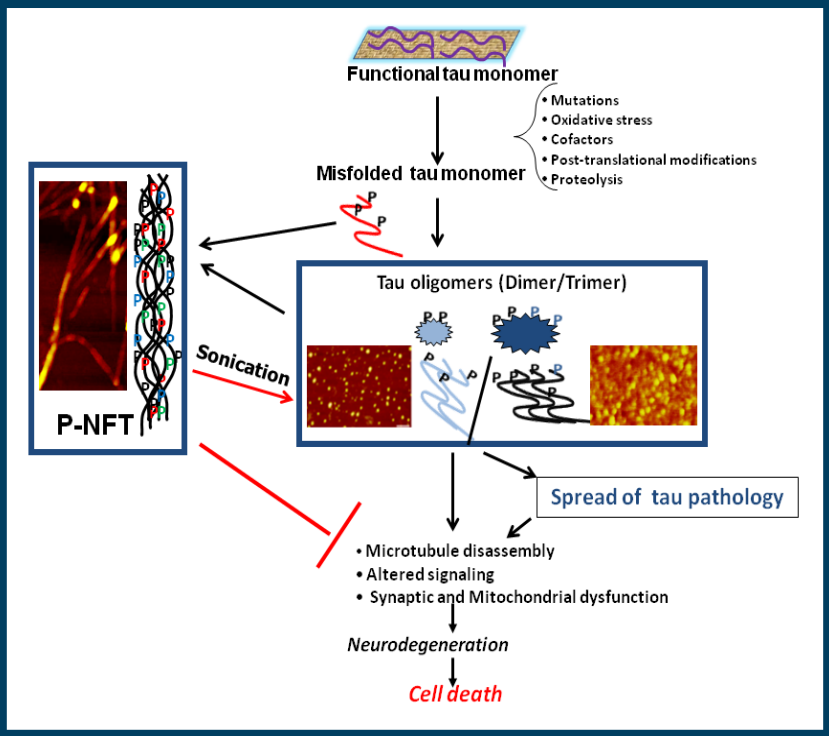
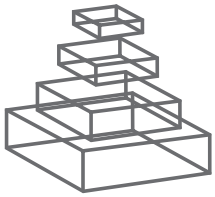


TAU OLIGOMERS

Topic Editors
Naruhiko Sahara and Jesus Avila

frontiers in NEUROLOGY



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TAU OLIGOMERS

Topic Editors:
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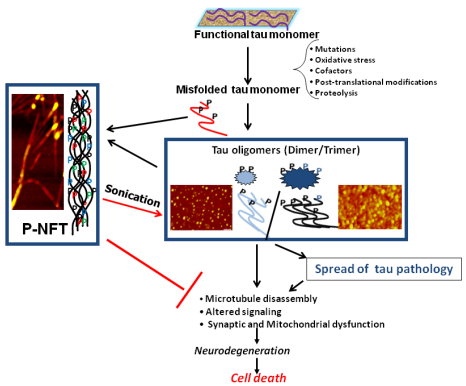

Schematic illustrating the central role of tau oligomers in tauopathies.
Image taken from: Gerson J.E. and Kayed R. (2013) Formation and propagation of tau oligomeric seeds. *Front. Neurol.* 4, 93. doi: 10.3389/fneur.2013.00093.

Neurofibrillary tangles (NFTs) composed of intracellular aggregates of tau protein are a key neuropathological feature of Alzheimer's Disease (AD) and other neurodegenerative diseases, collectively termed tauopathies. The abundance of NFTs has been reported to correlate positively with the severity of cognitive impairment in AD. However, accumulating evidences derived from studies of experimental models have identified that NFTs themselves may not be neurotoxic. Now, many of tau researchers are seeking a "toxic" form of tau protein. Moreover, it was suggested that a "toxic" tau was capable to seed aggregation of native tau protein and to propagate in a prion-like manner. However, the exact neurotoxic tau species remain unclear. Because mature tangles seem to be non-toxic component, "tau oligomers" as the candidate of "toxic" tau have been investigated for more than one decade. In this topic, we will discuss our consensus of "tau oligomers" because the term of "tau oligomers" [e.g. dimer (disulfide bond-dependent or independent), multimer (more than dimer), granular (definition by EM or AFM) and maybe small filamentous aggregates] has been used by each researchers definition. From a biochemical point of view, tau protein has several unique characteristics such as natively unfolded conformation, thermo-stability, acid-stability, and capability of post-translational modifications. Although tau protein research has been continued for a long time, we are still missing the mechanisms of NFT formation. It is unclear how the conversion is occurred from natively unfolded protein to abnormally mis-folded protein. It remains unknown how tau protein can be formed filaments [e.g. paired helical filament (PHF), straight filament and twisted filament] in cells albeit in vitro studies confirmed tau self-assembly by several inducing factors. Researchers are still debating whether tau oligomerization is primary event rather than tau phosphorylation in the tau pathogenesis. Inhibition of either tau phosphorylation or aggregation has been investigated for the prevention of tauopathies, however, it will make an irrelevant result if we don't know an exact target of neurotoxicity. It is a time to have a consensus of definition, terminology and methodology for the identification of "tau oligomers".

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"Tau oligomers," what we know and what we don't know

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Keywords: tau protein, tauopathy, neurodegenerative disease, propagation, tau phosphorylation

Neurofibrillary tangles, composed of intracellular aggregates of tau protein, are a key neuropathological feature of Alzheimer's disease and other neurodegenerative diseases, collectively termed tauopathies. Tau research has become one of the central players in the investigation of neurodegenerative diseases. Tau protein has several unique characteristics such as natively unfolded conformation, thermo-stability, acid-stability, and capability of post-translational modifications. We still do not know whether tau itself is toxic. With certain triggers, tau may transit into toxic forms. Researchers are now looking for "tau oligomers" as toxic components. Because "tau oligomers" contain variable species of tau protein [e.g., dimer (disulfide bond-dependent or -independent), multimer (more than dimer), granular (defined as EM or AFM) and perhaps small filamentous aggregates] (**Figure 1**), it is important to have a consensus regarding the definition, terminology, and methodology for the identification of "tau oligomers" (1–6).

Recently, "prion-like" toxicity and propagation mechanisms underlying the progression of disease have been proposed. With this concept, tau may have the ability to translocate between neurons and amplify toxic components (7). Although we do not know the exact forms of toxic tau oligomers, accumulating evidence has shown the probability of tau oligomer propagation (6).

Tau is an intracellular microtubule-associated protein. The mechanism of tau transmission from cell to cell is still unknown. Research focusing on extracellular tau will open potential new avenues for discovering the mechanism of tau propagation (8).

Abnormally hyperphosphorylated tau is a key feature of human tauopathies. Although we are not sure whether phosphorylation rather than oligomerization of tau is an initial molecular event in

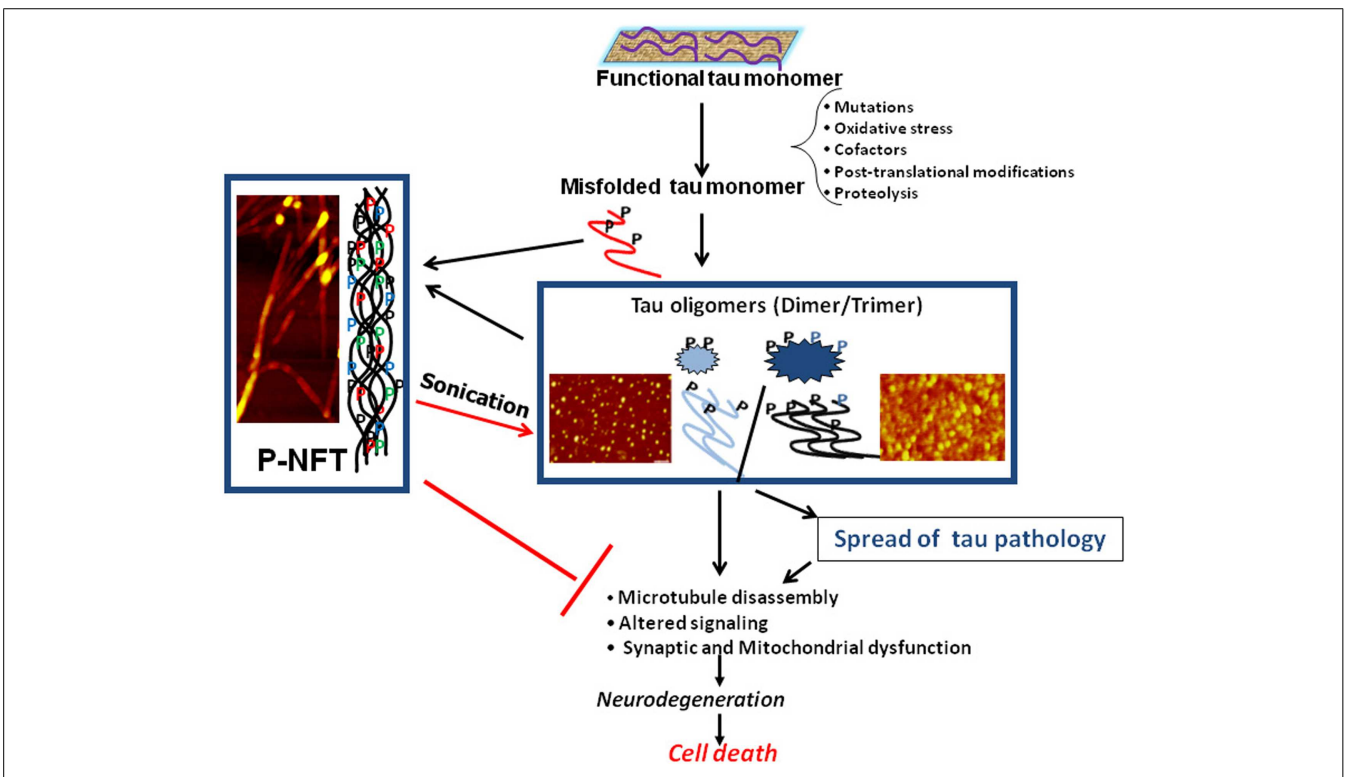

FIGURE 1 | Schematic illustration of the central role of tau oligomers in tauopathies. Figure taken from Gerson and Kayed (1).

tau pathogenesis, investigating the regulatory mechanisms of tau phosphorylation will be essential (9–11).

Here, we provide an overview of the current understandings of "tau oligomers" (1–12). Efforts toward the identification of neurotoxic tau species will ultimately lead to the translational research for developing novel therapeutic strategies for tauopathies.

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Are tau aggregates toxic or protective in tauopathies?

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Aggregation of highly phosphorylated tau into aggregated forms such as filaments and neurofibrillary tangles is one of the defining pathological hallmarks of Alzheimer's disease and other tauopathies. Hence therapeutic strategies have focused on inhibition of tau phosphorylation or disruption of aggregation. However, animal models imply that tau-mediated dysfunction and toxicity do not require aggregation but instead are caused by soluble hyper-phosphorylated tau. Over the years, our findings from a *Drosophila* model of tauopathy have reinforced this. We have shown that highly phosphorylated wild-type human tau causes behavioral deficits resulting from synaptic dysfunction, axonal transport disruption, and cytoskeletal destabilization *in vivo*. These deficits are evident in the absence of neuronal death or filament/tangle formation. Unsurprisingly, both pharmacological and genetic inhibition of GSK-3β rescue these tau phenotypes. However, GSK-3β inhibition also unexpectedly increases tau protein levels, and produces insoluble granular tau oligomers. As well as underlining the growing consensus that tau toxicity is mediated by a highly phosphorylated soluble tau species, our findings further show that not all insoluble tau aggregates are toxic. Some tau aggregates, in particular tau oligomers, are non-toxic, and may even be protective against tau toxicity *in vivo*. This has serious implications for emerging therapeutic strategies to dissolve tau aggregates, which might be ineffective or even counter-productive. In light of this, it is imperative to identify the key toxic tau species and to understand how it mediates dysfunction and degeneration so that the effective disease-modifying therapies can be developed.

Keywords: Alzheimer's disease, dimer, oligomer, filament, neurofibrillary tangle, insoluble tau

INTRODUCTION

TAU PROTEIN IN ALZHEIMER'S DISEASE AND OTHER TAUOPATHIES

Deposits of insoluble tau within neurons are defining pathological hallmarks in the group of neurodegenerative diseases known as tauopathies. Tauopathies include Alzheimer's disease (AD), Fronto-temporal Dementia with Parkinsonism on chromosome-17 (FTDP-17), Pick's disease, Corticobasal Degeneration (CBD), Progressive Supranuclear Palsy (PSP), and others (1). In all of these conditions, tau becomes both abnormally hyper-phosphorylated and deposited in insoluble aggregates [reviewed in Ref. (1, 2)]. These diseases differ in their clinical features, differentially-affected neuronal populations, and the distinct forms taken by the insoluble tau. Indeed, even within one disease state, the insoluble tau may be found in many distinct morphological forms; some *en route* to the final form of that disease's tau deposits, and others possibly on a different pathway.

In this review we will focus primarily on the forms of insoluble tau observed in AD, since they have been more widely studied. We will describe the different species of insoluble tau that have been identified; briefly review the factors that might promote tau aggregation; and then assess the evidence for and against the toxicity of each type of tau aggregate. Inevitably, this cannot be a comprehensive account of the extensive literature on this subject in the interests of space. Therefore we have selected papers which we believe represent the balance of evidence for and against toxicity, with apologies to those whose work we have not included. In this context we will use the term toxicity rather broadly, meaning either neuronal death, or neuronal dysfunction without death.

PHYSIOLOGICAL AND PATHOLOGICAL SPECIES OF TAU

This section briefly describes the major forms that tau has been shown to take in AD. These different species are treated in approximate order of size, from smallest to largest (**Table 1**). However, there is no intention to imply that each one goes on to form the next in a clear pathway.

MONOMER

Monomers of tau are highly soluble proteins of 55–74 kDa in size [depending upon splice variant and phosphorylation status – (3)]. There are six splice variants which contain either three or four microtubule-binding repeats, as well as either zero, one, or two N-terminal domains. These isoforms are usually denoted tau^{0N3R}, tau^{1N3R}, tau^{2N3R}, tau^{0N4R}, tau^{1N4R}, and tau^{2N4R}. They usually acquire a predominantly random coil structure under normal physiological conditions (4). Partially folded forms of tau monomers have also been described which are distinct from native tau monomers, and have a reduced level of random coiling but an increased level of β-sheet structure (5). Interestingly, such molecules are immediately positive for Thioflavin (which binds β-sheet). Compact monomers have also been characterized displaying intra-molecular disulfide bonds (6). Only the three isoforms of four-repeat tau can form these compact monomers, since

Table 1 | Summary of the major forms of tau identified.

Species of tau	Abnormally phosphorylated?	Toxic?
Monomer	Sometimes	Probably only when aberrantly phosphorylated
Dimer/trimer	Sometimes	Some types shown to be sufficient for toxicity
Small soluble oligomer	Sometimes	Some types shown to be sufficient for toxicity
Granular Tau oligomer	Sometimes	Not always
Filament	Yes	Might comprise of toxic tau, yet filaments themselves are probably neither necessary nor sufficient for toxicity
Neurofibrillary tangle	Yes	Might comprise of toxic tau, yet tangles themselves are probably neither necessary nor sufficient for toxicity
Ghost tangle	Yes	Unlikely

the second cysteine required for an intra-molecular interaction is in the extra repeat domain.

DIMER/TRIMER

Dimers are composed of two tau monomers in anti-parallel orientation linked by disulfide bonds. Tau dimers can be observed by electron microscopy (EM) as rod-like particles 22–25 nm long, which is similar in appearance to the monomers (7). Dimers can form from any isoforms of tau. Within that, however, two distinctly different forms of dimers have been described (8). One is cysteine-dependent and reducible; while in contrast the other is cysteine-independent, non-reducible, and has inter-molecular disulfide bridging at the microtubule-binding domain. Both forms have been identified *in vitro*, and in tau transgenic (JNPL3) mice (8). Preparing small oligomers from recombinant tau *in vitro*, dimers have been reported with apparent sizes of 180 kDa (9) and 130 kDa (10), as well as trimers with an apparent size of 120 kDa (11). In human tau transgenic mice, soluble tau species of 140 kDa have been described (8, 12). Small soluble tau species of approximately dimer and trimer size, and probably including tau fragments, have also been isolated from synapses in AD brains (13). It is unclear whether these variously reported dimers and trimers are indeed different tau species or whether they represent subtle variations of the same structure.

SMALL SOLUBLE OLIGOMER

Small soluble oligomers of tau of very many different sizes have been described *in vitro* and *in vivo*. Often, however (perhaps because of differences in post-translational modifications leading to different apparent sizes on PAGE), it can be difficult to determine if small oligomers described by different groups represent the same species or not. In one study, the soluble dimers described above were shown *in vitro* to develop into small soluble oligomers containing six to eight tau molecules (approximately 300–500 kDa in size) (8). JNPL3 mice, which over-express human tau with the P301L mutation ($tau^{0N4R\text{-}P301L}$) and harbor neurofibrillary tangles (NFTs), additionally have small tau oligomers which run at a wide range of sizes by PAGE [Sahara et al. (8)].

INSOLUBLE GRANULAR TAU OLIGOMER

Granular tau oligomers (GTOs) are electron-dense granular or globular aggregates of tau. They have been isolated from AD brains, mostly at early and moderate Braak stages (14). GTOs are composed of an average of 40 densely packed tau monomers. This corresponds to a size of 1800 kDa, or 20–50 nm in diameter when observed by EM or by atomic force microscopy (AFM) (15). It is important to note that, on the scale of insoluble protein aggregates generally, this is extremely small. Standard protocols for the sedimentation of insoluble proteins, such as 100,000 × *g* spin for 30–60 min [e.g., Ref. (16)], would fail to sediment GTOs which would remain in suspension in the "soluble" fraction, despite their demonstrable insolubility in SDS (15). Instead, sedimentation of GTOs requires a 200,000 × *g* spin for 2 h (15). The same authors developed a rigorous fractionation/purification protocol for GTOs. They further characterized the GTOs as being positive for MC1 and for Thioflavin, despite clearly being not filamentous in any way. They conclude that GTOs have β-sheet structure, and suggest that they may be composed of the partially folded form of tau monomer (15).

FILAMENT

It is well known that tau is capable of polymerization into filamentous forms. In AD, the predominant filaments are Paired Helical Filaments (PHF) and Straight Filaments (SF). In other tauopathies such as FTDP-17, however, there is variability in the morphology of tau filaments depending upon the tau mutations and/or tau isoforms involved. Here, filaments may take on other shapes such as twisted ribbon-like and rope-like filaments (17). A straight filament strand is 10 nm wide, and thus PHFs display alternating widths of 10 and 20 nm, with a half-periodicity of 80 nm (18, 19). Tau filaments exhibit β-sheet structure (20) which forms through the MT-binding repeat region (7, 21). Tau filaments from human AD brain have been shown to contain all six tau isoforms (22), although *in vitro* they can also be formed from single isoforms. They can be considered an amyloid (23, 24).

PRETANGLE

The pretangle is a slightly confusing concept that historically may have referred to a variety of species of tau, or even the status of a neuron. Generally speaking, a pretangle neuron is one that is positive for abnormal tau epitopes (misfolded and/or hyperphosphorylated), in some insoluble format large enough to be visible by light microscopy, yet free from mature fibrils or tangles by morphology. Bancher et al. (25) helpfully classified tangles into four stages (0–3). In this system[1], stage 0 tangles (later referred to

[1]For reference, stage 1 in this system is filamentous silver-stained tangles composed primarily of PHF; stage 2 is a classic neurofibrillary tangle and stage 3 is a ghost tangle (See "Neurofibrillary Tangle" and "Ghost Tangle").

by others as pretangles) are identified by cytoplasmic non-fibrillar (granular or diffuse) tau immunoreactivity, visible at the light microscope level. When viewed by EM, the labeled material was found to consist of PHFs, SFs, and smaller granular electron-dense material. Where pretangles were observed as granular via light microscopy, this probably represents non-filamentous clumps of PHFs, SFs, and the ultrastructural granules. Other researchers have described immunoreactivity for certain abnormal AD-associated tau epitopes in neurons containing no fibrils, and have deemed the neurons so labeled to be at a pretangle stage [for example Alz50 (26), the 12E8 epitope S262/S356 (27, 28), and T231 (27, 29)]. Confusingly, there are a number of conflicting reports in the literature as to whether "pretangles" are silver-staining, thioflavin-positive, and whether or not they contain β-sheet structure. It seems probable that these discrepancies arise from (a) a heterogeneity of what is meant by "pretangle" and (b) a sensitivity issue in regard to the assays for β-sheet. Pretangles should surely be positive for markers of β-sheet, since even the earliest partially-folded monomer (5) and certainly tau filaments (30) demonstrably contain β-sheet structure.

OTHER LARGE NON-FIBRILLAR TAU AGGREGATES

There are other forms and morphologies of pathological insoluble tau found in human brains which are large enough to be seen with the light microscope, and may be filamentous, yet are non-fibrillar in structure. Such aggregates include Hirano bodies, Pick bodies, and argyrophilic grains.

Hirano bodies have been described in AD, Pick's disease and other tauopathy brains (31, 32). Hirano bodies are large intraneuronal paracrystalline structures of 5–10 μm in width by 10–30 μm in length, composed of 7 nm filament arrays (32). They contain tau, other microtubule-associated proteins, actin, cofilin, other actin-binding proteins, and a fragment of APP.

Pick bodies are the characteristic morphology assumed by tau filaments in Pick's disease, in which they accumulate in limbic and cortical neurons. They are large structures that vary in size in different neuronal types, but are approximately the size of the nucleus. Pick bodies are formed of disorganized bundles of filaments which comprise only the three 3-repeat isoforms of tau, in contrast to the PHFs and SFs formed in AD which are made of all six isoforms [reviewed in Ref. (33)].

Argyrophilic grains are found in Argyrophilic Grain disease, where they accumulate in both neuronal processes and oligodendrocytes (34). Argyrophilic grains are structures that may be spherical, oval, comma-shaped, or spindle-shaped. As the name suggests, they are readily detectable by conventional silver-staining and light microscopy. The grains are much smaller than Hirano bodies, Pick bodies, and NFTs at approximately 4–9 μm in size. Argyrophilic grains are comprised of four-repeat tau in 9–18 nm SF and bundles of 25 nm smooth tubules. They never contain PHFs and ribbon-like filaments (34–36).

NEUROFIBRILLARY TANGLE

Neurofibrillary tangles are the classic tangles first described by Alzheimer in 1907. Classified by Bancher et al. (25) as stage 2 tangles and often described as "flame-shaped," they are large bundles of fibers consisting of both PHFs and SFs which may fill the entire neuronal cytoplasm. The fibers are silver-staining. Brief mention should be made here also of neuropil threads, which are bundles of SFs and PHFs occupying dendrites and largely displacing the cytoskeleton (37).

GHOST TANGLE

Ghost tangles are the structures that remain when the neuron within which the tangle formed has degenerated. They comprise large extracellular bundles of loosely arranged tau filaments. Compared to NFTs, ghost tangles stain more weakly for tau and more strongly for ubiquitin (25). It is thought that ghost tangles have undergone substantial proteolysis, and that thus the filaments are comprised predominantly of tau fragments, again in contrast to NFTs (38).

THE SEQUENCE OF EVENTS IN TAU AGGREGATION

There is some evidence to suggest that larger tau aggregates like PHFs and NFTs evolve from the successive aggregation of smaller tau species like monomers and soluble oligomers (**Figure 1**). One missing link appears to be whether small oligomers can form directly into GTOs in a linear pathway, or whether they represent two different pathways from monomers to PHFs and NFTs.

MONOMER → DIMER

There is evidence from the kinetics of tau polymerization that, once the partially folded conformation of the monomer has formed (however that may be triggered), then the process from monomers to dimers (and thence to oligomers) is energetically favorable and proceeds spontaneously (5). For monomers to be able to form dimers requires the PHF6 hexapeptide in the third microtubule-binding repeat domain (8, 39). However, the compact form of the tau monomer does not participate in this form of aggregation (40).

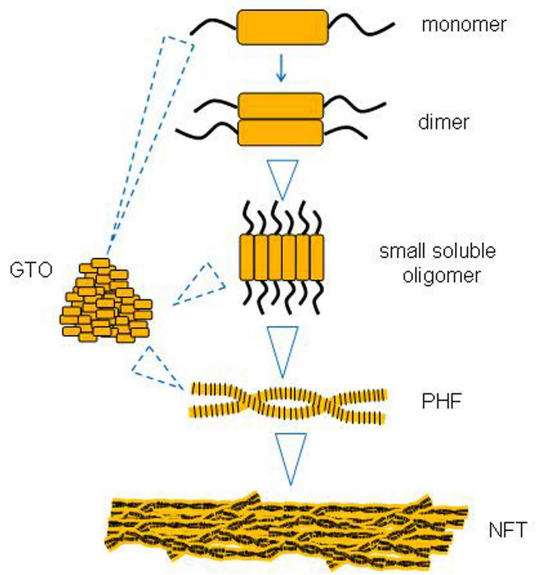

FIGURE 1 | A putative sequence of events in tau aggregation into neurofibrillary tangles (NFTs).

DIMER → SMALL SOLUBLE OLIGOMER

The tau dimer, in particular the cysteine-independent, non-reducible form (8), is thought to be an important intermediate which is involved in controlling the rate of formation of larger intermediates and fibrillization (6, 7, 41). In addition, more than one group has demonstrated that *in vitro* generated tau dimers aggregate to form larger tau oligomers (8, 9).

SMALL SOLUBLE OLIGOMER → GTO

We are not aware of any direct evidence that small oligomers proceed to form GTOs. There is, however, evidence that tau monomers *in vitro* can form GTOs (15), as well as that both monomers and GTOs can form PHFs. However, whether the sequence always proceeds from monomer → dimer → small oligomer → GTO → filament, or whether GTOs and other types of tau oligomers can be on different pathways, is not clear. In general, it is believed that when tau forms larger structures such as filaments of differing morphology, the interactions between tau molecules remain the same, and subunit packing follows the same plan (40). On these grounds it is plausible that GTOs might be part of the same pathway.

GTO → PHF

Increasing the concentration of GTOs *in vitro* causes them to form filaments, whereas the constituent soluble tau does not. On the basis of this, it is suggested that GTOs are precursors of PHFs (15).

MONOMER/DIMER → PHF (POSSIBLY VIA THE OTHER INTERMEDIATES)

There is a wide variety of evidence showing that monomers can polymerizes into PHF, but that does not address whether this is via the oligomeric intermediates or not. Such evidence includes the early *in vitro* demonstrations that tau at high concentrations will self-assemble into PHFs (42–44), and evidence that dimers are normally rate-limiting intermediates in this process *in vitro* (4, 6). There followed from these studies a large body of work delineating important details such as which motifs within tau are required for fibrillization, in which monomeric tau clearly formed into PHFs (39, 45–47). However, as in the early studies, whether oligomers were formed on the way was not necessarily assessed directly.

The mechanism for PHF formation requires two hexapeptide motifs in the microtubule-binding region of tau. These are PHF6 [(306)VQIVYK(311)] and PHF6* [(275)VQIINK(281)]. Formation of PHFs involves these two motifs changing conformation from random coil to β-sheet structure (24, 39). It should be noted that mutant tau containing no cysteines is still able to form PHFs *in vitro*, even though much more slowly than WT tau (40). This means that cysteine-dependent (covalent) dimers are not a requisite stage between monomer and PHF.

PHF → NFT

It is well established that NFTs *in vivo* are composed of PHFs and SFs (25). Furthermore, there is also direct *in vitro* evidence that filaments will spontaneously clump together into NFTs (48). Thus it is highly likely that NFTs are formed by the accumulation of tau filaments.

WHAT PROMOTES TAU AGGREGATION?

Little is known about what first triggers the initiation of tau aggregation. It is known that normal monomers do not spontaneously seed aggregation, and that some sort of trigger is needed (30, 49). However, numerous factors have been identified that can promote or increase tau aggregation, at least *in vitro* [reviewed in Ref. (50)].

Enzymatic cleavage of the tau monomer is one such factor. Truncation of the tau protein at Glu391 (51, 52), truncation by caspases at Asp421 (53), cleavage by thrombin (54), removal of the C terminus of the protein (55, 56), or deamination at asparagine or glutamine residues (57) have all been shown to promote tau aggregation [Reviewed in Ref. (58)].

Local concentration of tau can be key. Tau at high concentrations *in vitro* forms PHFs (42–44). Moreover, the transition of tau from random coil to β-sheet is also known to be concentration dependent (39), further supporting the idea that excessive local accumulation of tau may promote its aggregation (especially if other pro-aggregating factors – such as those discussed below – are also in the near vicinity).

Controversially, tau phosphorylation has been postulated to both stimulate and repress its subsequent aggregation into filaments. Circumstantial evidence for stimulation includes the seminal fact that filamentous tau is highly hyper-phosphorylated (59) at many sites. More direct but *in vitro* evidence shows that tau phosphorylated at AD sites polymerizes more readily into tangles of PHF/SF; dephosphorylation abolishes tau's self-assembly; and hyperphosphorylation of recombinant tau by brain kinases induces its self-assembly into tangles of PHF/SF (60, 61). In a cellular model, it has been shown that all three kinases GSK-3β, MEKK, and JNK3 are required for tau aggregation (62). Phosphorylation of tau specifically at Thr231, Ser396, Ser422, and Ser404 promotes self-aggregation of tau into filaments (55, 63, 64). *In vivo*, overexpression of the kinases GSK-3β or Cdk5 can promote tau aggregation (65, 66). On the other hand, *in vitro* studies have shown that tau phosphorylation is not necessary to drive tau into PHFs (41, 67). On the contrary, phosphorylation of KXGS motifs in the repeat region *inhibits* tau aggregation *in vitro* (54, 68). Furthermore, more recently emerging data showing that tau aggregates made up of recombinant non-phosphorylated tau can "seed" further tau aggregation in cells (discussed below) also supports the idea that phosphorylation is not required to promote aggregation (69, 70). However, it is not yet known whether phosphorylated tau would seed and promote aggregation at a different rate.

Some of the missense and deletion mutations found in tau in cases of fronto-temporal dementia (FTDP-17), when expressed in models, display enhanced aggregation compared to normal tau. *In vitro* PHF formation is faster for recombinant tau harboring one of various such mutations. Human tau with each one of the point mutations G272V, N279K, V337M, or R406W shows significantly faster *in vitro* PHF formation than WT full-length human tau, while the ΔK280 and P301L mutants form PHFs at dramatically greater speeds (46). This phenomenon has been confirmed *in vivo*: mice expressing mutant human tau^{P301L} develop pathology more readily than those expressing WT human tau, both on 0N4R and 2N4R tau backgrounds (71–73).

Many polyanionic cofactors of all kinds can promote PHF assembly. These include glycosaminoglycans (GAGs) such as

heparin and neuroparin (40, 74, 75); sulphoglycosaminoglycans (sGAGs) like keratins or chondroitin sulfates (76), RNA (41); polyglutamic acid (30, 41, 74); fatty acids such as arachadonic acid (77, 78) and alkyl sulfate detergents (79).

Other factors which may promote tau aggregation include tissue transglutaminase (80), Congo red (81), ferritin (82), H_2O_2 in the presence of iron (Fenton's reaction) (83), and quinones (84).

Despite this wealth of data over many years regarding factors that promote aggregation, questions still remain about what initiates tau aggregation *in vivo* in health and disease. However, once tau aggregation has been initiated, it is believed to promote further "prion"-like "seeding" and propagation of tau aggregation and pathology (85). This was first demonstrated *in vivo* where stereotaxic injections of brain homogenate containing tau aggregates led to induction and propagation of tau aggregation in tau transgenic mice (85). Supportive data also emerged from studies in cell culture showing that incubation of tau-expressing cells with fibrils of recombinant tau leads to induction of tau aggregation in the recipient cells (69, 70).

ARE NEUROFIBRILLARY TANGLES TOXIC?

NFTs: EVIDENCE FOR TOXICITY

The evidence that associates NFTs with neuronal dysfunction and neurodegeneration is largely correlative in nature. Studies of human post-mortem brains initially implicated NFTs in toxicity by showing a strong spatial and temporal correlation between NFTs and severity of dementia, and between NFTs and neurodegeneration or neuronal death (86–92). Some tau transgenic mouse models display neuron loss in the same timeframe and/or location as NFT formation. For example, expression of tau^{P301L} under the thy1.2 promoter causes neuronal apoptosis at the same age as filaments and NFTs (93); while tau^{P301L} under the prion promoter causes NFTs in spinal cord, brainstem, and pretangles in cortex, at the same time as loss of motor neurons (71). Furthermore, in tau mouse models, there is a correlation between reduction of NFT and improvement in cognition (94). The limitation of these correlative studies between reduced NFTs and reduced impairments is that, in many cases, other smaller tau species may be reduced also. This leaves open the question of whether it is the reduction of the NFTs or the smaller species which has been beneficial.

More direct evidence in favor of NFT toxicity comes from mice conditionally expressing human tau fragments harboring pro-aggregation or anti-aggregation mutations (95, 96). Pro-aggregation mice develop PHFs, "pretangles," and NFTs early; followed by synaptic and neuronal loss. In constrast, mice expressing the same tau molecule but with the anti-aggregation mutation never develop aggregates or neuronal pathology. This was verified in a *C. elegans* model of tauopathy which went further to show that treatment with anti-aggregation inhibitors protected against tau-mediated toxicity (97). This suggests that tau aggregation in the form of PHFs or larger has been the cause of cell death. Moreover, when expression of human tau is suppressed, mice are rescued from toxicity in terms of both cell death and cognition. This represents very strong evidence in favor or PHFs and/or NFTs as the toxic species. These conclusions were difficult to reconcile with a body of evidence detailed below proving that PHFs and NFTs are neither necessary nor sufficient for toxicity. However, the authors subsequently showed that in the animals expressing pro-aggregant tau, toxicity might in fact be mediated by a species of tau smaller than PHFs and NFTs (98, 99).

NFTs: EVIDENCE AGAINST TOXICITY

There is now strong evidence from a number of models that NFTs are not required for tau-induced neuronal dysfunction and toxicity. In most *Drosophila* models of tauopathy, neuronal NFTs are usually not formed at all, despite clear neurodegeneration, and functional phenotypes (100–104). In some mouse models where NFTs do form, cognitive/behavioral impairments and cell death can be demonstrated *earlier* in the time course of the disease prior to NFT formation (105). In a different mouse model [PrP44: the shortest human tau isoform (tau^{0N3R}) under the prion promoter] the formation of tau filaments coincides in the time course of the disease with phenotypes such as neurodegeneration and motor deficits, while NFTs form later (106, 107).

There is also compelling evidence that NFTs are not sufficient for toxicity, from mice that conditionally express human tau (tau$^{0N4R\text{-}P301L}$). These mice display age-dependent development of NFTs, neuronal loss, and progressive motor deficits. When tau expression is switched off after the onset of memory impairments and NFT formation, memory improves and cell loss is stabilized, yet NFTs remain (108, 109). Furthermore, when tau is turned off at a timepoint when there are pretangles but no NFTs yet, the pretangles stay stable. This indicates that they also are insufficient for toxicity. This is corroborated in a different study using the same mice, in which a successful treatment reduced motor deficits despite failing to reduce NFTs (110). Furthermore, tangle-bearing neurons in this model were shown to be just as active in a functional hippocampal circuit as non-tangle bearing neurons (as evidenced by expression of the immediate early gene *Arc* in response to environmental cues) (111). Further investigations of the mouse conditionally expressing pro- and anti-aggregant form of tau, mentioned in the preceding section, also supports this view. The pro-aggregation mice develop learning and memory deficits from which they recover after tau expression is turned off (98). However, it transpires that after an extended period of tau suppression, NFTs still remain, as in the tau$^{0N4R\text{-}P301L}$ mice. This implies that it is a smaller species of tau (soluble or insoluble) which has decreased in correlation with behavioral improvement in these studies (98).

In the light of such evidence, it has been suggested that formation of NFTs is a protective response that ultimately fails (58). This review describes a scenario in which caspases, having become activated because the cell contains toxic tau and is thus under stress, cleave the tau making it more fibrillogenic. Cleavage is unlikely to be the only trigger, since the initial steps of aggregation can involve primarily full-length tau isoforms (5, 112). Either way, the idea is that once tau aggregates seed, they can sequester toxic tau and thus delay cell death. However, the trade-off is that axonal transport is compromised and cellular protein degradation pathways become clogged, and thus the neuron gradually becomes dysfunctional (58). This is supported by evidence that NFT-bearing neurons appear to survive for decades (113) and maintain markers of normal gene expression (111), and may be in fact be longer lived than

those neurons without NFTs in the AD brain that would have died at earlier time-points and hence were not evident at post-mortem.

In conclusion, despite NFTs being a vital historical clue to the involvement of tau in neurodegenerative disease, a wide variety of strong evidence now exists that NFTs themselves are neither necessary nor sufficient to cause tau-induced toxicity and dysfunction.

ARE TAU FILAMENTS TOXIC?

Some of the evidence regarding NFTs is also applicable as indirect evidence about tau filament toxicity. That is, some of the evidence in favor of large aggregates being toxic could really apply to either NFTs or PHFs/SFs or both. Further, the evidence that NFTs are not toxic naturally casts suspicion onto smaller pathological species such as filaments. However, direct evidence for PHFs as the primary toxic species, rather than something smaller, is sparse.

FILAMENTS: EVIDENCE FOR TOXICITY

As with the evidence in favor of NFT toxicity described above, much of the evidence that implicates filaments as a toxic species of tau is correlative in nature. For example, in some mouse models of tauopathy, filaments coincide in the time course of the disease with phenotypes such as neurodegeneration and motor deficits (106), while NFTs form later (107). Similar results were seen in one *Drosophila* model of tauopathy in which tau filament formation was reported (114). Furthermore, mutations in tau which are responsible for FTDP-17 also promote faster tau filament formation (46), thus circumstantially implicating tau filaments in the disease process.

An immunotherapy study targeting tau in a mouse model provided some more direct evidence in favor of PHF toxicity. In this study, immunization of JNPL3 mice (with a short phosphorylated tau fragment) served to reduce tau aggregation (into PHFs or larger aggregates) and the associated behavioral phenotypes, but failed to reduce smaller species of tau. This suggests that that PHFs or larger tau aggregates are the toxic species in this model (115). In another study, tau filaments but not monomers (at physiological concentrations) were shown to selectively impair anterograde transport in isolated squid axoplasm (116, 117).

FILAMENTS: EVIDENCE AGAINST TOXICITY

As with the NFT situation, some animal models have impairment but no filaments, or at least not until later in the disease progression. For example, *Drosophila* (100–104) and *C. elegans* (118) expressing human tau display behavioral phenotypes indicative of neuronal dysfunction and toxicity without forming tau filaments or larger aggregates. Even in mice, in the transgenic tau model expressing the longest human tau isoform (tau^{2N4R}), brains contain some form of insoluble tau but nothing as big as PHFs (or NFTs), while the animals display a motor impairment (119). Such evidence indicates that filaments are not necessary for tau-induced toxicity.

There is also evidence that filaments are not sufficient for toxicity, since they continue to form in the conditional tau mice mentioned earlier, in which transgenic tau expression has been turned off and deficits thereby rescued (108). In another mouse model, Andorfer et al. showed that, while there was widespread neurodegeneration, the PHF-containing neurons appeared "healthy" in terms of nuclear morphology, suggesting that the polymerized protein was probably neuroprotective (105). In an *in vivo* lamprey model (120), administration of a benzothiazole derivative drug (purported to break up tau filaments) successfully improved tau-induced phenotype, but apparently did so without actually breaking up the tau filaments. This provides further evidence that filaments are not sufficient for toxicity. *In vitro*, polymerization of hyper-phosphorylated tau into PHFs abolishes its toxic activity to sequester other MAPs (121). Unlike the soluble form of hyper-phosphorylated tau, the filamentous form of tau does not bind MAPs and does not disrupt microtubules *in vitro* (121).

In an inducible cell line, the repeat domain of wild-type tau was non-toxic, whereas a similar construct harboring a point mutation that induced aggregate formation (eventually PHFs) caused cell death (54). Crucially, however, increased cell death was observed *before* PHF formation in the aggregate-prone mutant, demonstrating that PHFs were not necessary for toxicity, and that in fact a smaller form of aggregate was the toxic species.

While the *in vivo* evidence is not quite as extensive as for NFTs, one can also conclude that tau filaments are neither necessary nor sufficient for tau-induced toxicity, and that something smaller than filaments is the most toxic form of tau.

SPECIES OF TAU FOUND IN FILAMENTS

When the insoluble protein fraction from brains of AD patients or animal models, containing any tau filaments or larger tau aggregates, is solublized using urea or formic acid, the tau species which were building blocks of these large insoluble aggregates can be identified. These species include monomers of 55, 60, 64, and 68 kDa in size (67), and a 170-kDa species. The 64 and 170 kDa species in particular have been implicated in toxicity. The 64-kDa species represents a hyper-phosphorylated monomer. It is found in brains of the $tau^{0N4R\text{-}P301L}$ transgenic mouse (122), and increases with age in the insoluble fraction at the expense of the 55-kDa monomer (which is found in both soluble and insoluble fractions).

In the same study that showed NFTs were not necessary for toxicity [because they did not decrease in successfully treated tau mice – (110)], the successfully treated mice displayed a significant reduction in 64 kDa tau from the high-speed insoluble fraction. Confusingly, subsequent commentators have described this species as "soluble aggregated tau" [e.g., Ref. (123)]. However, this 64 kDa species is always a component of an aggregate that sediments at 150,000 × *g*, which is therefore bigger than a GTO. Another study in a conditional mouse model found that three distinct tau species correlated with neuronal dysfunction (12). Two of these species were in a sarkosyl-insoluble fraction (from which NFTs had been previously cleared) which must represent GTOs or filaments: a 64-kDa hyper-phosphorylated monomer and a 170-kDa hyper-phosphorylated oligomer. The third species was a 140-kDa oligomer from the soluble fraction. All of these species arose early in the disease progression, and increased with increasing learning and memory deficits. Conversely, all three decreased upon suppression of transgenic tau expression and recovery from neuronal dysfunction. These results clearly implicate one or more of the three species in toxicity; however, it is not clear whether the culprit is the soluble or insoluble components or both. The same 64 kDa species has also been described in a sarkosyl-insoluble

fraction from transgenic tau$^{0N4R\text{-}301L}$ mice that represents SFs and NFTs (71, 108). It is not toxic in this circumstance because it continues to increase after tau expression has been turned off and animals are recovering (108).

In summary, these two species of tau, a hyper-phosphorylated 64 kDa monomer and a 170-kDa oligomer, have frequently been demonstrated as components of an insoluble filament fraction. There is some evidence, although not conclusive, that these species may be associated with toxicity.

ARE (INSOLUBLE) GRANULAR TAU OLIGOMERS TOXIC?

Part of the difficulty in acquiring evidence about GTOs from the existing literature is that, as mentioned earlier, in standard insoluble protein fractionation protocols any GTOs in the sample will be lost. Even though fully insoluble, they are too small to sediment with a standard "high-speed insoluble" fraction (15). They remain in suspension in the "soluble" fraction; and yet as they are around 1800 kDa in size and not readily reducible without 8 M urea, they will not enter a standard PAGE resolving gel and be detected (Cowan, unpublished observation). Therefore when one reads, for example, studies in tau mouse models showing small oligomers and PHFs and drawing conclusions about toxicity, one cannot usually conclude anything about the presence or absence, toxicity or otherwise, of GTOs.

GTOs: EVIDENCE FOR TOXICITY

In the years following the identification of GTOs, when evidence was beginning to accumulate that PHFs and NFTs might not be toxic, some evidence remained that some insoluble form of tau must be toxic. There was speculation in reviews that insoluble oligomers of tau, perhaps GTOs, might be the most toxic species. However, there is no direct evidence for this. Clues that GTOs might be associated with toxicity come from studies showing that numbers of GTOs increase with progression of AD, that fewer GTOs are observed at Braak stage 0 than at stage 1, and that their peak precedes that of NFTs (14). GTOs are generally believed to consist of toxic phosphorylated species of tau because phosphorylated tau levels are high in the AD brain at the time-points when GTOs are abundant. Such clues have led to the suggestion that reducing GTOs may prove to be a promising therapeutic strategy; however, the authors of these publications acknowledge that the effect of GTOs on neuronal vulnerability is unknown.

There is also evidence that an insoluble oligomer(s) of some sort is probably the culprit, without clear evidence that it is GTOs. For example, in the study mentioned as evidence against PHF toxicity in an inducible cell line (54), the soluble tau construct was not toxic, whereas the aggregate-prone mutant tau species caused cell death *prior* to PHF formation. This therefore represents evidence against both soluble tau toxicity and PHF toxicity, but rather implicates some intermediate species. Similarly, in another study, very small insoluble tau oligomers (of up to a few hundred kDa) were isolated from synaptosomes derived from AD brains and were associated with impaired ubiquitin proteasome function (124). If any GTOs had been present in this preparation, they might not have been observed with the protocols used. Clearly small insoluble tau oligomers exist and are associated with toxicity but whether they can be classified as GTOs or indeed their precursors is not clear.

GTOs: EVIDENCE AGAINST TOXICITY

We have recently observed, in a *Drosophila* model of tauopathy, the formation of GTOs which are non-toxic (125) When flies express human tau^{0N3R} in neurons they exhibit a clear behavioral phenotype, but no insoluble tau. However, upon pharmacological or genetic manipulations which inhibit GSK-3β, the phenotype is rescued and GTOs are produced. We demonstrate that these GTOs produced in flies are the same size as those isolated from human brain and comprise of non-phosphorylated full-length tau monomers. Like us, another group has also demonstrated the formation of large insoluble tau oligomers in *Drosophila,* in conditions associated with rescue of tau-mediated toxicity (126). They showed that rescue of human tau^{0N4R} or tau$^{0N4R\text{-}R406W}$ induced neurodegeneration and behavioral deficits by co-expression of Nicotinamide mononucleotide adenylyltransferase (NMNAT) also led to increased formation of insoluble tau oligomers. However whether these insoluble tau oligomers were the same as the GTOs that we described in our model is not clear. Nonetheless, both our studies collectively imply that tau aggregation can correlate with rescue from tau-induced phenotype. However, whether this is because of sequestration of smaller toxic tau species, or the presence of non-phosphorylated tau in the conditions in which the GTOs form, or something else about their structure is not clear.

Overall, the pathological significance of GTOs has yet to be fully understood. It is possible that different GTOs form in different circumstances and the phosphorylated status of their constituent tau proteins and/or the extent of β-sheet structure may play a role in determining their toxicity.

ARE SOLUBLE (MONOMERIC OR OLIGOMERIC) TAU SPECIES TOXIC?

As has already been alluded to, there are numerous species of tau that are soluble, and it seems probable that they possess very different properties. Just considering monomers, there is clearly a multiplicity of species: the three major conformations described (regular, compact, and partially folded); the six splice variants; and of course the array of phosphorylation combinations, both demonstrated and possible, which have been barely touched upon here. Then there are at least two conformations of dimer (cysteine-dependent and independent), as well as trimers, and small oligomers of various sizes and phosphorylation states, as described earlier. We would speculate that there are probably many variations of soluble oligomeric tau species occurring in nature in the brain that have not yet been specifically described. Further, some of the evidence regarding the toxicity or otherwise of soluble tau cannot (or did not) differentiate between these species.

SOLUBLE TAU: EVIDENCE FOR TOXICITY

There are many examples of studies conducted *in vivo* and *in vitro* showing that soluble tau is sufficient to cause dysfunction and toxicity. To give a few examples, pseudophosphorylated tau causes cell death when virally expressed in hippocampal slices, without becoming SDS-insoluble (127). Soluble tau monomer applied

extracellularly to cells in culture causes intracellular calcium increase and cell death (128, 129). Additionally, some tauopathy models in *Drosophila* (100–102, 130) exhibit significant neuronal dysfunction and degeneration and yet contain no insoluble tau providing strong evidence that soluble tau is sufficient to cause dysfunction and toxicity. Similar evidence exists in mouse models: for example, mice expressing WT tau^{2N4R} under the CamK-II promoter (131) as well as those expressing mutant tau^{N279K} (132) both display learning defects but no NFTs or insoluble tau and no cell death. However, these mouse studies are subject to the caveat discussed in Section "Are (Insoluble) Granular Tau Oligomers (GTOs) Toxic?" that if there were any GTOs present they would not have been detected by the protocols used. Therefore, strictly speaking, we feel that the conclusion from such studies is: either soluble tau or small insoluble tau oligomers are sufficient for toxicity.

Further evidence supporting soluble tau toxicity comes from many studies showing rescue of tau-mediated phenotypes after suppression of tau expression which leads to reductions in soluble tau but persistence of tangle pathology (58, 108, 111, 133). In one such study, transgenic tau^{P301L} mice were treated with methylene blue at a dose sufficient to rescue their memory deficit, and reduce total and soluble tau without affecting insoluble tau aggregates (134). This study went one step further in showing that the reduction of soluble tau is required for the rescue of phenotype.

Whether the toxic soluble species of tau is monomeric or oligomeric (or both) in these studies is not always clear. There is a convincing body of evidence showing that certain specific dimers, trimers, and other very small soluble oligomers are sufficient to cause toxicity both *in vitro* and *in vivo*. Patterson et al. (135) show that the 180-kDa dimers that they produced *in vitro* can suppress fast axonal transport in a squid axoplasm model. The 120-kDa trimers produced by Lasagna-Reeves et al. have been shown to be toxic *in vitro* (11) and *in vivo* (136). These trimers cause significantly more cell death than tau monomers or filaments when applied to SH-SY5Y cells in culture (11). Intra-hippocampal injections of trimers cause significant loss of synapses and neurons resulting in memory deficits, whereas injections of tau monomers or fibrils do not (136). Overall, these findings show that dimers and trimers of tau can be toxic.

Tau species of this size range have also been demonstrated *in vivo,* indicating that they are physiologically relevant. Both the Kayed and the Binder laboratories have used the oligomers that they made *in vitro* to raise oligomer-specific anti-tau antibodies, TOC-1 (9) and T22 (137), which they demonstrate recognize tau *in situ* in the post-mortem AD brain. Both oligomer-specific antibodies react with "pretangles" in early Braak stages, and co-localized with some disease-associated phospho-tau epitopes.

Small soluble oligomers also arise in many transgenic tau animal models in a context that implicates them in toxicity. For example, transgenic *Drosophila* expressing either human tau^{0N4R} or tau$^{0N4R\text{-}S406W}$ in brain display soluble tau oligomers of 150–250 kDa in size (114, 126) and in both studies oligomer formation was associated with degeneration. Berger et al. (12) and Sahara et al. (8) independently identified small soluble tau oligomers of approximately 140 kDa (believed to be dimers) in brain homogenates of tau$^{0N4R\text{-}P301L}$ transgenic mice. The oligomers detected by Berger et al. (12) (in the inducible tau$^{0N4R\text{-}P301L}$ mice) appeared at very early stages of disease when memory deficits were evident in the absence of tangle formation or neuronal loss. Either this 140 kDa soluble species and/or the two small insoluble species of tau discussed in Section "Species of Tau Found in Filaments" are implicated in causing toxicity in this model.

Like the oligomers identified by the Binder and Kayed laboratories described above, the 140-kDa oligomers identified by Berger et al. (12) in tau transgenic mice are also detectable in the brains of AD and FTDP-17 patients. It is not clear whether all of these oligomers are one and the same tau multimer or whether they represent tau oligomers at different stages of maturation during the disease process. In addition, it has not been determined whether aggregation into larger oligomers alters the toxicity of the small tau oligomers.

SOLUBLE TAU: EVIDENCE AGAINST TOXICITY

Evidently, not all soluble phosphorylated tau can be toxic. Soluble tau of many species is obviously found physiologically in healthy individuals. One specific example of a soluble species known to be non-toxic is the compact monomer with intra-molecular disulfide bonds, which appears to be a species that is relatively protective and, notably, does not go on to form larger aggregates (40).

In the tau immunization study mentioned earlier as evidence in favor of PHF toxicity (115), it was found that immunization causes reduced aggregation of tau into PHFs or larger aggregates, and was associated with a reduced behavioral phenotype. However, this also causes an *increase* of PHF-1 immunoreactivity in the "high-speed soluble" fraction, which in this case would represent any species of GTO size or smaller. This suggests not only that PHFs might be toxic, but also that the soluble species present were not sufficient for toxicity (115).

The inducible pro-aggregation and anti-aggregation mutants of the tau repeat regions created by the Mandelkow laboratory argue against toxicity of soluble tau. In both the cell lines [the study mentioned as evidence against PHF toxicity: (54)], and in the mice (95, 98), the common theme was that the anti-aggregation mutant, which would always be a soluble form of tau, was never toxic. The mouse lines provide some additional correlative evidence against toxicity of any form of tau smaller than PHFs, in that the levels of "soluble" tau were constant between the pro-aggregation mouse, which experienced neurotoxicity, and the anti-aggregation mouse, which did not.

Clearly, physiological tau is soluble and non-toxic. However, under pathological conditions tau may undergo changes that render it toxic, even though it may remain soluble.

DISTINGUISHING BETWEEN SMALL (SOLUBLE) TAU OLIGOMERS AND TAU MONOMERS

In the above paragraphs, a general trend is that the evidence for soluble tau toxicity centers on some reasonably well-defined dimers and trimers which are demonstrably sufficient for toxicity; whereas the evidence against either has not distinguished between the myriad species, or has only said that one particular type of soluble tau

is not toxic. In that sense, the evidence against is not conclusive. While the specific data cited in favor of dimer and trimer toxicity is compelling, we still know very little about the properties (or indeed existence) of all the other soluble forms.

CONCLUSION

In conclusion, there is a body of evidence demonstrating that small soluble tau oligomers are the most toxic form of tau. Filamentous and fibrillar tau is neither necessary nor sufficient for tau-induced toxicity, and may very well represent a neuroprotective strategy. Such ideas are not new, and a number of reviews over the past few years have drawn the same conclusions (138–140). Nevertheless, this conclusion is still not broadly accepted. Even if it were to be accepted, many questions remain. We still have little idea which of the multiplicity of soluble tau species is the culprit or culprits: is it monomers, dimers or trimers or all three, and in which conformation(s) and phosphorylation state(s)? Also, we have incomplete information about the sequence of events on the pathway(s) of tau aggregation (**Figure 2**). Especially, where do GTOs fit in? Are they made gradually from increasing sizes of smaller oligomers? Or do they have a different conformation that makes them so compact and insoluble? Can they really go on to form PHFs *in vivo*? Such questions are important for informing the strategies to be implemented when developing treatments for AD and tauopathies. We do not yet know which species of tau would represent the best target for tau-based therapies. If a certain specific set of small soluble tau oligomers are toxic, while insoluble GTOs and larger insoluble tau species are not, then perhaps strategies aimed at breaking up large insoluble tau aggregates might prove ineffective. Especially if it transpires that GTOs are not only non-toxic but on an alternative pathway to PHFs, then perhaps *encouraging* GTO formation might even turn out to be a valid approach. Alternatively, it might not be size and solubility alone of the tau species that are the key toxicity-determining factors: levels of β-sheet structure and phosphorylation at certain sites may also be influential.

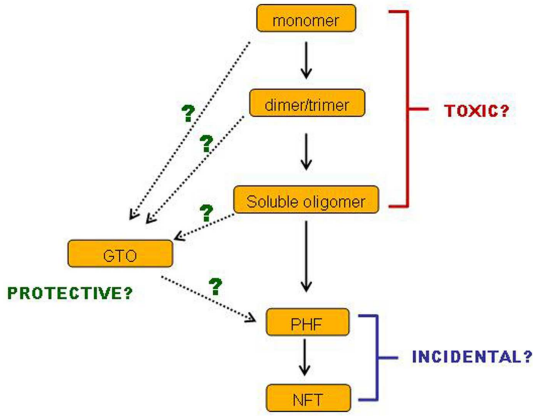

FIGURE 2 | Cartoon of remaining questions.

There is a precedent from other proteinopathies for a small soluble species being the most toxic, while the smallest insoluble form is relatively protective. This has been demonstrated for huntingtin protein in Hungtington's disease (141, 142), alpha-synuclein in Parkinson's disease (143), and amyloid beta in AD (144): however, evidence for this phenomenon in the case of tau aggregation is only beginning to emerge now.

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Characteristics of tau oligomers

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In Alzheimer disease (AD) and other tauopathies, microtubule-associated protein tau becomes hyperphosphorylated, undergoes conformational changes, aggregates, eventually becoming neurofibrillary tangles (NFTs). As accumulating evidence suggests that NFTs themselves may not be toxic, attention is now turning toward the role of intermediate tau oligomers in AD pathophysiology. Sarkosyl extraction is a standard protocol for investigating insoluble tau aggregates in brains. There is a growing consensus that sarkosyl-insoluble tau correlates with the pathological features of tauopathy. While sarkosyl-insoluble tau from tauopathy brains has been well characterized as a pool of filamentous tau, other dimers, multimers, and granules of tau are much less well understood. There are protocols for identifying these tau oligomers. In this mini review, we discuss the characteristics of tau oligomers isolated via different methods and materials.

Keywords: tau, oligomers, dimer, sarkosyl-insoluble, antibody

INTRODUCTION

Tau is a phospho-protein that belongs to the family of microtubule (MT)-associated proteins. The primary function of tau protein is to modulate MT dynamics for maintaining neuronal processes and regulating axonal transport. During pathogenesis, tau protein abnormally aggregates into intracellular, filamentous inclusions, or neurofibrillary tangles (NFTs) in the brains of individuals with neurodegenerative disorders. These are termed tauopathies [reviewed in Ref. (1)].

In human tauopathies, intracellular aggregates of abnormally hyperphosphorylated tau protein and neuronal cell loss typically coincide within the same brain regions (2). Several transgenic mouse models that overexpress human tau protein have demonstrated how tau pathology and neuronal loss progresses [mouse models are reviewed in Ref. (3)]. However, recent data suggest that tau is involved in neuronal dysfunction before NFTs are formed (4, 5). *In vitro* tau polymerization studies indicated that NFT formation consists of several steps: dimerization, multimerization, oligomerization, and protofibril formation (6–11).

About two decades ago, tau aggregation intermediates (also referred to as AD P-tau) were isolated from the buffer-soluble fraction derived from brains of AD patients (12). More than 10 years later, attention has focused on oligomeric tau species in human (13) and transgenic mouse (14) brains in order to identify the exact neurotoxic components of tau protein. However, the potential role of tau oligomers is poorly understood because they exist in various states (e.g., dimers, multimers, and granules). Here, we review various protocols used to isolate tau oligomers and propose a general outline for the identification of tau oligomers.

SOLUBLE PRE-FIBRILLAR TAU IN HUMAN AD BRAINS

Greenberg and Davies first reported to isolate sarkosyl-insoluble tau from paired helical filament (PHF)-enriched fraction from human AD brain homogenates (15). Cortical gray matter was homogenized in buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 0.8 M NaCl, and 10% sucrose, and then centrifuged at 27,200 × *g*. PHF-associated tau was enriched from the supernatant by taking advantage of their insolubility in the presence of a detergent, sarkosyl. PHF-associated tau migrated at around 57–68 kDa on one-dimensional PAGE gels. This enriched supernatant was more acidic on two-dimensional PAGE gels compared to extracts from normal brains. Although this PHF-associated tau was not extracted from highly insoluble fraction containing NFTs, the sarkosyl-insoluble tau displayed the same structural and antigenic properties as PHFs isolated from NFTs (16–18) and was distinguishable from normal, soluble tau proteins.

Kopke et al. isolated non-PHF hyperphosphorylated tau from AD brains (12). In their protocol, cortical gray matter was homogenized in buffer containing 20 mM Tris-HCl (pH 8.0), 0.32 M sucrose, 10 mM β-mercaptoethanol, 5 mM EGTA, 1 mM EDTA, 5 mM $MgSO_4$, and proteinase inhibitors. The homogenate was then subjected to differential centrifugation, and the fraction resulting from centrifugation between 27,000 and 200,000 × *g* was collected. This fraction was further extracted with 8 M urea to separate out the PHF-enriched pool. The supernatant contained abnormally phosphorylated non-PHF tau. These tau species were named AD P-tau and had a molecular weight of 67–70 kDa. They were three to fourfold more phosphorylated than tau extracted from control brains and could be detected by Tau1 antibody after alkaline phosphatase treatment. These highly phosphorylated AD P-tau proteins lost their normal MT assembly-promoting activity, which could be recovered upon dephosphorylation with alkaline phosphatase (19). Moreover, AD P-tau could sequester normal tau into filamentous tau aggregates, resulting in MT de-polymerization (20).

These studies suggest that a pool of intermediate pathological tau species exists and that this pool can be recovered in

buffer-soluble fractions. The physiological activity and function of these tau species is reduced compared to normal tau species due to hyperphosphorylation. Since the intracellular mobility dynamics of these intermediate tau species is much greater than that of condensed tau aggregates in NFTs, it is possible that intermediate tau species induce neuronal death and/or synaptic dysfunction. Therefore, the isolation and characterization of these tau species is paramount for understanding the pathogenesis of AD and for searching therapeutic methods.

TAU OLIGOMERS IN MOUSE MODELS OF TAUOPATHY

The first tau transgenic mouse model of frontotemporal dementia with Parkinsonism linked to tau on chromosome 17 (FTDP-17-Tau) was the JNPL3 line, which overexpresses P301L mutant 4R0N tau (21). The biochemical characterization of insoluble tau in these mice was done by a modified Greenberg and Davies method (21, 22). In this protocol, mouse brains were homogenized in buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, phosphatase inhibitors, and protease inhibitors. A pellet collected from 150,000 × *g* centrifugation was re-homogenized in high-salt/sucrose buffer [10 mM Tris-HCl (pH 7.4), 0.8 M NaCl, 10% sucrose, 1 mM EGTA, 1 mM PMSF] and centrifuged again at 150, 000 × *g*. The resulting supernatant was incubated with 1% sarkosyl, and then centrifuged at 150, 000 × *g*. The pellet was re-suspended in TBS as the sarkosyl-insoluble fraction. A 64-kDa tau predominantly existed in the sarkosyl-insoluble fraction; this tau was phosphorylated at multiple sites. Most notably, the amount of 64 kDa tau increased in an age-dependent manner, correlating well with the pathogenesis in JNPL3 mouse brain.

Noble et al. proposed a slightly different protocol for tissue homogenization using RIPA buffer without SDS (23). This modification allows for the study of both cytosolic and membrane-associated proteins involved in AD pathogenesis, such as amyloid precursor protein (APP), in the same extracts (24).

Another broadly used tauopathy mouse model is rTg4510 mice, which express repressible P301L mutant 4R0N tau and develop progressive age-related NFTs, neuronal loss, and behavioral impairment (5). Using a protocol similar to the one for JNPL3 mice with an additional 13,000 × *g* centrifugation as the first step, Berger et al. identified 140 and 170 kDa multimeric tau species in rTg4510 mouse brains (14). The 140 kDa tau was recovered in the supernatant fraction resulting from 150,000 × *g* centrifugation, while the 170 kDa tau was mostly in the sarkosyl-insoluble fraction. Both multimers were not affected by the presence or absence of reducing agent, indicating that the multimers are disulfide-bond independent. Importantly, the accumulation of 140 kDa tau coincided with the behavioral impairments of rTg4510 mice (14). Although this finding has had a huge impact on our understanding the neurotoxic mechanisms of tau oligomers, it is still unclear whether 140 and 170 kDa tau multimers can induce neuronal dysfunction. This is because these multimers comprise such a small proportion of the total tau pool (roughly < 0.1% of total tau, as estimated by Western blot signal). It should be noted that tau multimers with apparent molecular weights of ∼140 and ∼170 kDa are in fact tau dimers of ∼120 and ∼130 kDa, based on Bis-Tris or Tris-acetate SDS-PAGE migration (11, 25). This was further supported by mass spectrometry analysis of cross-linked tau dimers (26).

Most recently, we demonstrated that TBS-extractable 64 kDa tau species represents better the species involved in the progression of brain atrophy than does the sarkosyl-insoluble tau species (25). These 64 kDa tau species can be recovered in the supernatant following centrifugation of brain homogenates at 27,000 × *g* and further separation from normal tau by 150,000 × *g* centrifugation. TBS-extractable 64 kDa tau and normal tau are similar in thermo-stability but differ in other properties. Under non-reducing gel electrophoresis conditions, nearly all 64 kDa tau species are detected as dimers (∼130 kDa, according to size of molecular markers), whereas most normal tau proteins are detected as monomers. Immuno-electron microscopy revealed that the TBS-extractable 64 kDa tau enriched fraction contains tau-positive granules and filaments (25). This morphological finding was supported by MC1 immunoreactivity and Ab39 insensitivity (25). The MC1 antibody recognizes an early pathogenic conformation of tau (27), while the Ab39 antibody only detects mature tangles (28, 29). Overall, the characteristics of TBS-extractable 64 kDa tau are similar to AD P-tau from human brains.

IN VITRO TAU OLIGOMERIZATION

With tau assembly modeled *in vitro*, unphosphorylated recombinant tau can be polymerized by inducers such as heparin, heparan sulfate, polyunsaturated fatty acids, RNA, or quinones (30–34). Using the heparin-induced tau self-assembly method, we produced and isolated granular-shaped tau oligomers from soluble tau and filamentous tau by sucrose density gradient ultracentrifugation (10). These granular tau oligomers were morphologically defined by atomic force microscopy (AFM) to be 15–25 nm granules, and their molecular mass corresponded to about 40 tau molecules (10). Once formed, granular tau can continue to form filaments without any inducers in a concentration-dependent manner (10).

More recently, Lasagna-Reeves et al. proposed a method to prepare tau oligomers by using amyloid seeds (35). In their protocol, tau oligomerization can be induced in a relatively short period (1 h incubation at room temperature) after adding Aβ42 oligomers. After a total of three rounds of seeding procedures, Aβ42 seeds could be diluted to below the detection limit (35). Examination of these tau oligomers by transmission electron microscopy or AFM revealed a spherical morphology (35). This unique method of producing tau oligomers is a reasonable representative model supporting the amyloid hypothesis (36, 37), in which Aβ oligomers trigger NFT formation. Interestingly, tau oligomers, but not tau monomers or tau fibrils, can cause memory impairment in wild-type mice (13) and can decrease long-term potentiation in hippocampal brain slices (38).

The production of granular tau oligomers must be initiated by dimerization of tau monomers. Heparin-induced tau polymerization allows us to detect initial dimers because of the

relatively slower kinetics compared to arachidonic acid-induced tau polymerization (6, 39). It begins by increasing the formation of cysteine-dependent dimers, which occur prior to the detection of thioflavin T (ThT) binding (11). The kinetics of tau polymerization is dependent on oxidative/reducing state. Higher-order oligomers and aggregates assemble more rapidly in the absence of the reducing agent dithiothreitol (DTT) (11). However, cysless-tau (4R tau with both C291A and C322A mutations) forms dimers, which eventually aggregate into fibrils after 24 h incubation with heparin, suggesting that tau aggregation occurs without disulfide-bond formation (11).

Two distinct tau dimers (i.e., cysteine-dependent and cysteine-independent dimers) have been identified in tauopathy mouse models, including JNPL3 mice (11) and rTg4510 mice (14, 25). These dimers have also been shown in cell cultures (11, 40). Dimer formation is an essential step for their further assembly into higher-order oligomers. Although these dimers themselves may not exist in a steady state, it is important to detect the initial step of tau dimerization.

GENERATION OF TAU OLIGOMER-SPECIFIC ANTIBODIES

A monoclonal antibody that selectively recognizes tau dimers and higher-order oligomers has been generated by Binder's group (26). This antibody, named tau oligomeric complex 1 (TOC1), was made against benzophenone-4-maleimide cross-linked recombinant tau dimers (26). Immunogold labeling and dot-blot analysis of aggregated recombinant tau revealed that TOC1 selectively labels tau dimers or oligomers but not filaments (26). Based on their preliminary mapping of the TOC1 epitope, the proline-rich region (Gly155-Gln244) and the C-terminal portion (Leu376-Ser421) were identified as potential binding segments for forming cross-linked tau dimer (26). Since these two regions are on the opposite sides of the MT-binding domains, Patterson et al. advanced the idea of the formation of an antiparallel dimer (26).

TOC1 immunoreactivity is selectively detectable in the early stage of AD pathogenesis; however, TOC1 antibody fails to label mature tangles in AD brains (26). In rTg4510 mice, TOC1 immunoreactivity was observed in the TBS-extractable 64 kDa tau enriched fraction and linked to early pathological changes (Sahara, in preparation). Notably, the immunohistochemical staining pattern of TOC1 antibody was clearly different from those of MC1 and Ab39 antibodies (**Figure 1**). Since NFTs themselves might be protective [reviewed in Ref. (41)], other harmful tau species such as tau oligomers are currently of particular interest. If TOC1 antibody selectively interacts with tau dimers and higher-order oligomers but not tau filaments, and if those species cause neurotoxicity, this antibody can be a useful tool to track the pathway of tau neurotoxicity.

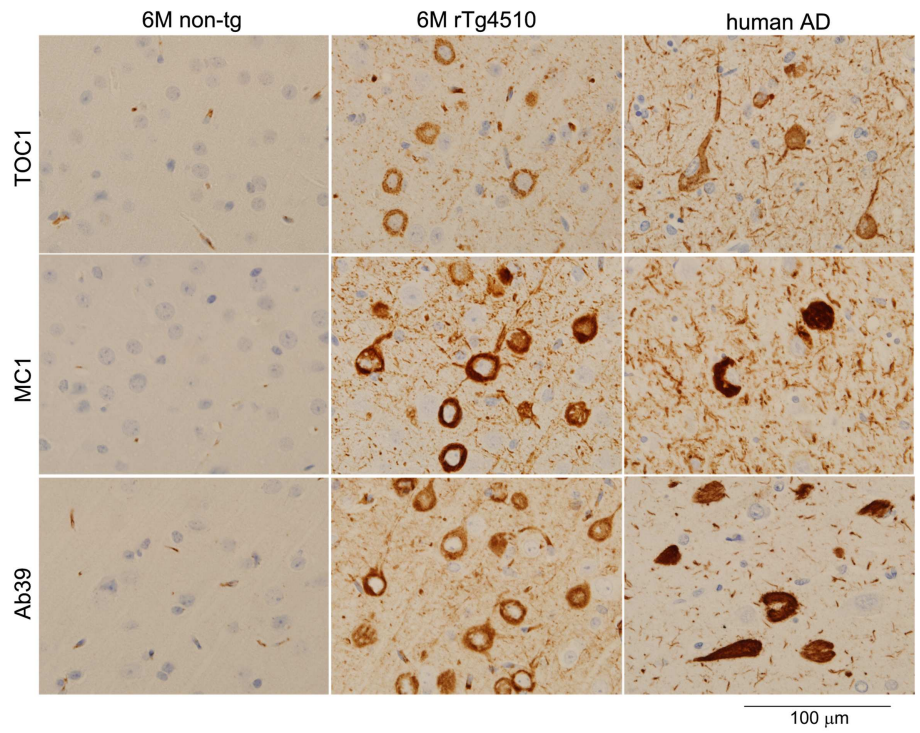

FIGURE 1 | Light microscopic images of immunostained brain sections from a non-transgenic mouse, a rTg4510 mouse, and human with AD. Formalin-fixed paraffin sections were stained with TOC1 (1:2500), MC1 (1:1000), and Ab39 (1:250) antibodies by the Dako Universal Autostainer (Dako, Carpinteria, CA, USA). The sections were then counterstained with hematoxylin. TOC1 diffusely stained cytoplasmic regions of neurons (top panels, rTg4510 and AD brains), while MC1 and Ab39 densely stained these neurons (middle and bottom panels, respectively). This difference in staining pattern is due to the specific binding of TOC1 antibody to premature tau aggregates. Scale Bar, 100 μm.

Table 1 | Summary of brain-derived tau oligomer preparation methods.

Product	Reference	Material origin	Detect method	Oligomer properties
AD P-tau	Kopke et al. (12), Alonso et al. (20), Alonso et al. (44)	AD patients	WB, EM	AD P-tau is isolated from the 27 K–200 K × *g* fraction, soluble in urea, hyperphosphorylated, no ubiquitin immunoreactivity, self-assembly into filaments, sequesters N-tau
140 and 170 kDa tau	Berger et al. (14)	rTg4510 mice JNPL3 mice	WB, SEC	Disulfide-bond independent, correlates with memory loss, 140 kDa tau is not hyperphosphorylated, 170 kDa tau is hyperphosphorylated, and has strong immunoreactivity with AT8
TBS-extractable 64 kDa tau	Sahara et al. (25)	rTg4510 mice	WB, IHC, EM	TBS-extractable 64 kDa tau is isolated from 27 K to 150 K × *g* fraction, thermo-stable, hyperphosphorylated, mostly disulfide-bond-dependent, correlates with brain atrophy, contains tau-positive granules/short filaments
T22-positive tau	Lasagna-Reeves et al. (38), Lasagna-Reeves et al. (42)	AD patients	WB, IHC, SEC, AFM	Hyperphosphorylated, not ubiquitinated at pretangle stage, contains oligomers with 4–8 nm diameters, propagate abnormal tau conformation of endogenous murine tau

WB, Western blot; EM, electronic microscopy; IHC, immunohistochemistry; SEC, size-exclusion chromatography; AFM, atomic force microscopy.

Another tau oligomer-specific antibody, T22, was generated by Kayed's group (42) against antigenic tau prepared from Aβ seeding of tau oligomers (35). The specificity of this antibody was confirmed by ELISA and dot blotting. It detects only tau oligomers but not tau monomers or PHF fibrils prepared by the heparin-induced tau polymerization method (42). Immunohistochemically, this antibody selectively stains pretangles, neuritic plaques, and neuropil threads but not ghost tangles in AD brain sections (42). Western blotting showed that T22 antibody recognizes higher-molecular-weight tau species (e.g., dimers, trimers, and tetramers) but not monomers (42). It should be noted that the SDS-PAGE samples were not denatured by boiling before running on gels and that T22 immunoreactivity was diminished by denaturing agents such as 8 M urea (42).

These novel tau oligomer-specific antibodies provide a new method to diagnose the early pathological changes that occur in tauopathy. It would be extremely useful to develop methods employing cerebrospinal fluid biomarkers combined with total tau, phosphorylated tau, tau oligomers, and other biomarker measurements to differentially diagnose dementias, such as AD, frontotemporal lobar degeneration, progressive supranuclear palsy, corticobasal degeneration, dementia with Lewy bodies, vascular dementia, and prion disease.

CONCLUSION

Abnormal tau aggregation is considered to be a critical pathological feature of tauopathy. However, the initial molecular event of tau pathogenesis is yet unclear. The hyperphosphorylation of tau is strongly suggested to be directly correlated with the severity of AD pathology (43). Iqbal and colleagues demonstrated that hyperphosphorylated tau extracted from AD brain reduces MT stabilization, sequesters normal tau from MT, and aggregates themselves in the absence of inducer molecules (20, 44). Many studies attempting to identify tau oligomers have demonstrated the existence of hyperphosphorylated tau oligomers in human and transgenic mouse brains (e.g., AD P-tau, 140 and 170 kDa tau multimers, TBS-extractable 64 kDa tau, and T22 antibody-positive tau oligomers) (**Table 1**). Thus, hyperphosphorylation of tau could be the initial event of NFT formation. However, the amount of 140 kDa tau multimer (normal tau dimer) correlates well with behavioral deficits (14), suggesting that hyperphosphorylated tau oligomers may not be essential for neurotoxicity. If antibodies can be generated to recognize non-phosphorylated and hyperphosphorylated tau dimers independently, we will be able to better identify toxic tau species and optimize potential oligomerization inhibitors as possible novel therapies. Standardized isolation methods of tau oligomers are in need to improve consistency between researchers.

In summary, accumulating evidence from biochemistry, immunology, and molecular imaging reveal the existence of tau oligomers as mainly buffer-soluble, non-filamentous, granular-shaped conformers. The neurotoxicity of these oligomers has been confirmed in both *in vitro* and *in vivo* experiments (4, 5, 14, 42, 45). The next step of tau oligomer research should investigate whether tau dimers and/or non-granular oligomers exist and functionally correspond to neuronal dysfunction.

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Formation and propagation of tau oligomeric seeds

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Tau misfolding and aggregation leads to the formation of neurofibrillary tangles (NFTs), which have long been considered one of the main pathological hallmarks for numerous neurodegenerative diseases known as tauopathies, including Alzheimer's Disease (AD) and Parkinson's Disease (PD). However, recent studies completed both *in vitro* and *in vivo* suggest that intermediate forms of tau, known as tau oligomers, between the monomeric form and NFTs are the true toxic species in disease and the best targets for anti-tau therapies. However, the exact mechanism by which the spread of pathology occurs is unknown. Evidence suggests that tau oligomers may act as templates for the misfolding of native tau, thereby seeding the spread of the toxic forms of the protein. Recently, researchers have reported the ability of tau oligomers to enter and exit cells, propagating from disease-affected regions to unaffected areas. While the mechanism by which the spreading of misfolded tau occurs has yet to be elucidated, there are a few different models which have been proposed, including cell membrane stress and pore-formation, endocytosis and exocytosis, and non-traditional secretion of protein not enclosed by a membrane. Coming to an understanding of how toxic tau species seed and spread through the brain will be crucial to finding effective treatments for neurodegenerative tauopathies.

Keywords: tauopathies, tau oligomers, oligomeric seeding, propagation of tau pathology, Alzheimer's

TAU OLIGOMERS ARE THE TOXIC TAU SPECIES IN NEURODEGENERATIVE TAUOPATHIES

The formation of tau aggregates and neurofibrillary tangles (NFTs) is one of the main pathological hallmarks of numerous diseases termed tauopathies, including the two most common neurodegenerative diseases, Alzheimer's Disease (AD) and Parkinson's Disease (PD) (1, 2). However, it is evident that cell death occurs initially prior to NFT formation in AD (3–6) suggesting that NFTs are not the pathogenic species responsible for the spread of the disease. Recent evidence points to the presence of multimeric tau species which are intermediates between tau monomers and NFT – known as tau oligomers – as the toxic species inducing synaptic dysfunction and cell death in neurodegenerative tauopathies (7–12).

Numerous researchers have investigated tau pathology using animal models, yielding a better understanding of the toxicity of different tau structures. A study in aged mice expressing native human tau (htau mice) found that while NFT formation occurred as animals aged, there was no correlation between the presence of tau filaments and cell death (13). Additionally, a study examining the P301S mouse model, which expresses mutant human tau, found that hippocampal synaptic dysfunction occurred prior to NFT formation (14). Studies using the rTg4510 mouse model, which conditionally expresses P301L mutant tau, found that cell death occurred prior to NFT formation and that cell loss and behavioral impairments could be suppressed by inhibiting tau expression without removing NFTs or preventing their continued accumulation (7, 15). In accordance with this finding, it has been shown that NFTs are protective in the same mouse model (16), and only pro-aggregate human tau mice (TauRD) show behavioral deficits (17). Another study in the same mouse model characterized tau oligomers biochemically that appeared early and correlated with cognitive deficits (8, 12). Similar results have also been seen in *Drosophila* AD models, where expression of mutant tau causes neurodegeneration, synaptic dysfunction, and axonal transport deficiencies in the absence of NFTs (18, 19). Usage of the protein nicotinamide mononucleotide (NAD) adenylyl transferase (NMNAT) was shown to decrease behavioral and morphological deficiencies in a frontotemporal dementia *Drosophila* model by decreasing levels of tau oligomers (20).

Biochemical analysis of human AD brain tissue has also yielded results suggesting that tau oligomers may initiate toxicity, rather than NFTs. When compared to control brains, levels of tau oligomers were found to be significantly increased in AD brains early in the disease, prior to when NFTs appear and clinical symptoms are evident (9, 21–23). In addition to correlative evidence for the importance of tau oligomers to toxicity, treatment with tau oligomers has also been shown to cause adverse effects in animals. Isolated tau oligomers, but not monomers or NFTs, induced memory impairments, synaptic dysfunction, and mitochondrial dysfunction when given intracerebrally to wild-type mice (24). Therefore, it is possible that NFTs are actually neuroprotective, sequestering toxic forms of tau into large aggregates with less flexibility and surface area to interact with cells. All of these studies form the framework for the model of the progression of neurodegenerative tauopathies beginning with the seeding and propagation of toxic tau oligomers (**Figure 1**).

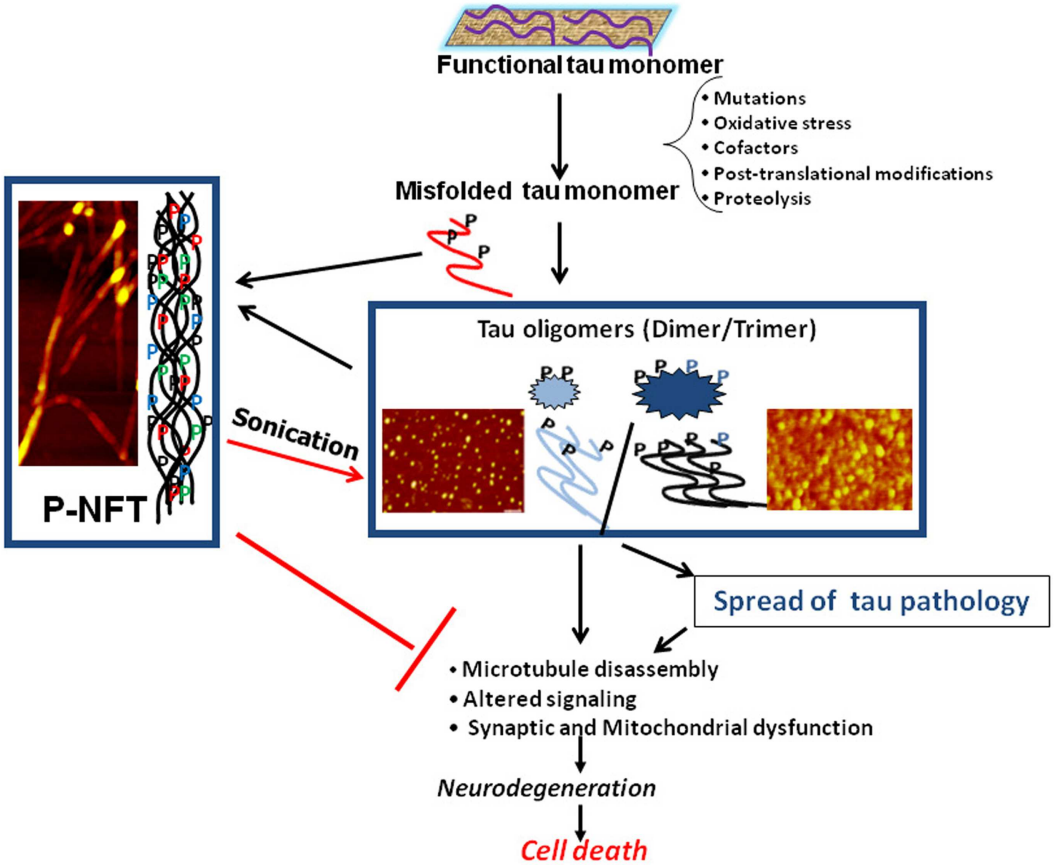

FIGURE 1 | Schematic illustrating the central role of tau oligomers in tauopathies. Tau intermediate soluble aggregates (tau oligomers) are the toxic tau entities and initiators of tau pathology and propagation in tauopathies, rather than monomeric tau or hyperphosphorylated NFTs (p-NFTs). Sonication of fibrillar tau generates toxic tau oligomers. Thus, tau oligomers represent the ideal target for anti-tau therapeutic approaches. AFM images are of brain-derived tau oligomers and NFT (72, 138).

TAU OLIGOMERS ARE SEEDS FOR THE PROPAGATION OF PATHOLOGICAL TAU

Recently, researchers have begun to make comparisons between the spread of neurodegenerative disease and prion disease, as studies suggest that misfolded protein templating, known as seeding, may underlie the progression of disease (25). Understanding how tau seeds pathological forms of the protein which propagate to different brain regions is critical to devising a solution to stop the spread of disease. There are two main models for the formation and seeding of pathological tau, oligomer-nucleated conformational induction – based upon the mechanism of action of prion protein, Sup35 (26) and amyloid β (Aβ) (27) – and template-assisted growth. Template-assisted growth proposes that tau fibrils act as template molecules for unfolded monomers. When monomers come in contact with filaments, they are integrated into the filament in organized, parallel stacked β sheets, optimizing hydrogen bonding for stabilization (28). It has been difficult, however, to find spontaneous tau aggregation which occurs experimentally. When fibrils are cleaved, leaving only three microtubule binding repeats, the fragments aggregate spontaneously *in vitro* (29). However, on its own, tau will not polymerize *in vitro* without the addition of certain reagents, post-translational modifications, such as phosphorylation, or induction of mutations.

In order for aggregation to occur, tau must be released from microtubules to reach a high concentration of free cytosolic tau, conformational changes must occur to allow for aggregation, possibly by increasing β sheet content, and dimerization must occur (30, 31). The addition of polyanions, such as heparin or RNA can induce fibrillization of tau (32), causing a conformational change from random coil structure to β sheet structure (33). Free fatty acids, such as arachidonic acid can also increase aggregation (34, 35) due to the presence of an alkyl chain, which induces micellization, and a negatively charged head group on the fatty acid to create a negatively charged surface on the micelle. In the presence of tau, the critical concentration for micelle formation is greatly decreased, allowing anionic micelles to attract tau to the negatively charged surface and thereby compensate for positive charges in tau and enable tau aggregation (36, 37). Phosphorylation may also play a role in fibrillization. Paired helical filaments (PHF) and straight filaments (SF), which make up the NFTs found in the brains of patients with AD, are comprised of hyperphosphorylated tau (38, 39). Phosphorylated tau has a higher tendency toward aggregation

than unphosphorylated tau and kinases involved in the phosphorylation of these sites in tau have been shown to be altered in AD (40). Hyperphosphorylated tau has been shown to aggregate *in vitro,* possibly due to the addition of negative charge which would increase aggregation, similarly to the addition of polyanions and free fatty acids. Furthermore, this process can be inhibited by dephosphorylation (41, 42). Phosphorylation may also induce aggregation by reducing the interaction of tau with microtubules and allowing it to interact instead with unphosphorylated tau and form aggregates (43–45). Mutations, such as those that lead to frontotemporal lobar degeneration (FTLD), can increase tau aggregation through different mechanisms. Many mutations lead to a decrease in microtubule assembly kinetics, which could lead to more free cytosolic tau and increase aggregation (46–49). Some mutations lead to a decrease in the dissociation constants (K_d) for dimer and filament formation, while others increase the rate of nucleation without affecting K_d (30). Mutations that cause increased formation of β-sheets lead to heightened aggregation due to an increase in hydrophobic interactions, deviating from the highly hydrophilic native tau protein (50, 51). Fibrillar tau can thereby be recognized by dyes which interact with β-sheets, such as Congo Red and thioflavin S (52). However, at high concentrations, these dyes can induce fibrillization due to an attraction between positive charges formed in the core of PHFs and negative charges of the anionic dyes (53–55).

In amyloid proteins in which seeding has been well-established, such as prion proteins and Aβ, oligomers have been shown to be the most potent seeds (56, 57), working by way of oligomer-nucleated conformational induction (26, 27). Due to the increased interest in the toxicity of tau oligomers, evidence has emerged in support of the oligomer-nucleated conformational induction model as more studies have begun to explore the importance of tau oligomers in the initialization of tauopathies. Oligomer-nucleated conformational induction entails oligomers or conformational changes irreversibly stabilizing the highest energy protein states, known as the nucleus, allowing stable monomers to aggregate into oligomeric structures. Oligomers are driven to further elongation to form lower energy, stable filaments (58). As opposed to template-assisted growth, monomers are not incorporated directly into fibrils, but are instead entirely aggregated into oligomers prior to filament formation (59). Tau dimerization increases the tendency to aggregate and can be induced by oxidation (60), which suggests that tau oligomerization may be an important step in the fibrillization pathway. The appearance of oligomeric species of other amyloid proteins has been observed on the path to fibril formation (61–63).

While the addition of reagents and mutations used to induce fibrillization has been integral to understanding how tau aggregation occurs, it does not explain how fibril formation may occur spontaneously in sporadic disease. The mechanism by which tau aggregation occurs physiologically has not yet been elucidated, however there have been some advances in the understanding of how certain steps in the process may occur. Release of tau from microtubules may occur following post-translational modifications, such as phosphorylation (43, 44). Localization to anionic surfaces, alternative splicing, and post-translational modifications stabilizing aggregated conformations may all act as enhancers to increase speed of nucleation (64). Under physiological conditions, nucleating cofactors can induce tau aggregation in a similar fashion to agents used *in vitro*. There is evidence that polyanionic species, such as tubulin, RNA, and α-synuclein can increase the tendency of tau to aggregate (65–67) The formation of disulfide bridges is critical for the initial creation of dimers from monomers, as well as intermolecular crosslinking of the microtubule binding domain independent of cysteine to continue oligomerization of three-repeat tau (68). Prior to monomer aggregation into oligomers, the free energy of solvation decreases, causing a shift in preference for peptide-solvent interactions toward peptide–peptide interactions, as water is evacuated due to poor interaction with the peptide backbone and sidechains. Water release increases entropy of the solvent, thereby balancing the loss in conformational entropy caused by aggregation. The interaction of side chains with the backbone in the form of hydrogen bonding leads to the creation of β-sheet structure and aggregate stabilization. While oligomers form a similar structure to fibrils, they are not as ordered, which likely increases their toxicity (69). Proteolytic processing by endogenous proteases has also been shown to create self-aggregating fragments, which nucleate and co-aggregate with full-length protein effectively enough for a small amount of fragment to seed PHFs (70). Direct interactions between misfolded tau and native protein may be the underlying mechanism of seeding as experiments have shown tau protein–protein interactions occur when tau aggregates enter cells containing native tau (71).

Tau oligomers – identified with the tau oligomer-specific antibody, T22, which does not recognize monomers or fibrils (59) – which have been seeded with oligomers derived from brain tissue have been shown to be highly toxic (23, 72). When tested with Bis-ANS, which recognizes exposed hydrophobic patches, oligomers had higher affinity for Bis-ANS than PHFs, which may underlie toxicity. The toxic effects of tau oligomers formed by seeding recombinant tau with oligomeric seeds, however, can be prevented when pre-treated with T22 (23, 72).

Some tauopathies, such as progressive supranuclear palsy (PSP), only have one pathogenic species involved in disease progression (1). However, most tauopathies contain other amyloid proteins in addition to tau, such as Aβ in AD and α-synuclein in PD. In such diseases, cross-seeding of heterologous protein species is an additional mechanism which is important for tau seeding (59, 67, 73–77).

TAU OLIGOMERS PROPAGATE FROM AFFECTED BRAIN REGIONS TO UNAFFECTED REGIONS

Aβ has been shown to propagate from affected brain areas to unaffected areas in mice over-expressing Aβ precursor protein that have been injected with Aβ isolated from the brains of AD patients and AD transgenic mice (78, 79), suggesting that perhaps tau could spread in a similar fashion. A few years later, a similar mechanism was demonstrated for the propagation of tau. When tau extracted from P301S mice was injected into the brains of mice over-expressing wild-type human tau (ALZ17 mice), which do not form tau aggregates, tau pathology was observed to have spread from the injection site to neighboring brain regions (80). Additionally, in transgenic mice which differentially express pathological tau in the entorhinal cortex, where tau pathology is first

observed in AD, human tau has been shown to spread to both neighboring and synaptically connected neurons which do not express human tau mRNA. Translocated human tau was able to seed mouse tau misfolding (81, 82). However, these studies did not specifically investigate which tau species specifically induced seeding and propagation of tau pathology and the usage of transgenic mouse models is not analogous to sporadic forms of AD. When wild-type mice were injected with both tau oligomers and PHFs isolated from AD brains, tau oligomers induced the spread of tau pathology from the injection site to neighboring brain regions and impaired memory, as measured by object recognition. Conversely, mice injected with PHFs only exhibited tau pathology near the injection site and did not exhibit any memory impairments on the behavioral task, suggesting that tau oligomers, but not fibrillar tau, is capable of seeding and propagating pathology (72). Furthermore, similar results have been found using primary neurons. Neurons were exposed to low molecular weight aggregates – recognized by the tau oligomer-specific antibody, T22, and examined via electron microscopy for oligomeric characteristics – as well as to fibrils formed *in vitro*, filaments formed *in vivo*, and monomers. Low molecular weight aggregates and short fibrils exhibited uptake into the cell, but monomers and filaments were not internalized (83). Other studies have shown tau aggregate uptake using cell culture, but did not specifically identify the type of aggregates being internalized. Neural stem cells treated with tau monomers and aggregates formed using the tau microtubule binding repeat region induced to fibrillize with arachidonic acid, exhibited significantly more tau aggregate uptake than monomer uptake. Additionally, aggregates, but not monomers, induced seeding of endogenous tau misfolding (84).

On the other hand, Guo and Lee hypothesized that seeding of pathological tau in cultured cells would be able to occur more quickly by seeding with pre-formed tau fibrils, thereby omitting the step where monomer must be converted to oligomer prior to fibril formation. Fibrillization of recombinant tau was induced with the addition of heparin and was verified using thioflavin T. Fibril-treated cells exhibited seeding and propagation of aggregates via endocytosis. However, following fibril confirmation with thioflavin T and prior to cell treatment, fibrils used in this study were sonicated (85). Previous research investigating Aβ seeding found that sonication increased seeding ability by fragmenting fibrils into smaller, soluble species (57) and sonicated prions have also been shown to have more potent seeding potentials than unsonicated fibrils (56). Since it has been shown that both prion and Aβ oligomers, rather than fibrils, are the seeds for pathological protein templating (26, 27), it is likely that sonication partially converts insoluble fibrils into soluble oligomeric forms. Sonication of tau fibrils has also been shown to cause shearing of filaments, particularly those in PHF form (86). Therefore, it is likely that sonicated tau fibrils used to treat cells in the previous study (85) also contained tau in oligomeric form, which may explain why seeding and propagation was successful.

Recently, Wu et al. studied propagation of tau in primary neurons using microfluidic chambers which allow somatodendritic compartments to be isolated from axonal compartments, enabling not only the analysis of tau uptake from the extracellular space into the cell, but also propagation within the neuron. They found that low molecular weight tau aggregates specifically recognized by tau oligomer-specific antibody, T22, propagate between isolated neuronal compartments both anterogradely and retrogradely (83). Importantly, tau is primarily found in the axons of healthy neurons (87), though tau may also be found in the dendrite where it colocalizes with the src kinase, fyn (88, 89). In AD, however, misfolded and hyperphosphorylated tau accumulates in the axon, dendrites, and the cell body (90), suggesting that intracellular transport may also be important for the spread of disease. In lamprey neurons expressing low levels of tau, tau was primarily localized to the axon and proximal dendrites, both regions consistent with tau functioning as a microtubule-associated protein. However, in neurons expressing high levels of tau, tau was found in distal dendrites and near the soma membrane, both areas lacking microtubules. High-expressing tau cells showed more degeneration and secretion of tau. Moreover, as tau can modulate activity of microtubule-associated motor proteins involved in dendritic transport, tau localized to the dendrite may have implications for its propagation (91). Phosphorylated tau localized at the synapse in AD brain samples appears to correlate with ubiquitin-proteasome system (UPS) dysfunction, suggesting that tau oligomer accumulation at the synapse impairs the UPS, which is a crucial player in the breakdown of tau. Accumulation of tau at the synapse may also suggest a mechanism for trans-synaptic tau propagation (92).

Phosphorylation clearly plays a role in the toxicity and localization of tau, however, its exact role in neurodegenerative disease is unknown and appears to be quite complicated. While hyperphosphorylated tau has been shown to have toxic results in the cell, increasing aggregation and abnormal tau localization (40, 90), dephosphorylated tau can also have harmful effects. Phosphorylated tau released into the medium of cultured neuroblastoma cells through muscarinic receptor activation that is dephosphorylated by tissue-non-specific alkaline phosphatase (TNAP) led to excitotoxicity, increasing calcium levels in nearby cells. Additionally, levels of TNAP are heightened in AD brains compared to control brains (93). Another study of primary cortical neurons also found that extracellular tau is largely dephosphorylated (94). Conversely, one study found that phosphorylation of tau increased its secretion from HeLa cells (95). Inflammation and activation of microglia has been shown to increase tau phosphorylation as well as aggregation, but is complicated by the fact that the opposite effect is seen in Aβ (96–99). The localization of tau in the cytosol, cell membrane, and the nucleus also appears to be important for tau toxicity, and is mediated by phosphorylation. Oxidative stress and heat shock induce the dephosphorylation of cytosolic tau and its transport into the nucleus. Once relocated to the nucleus, tau appears to protect neuronal DNA from damage under cell stress (100), which may be important in AD where DNA damage has been shown to occur (101). One possibility is that abnormal phosphorylation of tau in AD may prevent tau from being dephosphorylated and translocated to the nucleus to protect against DNA damage. The localization of tau to the cell membrane may also depend upon its dephosphorylation, as tau with lower levels of phosphorylation in its proline region was shown to be associated with the cell membrane, while phosphorylated tau was found in the cytoplasm (102, 103). However, interaction with membrane bound proteins, such

as aforementioned fyn may stabilize association of phosphorylated tau with the membrane.

MECHANISM OF TAU PROPAGATION

The entry of prion proteins, Aβ, and other amyloid proteins into the cell via different mechanisms has been well-established (104–107). One hypothesis for amyloid oligomer toxicity and entrance into cells is through protein interaction with the cell membrane. One model suggests that oligomers embed themselves into the cell membrane and form pores. However, it appears as though the formation of pore-like annular protofibrils occurs through a separate pathway from fibril formation (108). An alternative model suggests that oligomers interact with lipid rafts in the bilayer, causing membrane thinning and increased membrane permeabilization, which may play a role in oligomer toxicity, allowing non-specific ion entrance, as well as leakage of cellular compartments. Several types of amyloid oligomers have been shown to increase membrane permeability, including Aβ, α-synuclein, and prion protein (107, 109–111). Tau has been reported to interact with the lipid rafts in the cell membrane and undergo conformational changes leading to membrane stress (112–115). Additionally, permeabilization of the membrane could mediate internalization of oligomers into the cell.

There has also been evidence for endocytosis as a route of amyloid entry into the cell. Propagation of α-synuclein, prion protein, Sup35, and Aβ has been shown to be associated with the endosomal pathway (116–118). One study found that tau aggregates colocalize with dextran in neural stem cells, implying that entry into the cell occurs via macropinocytosis. However, the aggregate type was not specifically tested (84). Aggregates identified specifically as tau oligomers colocalized with fluid-phase endocytosis marker, dextran, as well as with early endosomal marker, Rab5, and late endosomal/lysosomal marker, Lamp1. When endocytosis was inhibited with dynamin inhibitor, Dynasore, tau uptake was blocked, while inhibition of clathrin-mediated endocytosis with Pitstop2B did not impact internalization (83). These studies together suggest a mechanism for tau propagation in which tau is internalized via pinocytosis and enters the endosomal pathway. Tau can move through the endosomal pathway to the lysosome where toxic species may be degraded or recycled back to the cell membrane, where they may be released to be internalized by adjacent neurons. More research is needed to determine how membrane-enclosed tau oligomers are released inside of the cell, though it appears likely that the majority are degraded in the lysosome, while those that avoid degradation may cause the endosomal membrane to burst and be released in the cytoplasm, where they can seed aggregation of healthy tau (83). While clathrin-mediated endocytosis did not appear to be involved in tau propagation, endocytosis inhibitors are often found to be non-specific (119), and therefore, the possibility of other types of endocytosis in tau spread bears more study.

Receptor-mediated endocytosis could be another route of entry into the cell as amyloids have been reported to bind to cell surface receptors. Internalization of α-synuclein has been shown to be dependent upon receptor-mediated endocytosis, potentially through caveolin-mediated endocytosis (117). Additionally, Aβ binds to NMDA, α7 nicotinic acetylcholine, and APOE receptors, inducing receptor endocytosis (120–124). Aβ oligomers also bind cellular prion protein, PrP^c, which is complexed with the Src tyrosine kinase, Fyn. This interaction has been shown to increase tau dysfunction and prevents native tau from binding to fyn (125). Under normal conditions, tau binds to fyn in oligodendrocytes (126) and in neurons, activating the Ras/MAPK pathway (103). Mutations to the microtubule binding region in tau lead to decreases in oligodendrocyte process number and length and disease-related missense mutations increase tau association with Fyn (127). Results indicate that the interaction between tau and fyn may be important for neurodegeneration, both through a loss in native tau interaction and through a gain in toxic tau function. Interaction with PrP^c complexed to fyn could also mediate tau entry into the cell as the PrP^c complex is associated with and endocytosed with caveolin (128). Tau may also enter the cell through a direct interaction with fyn. Lee et al. used a lamprey ABC tauopathy model in which tau is expressed in specifically identified ABC neurons to investigate the spread of tau. They report that tau phosphorylated at Y18, the site most commonly phosphorylated by fyn kinase, is associated with vesicular organelles. Additionally, when tau is overexpressed and localizes to the dendrite, dendritic vesicle accumulation is observed. Phosphorylated tau is colocalized with vesicles which bear resemblance to endosomes, as well as to fyn. Fyn has also been shown to colocalize with exosomes, suggesting a possible mechanism for fyn-tau transport in which fyn-associated tau is endocytosed, transported from early endosomes to late endosomal compartments, and then transported out of the cell via exosomes (91).

While one mechanism for tau oligomer release is through oligomer toxicity leading to cell death, causing the cell to lyse and release its contents (129), studies show that this likely does not account for the majority of tau release. In primary neurons treated with tau oligomers, extracellular tau only increases once levels as high as 40% cell death are reached, which does not correspond to physiological levels of cell death during the initial spread of neurodegenerative disease (130). Additionally, treatment with tau oligomers in primary neurons does not lead to significant levels of apoptosis (83). There has however been some evidence for non-apoptotic membrane blebbing as a possible secondary mechanism for tau release (91, 131).

Exocytosis has been implicated as a mechanism of amyloid spread as prion proteins and α-synuclein have been shown to be associated with exosomes in cell culture (132, 133). However, investigations of a similar mechanism for tau release have been unclear. Simón et al. found that when tau was overexpressed in kidney-derived cell lines, tau was secreted contained within membrane vesicles (129, 134). While tau secreted by neuroblastoma cells and tau in human CSF was found to be associated with exosomes in one study (135), another reported that tau was not detected in isolated exosomes from neuroblastoma cells (130). However, these studies used cell models where tau was overexpressed. In an attempt to approach more similar conditions to those seen physiologically, researchers cultured primary cortical neurons containing endogenous tau and found that tau was released by a mechanism unrelated to cell death and was regulated by AMPA receptor activation. Inhibition of synaptic vesicle release decreased extracellular tau, while tau was not found to be

associated with exosomes, indicating that release of tau through traditional synaptic exocytosis following AMPA receptor activation may be one mechanism of tau release (94). Another study found that cells constitutively release tau which is not contained within a membrane under conditions inhibiting cell death (136). Therefore, more research is warranted to investigate the conditions under which tau is associated with exosomes and the specific tau conformations found in exosomes. Tunneling nanotubes – long, temporary channels that allow for long-distance transport between cells – have recently been discovered as a transport mechanism for prion protein (137). While they have not yet been studied directly in the context of tau, similarities between the spread of prions and tau suggest that tunneling nanotubes may be another potential mode of tau propagation meriting study.

CONCLUSION

Determining how neurodegenerative tauopathies initiate and propagate toxic species will be crucial to finding a treatment for these diseases. Recent evidence suggests that tau oligomers, not NFTs, are the toxic tau species mediating the initiation, seeding, and propagation of neurodegenerative tauopathies and are the best target for anti-tau therapeutics. The mechanism by which tau seeding occurs remains to be elucidated, but oligomer-nucleated conformational induction, whereby native tau monomers are entirely converted to oligomers prior to aggregation into fibrils, appears to be a likely model. Tau oligomers can effectively enter cells, be transported intracellularly, and be released from cells to affect others. However, the mechanism by which propagation occurs is unclear. Tau likely enters the cell in one of two main ways, stressing the cell membrane or entering via endocytosis. Entrance through interaction with the membrane may occur through formation of pores or by interacting with lipid rafts causing membrane stress. Both macropinocytosis and receptor-mediated endocytosis have been implicated as possible mechanisms for tau entry. Tau secretion is likely not due simply to cell death, but may occur within exosomes, through synaptic vesicle release, or a non-traditional secretion pathway in which tau is not enclosed in a membrane. The elucidation of the mechanisms addressed will lead to a better understanding of neurodegenerative disease and may reveal new targets for treatment.

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Tau oligomers as potential targets for Alzheimer's diagnosis and novel drugs

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A cumulative number of approaches have been carried out to elucidate the pathogenesis of Alzheimer's disease (AD). Tangles formation has been identified as a major event involved in the neurodegenerative process, due to the conversion of either soluble peptides or oligomers into insoluble filaments. Most of recent studies share in common the observation that formation of tau oligomers and the subsequent pathological filaments arrays is a critical step in AD etiopathogenesis. Oligomeric tau species appear to be toxic for neuronal cells, and therefore appear as an appropriate target for the design of molecules that may control morphological and functional alterations leading to cognitive impairment. Thus, current therapeutic strategies are aimed at three major types of molecules: (1) inhibitors of protein kinases and phosphatases that modify tau and that may control neuronal degeneration, (2) methylene blue, and (3) natural phytocomplexes and polyphenolics compounds able to either inhibit the formation of tau filaments or disaggregate them. Only a few polyphenolic molecules have emerged to prevent tau aggregation. In this context, fulvic acid (FA), a humic substance, has potential protective activity cognitive impairment. In fact, formation of paired helical filaments *in vitro*, is inhibited by FA affecting the length of fibrils and their morphology.

Keywords: tau oligomers, PHFs, Alzheimer's disease, tauopathies, diagnosis and treatment

INTRODUCTION

Tau protein, a member of the microtubule-associated protein (MAPs) family, plays a fundamental role in the assembly and stabilization of microtubules, as well as on axonal transport and neurite outgrowth (1–3). In this context, tau protein plays an important role in the maintenance of neuronal polarity and in the stabilization of the morphology of differentiated neurons. In developing neurons, tau activity is crucial for the morphogenesis of the growth cones and, it has been suggested to play a key role in promoting axonal growth (4). Tau is encoded by a single gene located on chromosome 17 (17q21), possessing 16 exons in its primary transcript. Six different isoforms are expressed by post transcriptional modifications (alternative splicing) from the primary transcript. Mature protein length is about 352 up to 441 amino acid residues, and a molecular weights of 45–65 kDa depending on the tau isoforms (5, 6). The C-terminal region has a domain containing the microtubule-binding repeats, which is critical for microtubule assembly (2), whereas the affinity of tau for microtubules is finely regulated by an orchestrated set of phosphorylations. The motif KXGS is one of several different motifs located within these repeats susceptible to be phosphorylated (7). In turn, tau is characterized as an hydrophilic cationic protein, unfolded under native conditions, and with a low ordered secondary structure (8). In addition to roles in stabilization of microtubules, tau plays a major role in bridging the different cytoskeletal structures. Thus, besides microtubules, tau interacts with actin and intermediate filaments (2, 9).

Tau is located primarily in neurons, however, traces of this protein have been found in certain non-neuronal cells. Under pathological conditions, tau can be also expressed in glial cells (10). It is also possible to find tau or it's mRNA in several peripheral tissues such as heart, liver, lung, skeletal muscle, among others (11–13). Interestingly, tau variants have been also observed in human platelets (3, 14, 15).

Tau phosphorylation plays an important role in regulating its binding to microtubules and thereby regulating their stability within neuronal cells. However, under pathological situations, tau protein is abnormally phosphorylated or dephosphorylated in specific residues, perhaps due to the activities of various protein kinases and phosphatases (such as GSK-3β and Cdk-5, and PP1A and PP2, respectively) (16, 17). This change in the phosphorylation state of tau can lead to irreversible changes in the dynamics of microtubules, cellular dysfunction, ultimately triggering cell death of the neuron (6, 18). Moreover, tau protein may have other post-translational modifications, including: (i) glycosylations, (ii) ubiquitinations, (iii) truncations, and (iv) nitrations (6).

Hyperphosphorylated tau protein is the main component of abnormal protein aggregates found in the neurons of patients with neurodegenerative brain disorders known as tauopathies.

Those neurodegenerative diseases have in common the presence of intraneuronal aggregates of tau. These aggregates are known as Neurofibrillary Tangles (NFTs) and are made up of paired helical filaments (PHFs) and straight filaments mainly composed of hyperphosphorylated tau. The formation of PHFs from tau molecules may follow different steps and could involve tau phosphorylations, followed by limited proteolysis and conformational changes in tau protein (19), and finally its polymerization into aberrant polymers in neuronal cells. These neuronal disorders include: Pick's disease, corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), frontotemporal dementia with Parkinsonism linked to chromosome 17 (DFTP-17) and Alzheimer's disease (AD). AD is considered the most prevalent tauopathy worldwide (20). In AD, abnormal phosphorylations occur and specifically tag certain amino acids of tau protein: Ser202, Thr205, Ser235, and Ser404 (21). These post-translational modifications are catalyzed by two main protein kinases: the Cdk-5/p35 system and GSK-3β (6). In this paper, we focus on how tau oligomers became the focus for the search for new drugs, and also potential targets for accurate diagnosis of AD.

ARE TAU OLIGOMERS RESPONSIBLE FOR NEURONAL DEGENERATION? TECHNICAL APPROACHES

Insoluble aggregates of the MAP tau characterize a number of tauopathies. Although there is much evidence linking tau to neurodegeneration, the precise mechanism of tau-mediated neurotoxicity remains to be elucidated. In fact, tau oligomers appear to be the toxic form of tau in neurodegenerative disease. In agreement with this hypothesis, the presence of immunoreactive tau protein in neurons of AD brain tissue, previous to tangle formation, has been shown. Binder and coworkers have produced the novel monoclonal antibody TOC1 that recognizes non-fibrillary tau. This antibody is selective in terms of specifically labeling tau dimers and oligomers, but does not label tau filaments. Time-course analysis and antibody labeling indicates that oligomers appear as an early event in AD pathogenesis. Aggregated tau, but not monomeric tau, inhibited anterograde fast axonal transport. This inhibition requires a small stretch containing amino acids from the N-terminal region on tau, a phosphatase-activation domain. The molecular chaperone heat-shock protein 70 (Hsp 70) clearly affects tau oligomers formation and stability, as investigated in the squid axoplasm. Hsp 70 preferentially bound to tau oligomers over filaments and prevented anterograde axonal transport inhibition observed with a mixture of both forms of aggregated tau (22).

Tau aggregates comprise abnormally hyperphosphorylated and misfolded tau. Research has traditionally focused on understanding how hyperphosphorylated and aggregated tau mediates dysfunction and toxicity in tauopathies. Recent findings in *Drosophila* and rodent models of tauopathy suggest that large insoluble aggregates such as tau filaments and tangles may not be the key toxic species in these diseases (23). Thus, some investigators have shifted their focus to study pre-filament tau species such as tau oligomers and hyperphosphorylated tau monomers. Interestingly, tau oligomers can exist in a variety of states including hyperphosphorylated and unphosphorylated forms, which can be both soluble and insoluble. It remains to be determined which of these oligomeric states of tau are causally involved in neurodegeneration and which molecule signal the beginning of the formation of inert/protective filaments. It will be important to better understand this aspect so that tau-based therapeutic interventions can target the really toxic tau species.

Another interesting study showed that oligomers of recombinant full-length human tau protein are neurotoxic *in vivo* after subcortical stereotaxic injection into mice. Data showed that tau oligomers impaired memory consolidation, whereas tau fibrils and monomers did not. In this context it was assumed that tau oligomers can affects synaptic transmission. Thus, synaptic dysfunction seems to result from the action of tau oligomers, potentially reducing the activity of the synaptic vesicle-associated proteins synaptophysin (24). Some studies identify tau oligomers as an acutely toxic tau species *in vivo*, and suggest that tau oligomers induce neurodegeneration by affecting mitochondrial and synaptic function, both of which are early hallmarks in AD and other tauopathies (25). These results open new avenues for neuroprotective intervention strategies for tauopathies by targeting tau oligomers.

OLIGOMERS AND THEIR USEFULNESS FOR MONITORING AD

As mentioned above, tau oligomers are one of the neuropathological hallmarks of AD and other tauopathies. Regarding NFT accumulation in AD, there is evidence showing that progressive neuronal loss and cognitive impairment correlates with the accumulation of soluble species of tau in NFTs in AD mouse models (26). This etiopathological feature has become the target for potential treatments for AD, diagnosis and monitoring evolution of the disease. On this last point, some diagnostic biomarkers for AD based on tau have been developed: (1) biomarkers in cerebrospinal fluid (CSF) and (2) biomarkers in platelet tau (3). Since in the CSF significant exchange of substances with a varied neural environment appears to occur, tau variants are released to the fluid. In this context, one of the most reliable biomarkers reported was based on the ratio between normal tau and hyperphosphorylated tau (P-tau) in the CSF (27). This evaluation about tau and P-tau allows a better correlation with the later stages of synaptic dysfunction and early neurodegeneration (27, 28). Furthermore, the second biomarker is based on the detection of tau in blood platelets (15, 29). Alterations in platelets from AD patients, including modifications in platelets β-amyloid Precursor Protein (APP) have been described previously (30) and APP evaluations have been postulated as a potential biomarker for AD (31). Considering that information we focused our efforts in analyses of platelets tau protein. The innovative method was developed in our laboratory and is based upon the difference between the ratio of molecular species of high molecular weight tau (multimeric) versus low molecular weight (monomeric). In western blot experiments performed with platelet protein extracts obtained from peripheral blood from healthy subjects and patients with AD, it was observed that the latter had tau immunoreactive bands migrating at molecular weights much higher than expected by electrophoresis under denaturing and reducing conditions (SDS-PAGE) (15). These high molecular weight forms of tau (tau HMW) could be attributed to oligomeric

forms of the protein, which are increased in AD patients as compared to healthy elderly subjects (**Figure 1**). Therefore, platelets tau has been postulated as a biomarker for AD (29). Different tau species with variable stages of aggregation are visualized in the electrophoretic patterns. Subsequent studies by Farias et al. (15), have shown that there is a close correlation between the degree of platelet tau modification and level of cognitive impairment, which was measured using neuropsychological tests in patients with AD [for more detailed information about these biomarkers see Ref. (3)]. Moreover, correlations between HMWtau/LMWtau ratio and brain neuroimaging have been observed. This ratio, controlled for age and education, significantly correlated with a clusters of 717 voxels in the right parahippocampal cortex with peak at Talairach coordinates 16, −10, −23 (unpublished observations).

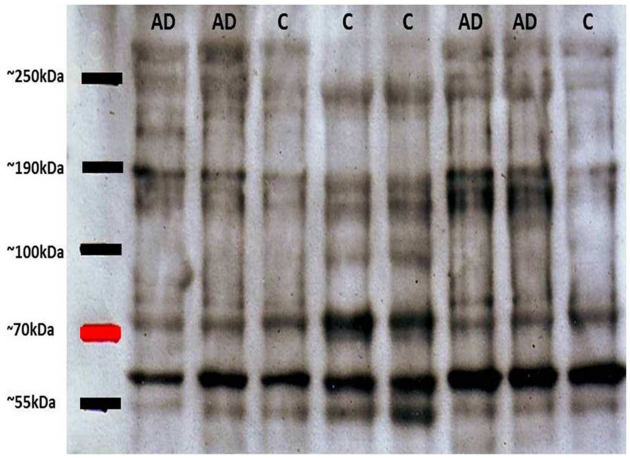

FIGURE 1 | Representative immunoblots of platelet tau with tau-5 antibody. High molecular weight tau bands (about 80 kDa) can be appreciated, with greater immunoreactivity in patients with Alzheimer's disease (AD) compared with control subjects (C). Subsequent densitometric analysis allows obtaining the relationship between HMWtau versus LMWtau.

TAU AS A THERAPEUTIC TARGET FOR ANTI-ALZHEIMER DRUGS

Since tau has been recognized as an important actor in neurodegenerative diseases, many molecules that act on tau pathology have been investigated as potential vectors for therapeutic approaches for AD, but also for other tauopathies such as PSP or CBD. One of the molecules that have attracted much attention is the phenothiazine methylthioninium chloride, better known as methylene blue (MB). MB has a very interesting property as an aggregation inhibitor for proteins that adopt beta sheet conformation (32).

Rember™ is the trade name of MB, and a Phase II study randomizing 321 mild to moderate AD patients with placebo or three different doses of Rember™ was presented in 2008 at the International Conference on AD in Chicago. Treatment effects of four points were described on ADAS-Cog on the treatment group as compared to placebo subjects, and also conservation on the cerebral blood flow and brain glucose used in SPECT and FDG-PET scans (33). However, as today, the lack of peer reviewed publications on this compound affect the reliability of the results. In animal models, the effects of MB on cognition appears not to be related to a reduction on NFTs but to a decrease in soluble tau levels (34) (**Figure 2**).

Other groups have focused their efforts on investigating tau kinases inhibitors (16) or tau phosphatase activators (17), as an indirect manner to halt tau hyperphosphorylation, recognized as leading to pathological aggregation of the protein. However, difficulties in finding specific inhibitors/activators with adequate safety profiles have impacted in the lack of new drugs in this area (32, 35). A possible way to decrease adverse effects of tau kinase inhibition might be the use of relatively broad specificity, low power compounds (36).

Davunetide is an eight aminoacids peptide that can be administered intranasally or intravenously and has been described as a tau hyperphosphorylation inhibitor as well as an inhibitor of caspase 3 activation (37). Davunetide has been successfully evaluated in a series of *in vitro* and *in vivo* models (38) for neurodegenerative diseases that include AD, PSP, and schizophrenia (38, 39).

An example of "non-traditional" approaches to tau aggregation regulation includes chaperones modulation. Hsp 70 chaperones assist protein-folding processes and are found up-regulated in several tumors, but also in neurodegenerative diseases such as Pick's disease, AD, and other tauopathies. Deregulation in Hsp 70 chaperones appears to be implicated in the processes of tau aggregation, so compounds that bind Hsp 70 chaperones are under investigation as possible treatment compounds for neurodegenerative diseases, since there is evidence that Hsp 70 inhibition leads to tau ubiquitination and clearance through ubiquitin-proteasome system (40).

There are some natural compounds that are able to inhibit tau aggregation and possibly, make an impact in neurodegenerative diseases. Shilajit is a natural phytocomplex that has been found in the Himalayan Mountains between India and Nepal and also in the Tibet and Afghanistan and has been used in ayurvedic medicine for centuries as a rejuvenating compound. Our laboratory has worked with the *Andean Compound* (or *Andean shilajit*), a natural compound that can be found in Andean Mountains. *Andean shilajit* is generated by a long-term degradation of certain plants by microorganisms, mostly fungi and is rich in Fulvic Acid (FA) and humic substances among others (**Figure 2**). This natural endemic phytocomplex, resulting from fossilized plants degradation through the years, was discovered in 2008 in the North of Chile and was named *Andean Compound* (41). *In vitro* assays and cell culture data show that Andean Compound and FA strongly interferes with tau aggregation, and interestingly an increase in neurites outgrowth has been observed in neural cell cultures exposed to this natural compound (42). In addition, a placebo-controlled pilot clinical study suggests that consumption of a nutraceutical formulation of *Andean Compound* plus B complex vitamins may produce stabilization of cognitive function in AD patients at a 24-weeks as determined with Global deterioration scale (GDS) and Neuropsychiatric inventory (NPI) measurements (42) (**Figure 2**). On the other hand the same *shilajit* – based compound has a very good safety profile when tested on healthy population (unpublished data).

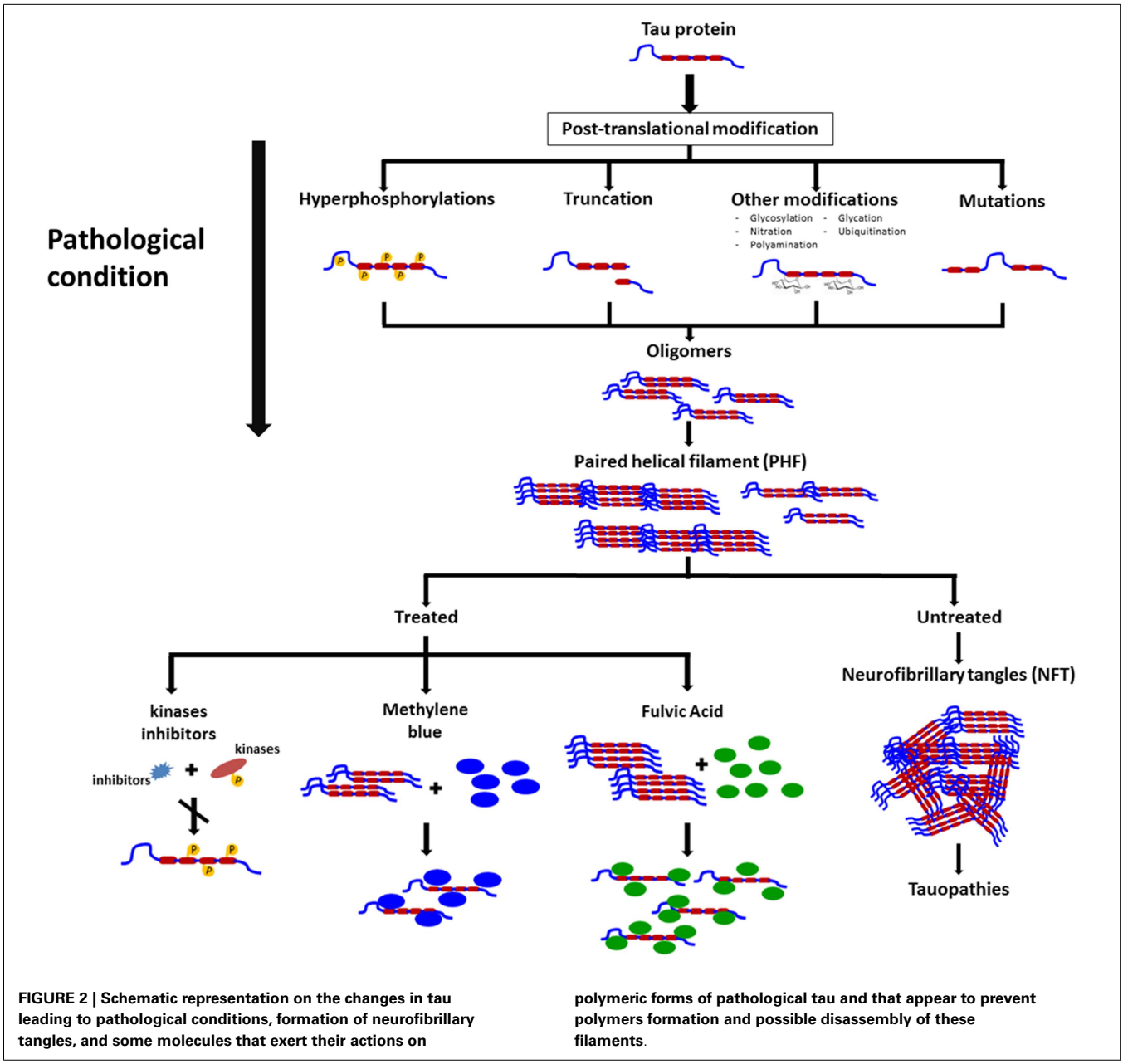

FIGURE 2 | Schematic representation on the changes in tau leading to pathological conditions, formation of neurofibrillary tangles, and some molecules that exert their actions on polymeric forms of pathological tau and that appear to prevent polymers formation and possible disassembly of these filaments.

CONCLUDING REMARKS

Currently, we do not know the exact cause of synaptic dysfunction and neurodegeneration in AD, however, in recent years it has become increasingly clear the importance of tau protein and its post-translational modifications in the pathophysiological processes of AD and other tauopathies.

In this context, determination of different forms of tau protein in brain, CSF (43) and also in blood (44) and peripheral cells (15) has been postulated as a powerful tool for detection and monitoring of the disease in different stages and there is clear evidence of a profile of tau and other biomarkers modifications during AD progression (45, 46). The presence of tau modifications in peripheral cells also points to the inference that AD may be a systemic disease not only confined to nervous tissue. But currently we do not have any information on the functional impact – if any – of oligomeric tau forms in peripheral cells. The latest criteria of AD consider tau-based biomarkers as reliable indicators of neuronal injury processes (47), but as of today there is not a widely available marker of tau modification for clinical use. Hopefully in the near future we will refine techniques for non-invasive assessment of tau. Thereon, are extremely interesting attempts to generate PET markers for tau deposition in brain (48, 49) and we already have promising data regarding measurement of different forms of tau in blood cells (15, 29).

On the other hand, in the absence of positive clinical results in studies with beta amyloid targeted therapies, the need to

evaluate therapies that can act on post-translational changes of tau protein has become evident. Particular interest has been paid to therapies that may modulate levels of phosphorylation and oligomerization of tau, whether by direct action on tau or by acting on other related proteins like kinases or heat-shock proteins.

Unfortunately we still need information from large multicenter studies about the usefulness of tau-focused therapies, however, this is a promising field that has attracted the efforts of multiple investigators, so we expect that new and exciting discoveries are right around the corner.

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What renders TAU toxic

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TAU is a microtubule-associated protein that under pathological conditions such as Alzheimer's disease (AD) forms insoluble, filamentous aggregates. When 20 years after TAU's discovery the first TAU transgenic mouse models were established, one declared goal that was achieved was the modeling of authentic TAU aggregate formation in the form of neurofibrillary tangles. However, as we review here, it has become increasingly clear that TAU causes damage much before these filamentous aggregates develop. In fact, because TAU is a scaffolding protein, increased levels and an altered subcellular localization (due to an increased insolubility and impaired clearance) result in the interaction of TAU with cellular proteins with which it would otherwise either not interact or do so to a lesser degree, thereby impairing their physiological functions. We specifically discuss the non-axonal localization of TAU, the role phosphorylation has in TAU toxicity and how TAU impairs mitochondrial functions. A major emphasis is on what we have learned from the four available TAU knock-out models in mice, and the knock-out of the TAU/MAP2 homolog PTL-1 in worms. It has been proposed that in human pathological conditions such as AD, a rare toxic TAU species exists which needs to be specifically removed to abrogate TAU's toxicity and restore neuronal functions. However, what is toxic in one context may not be in another, and simply reducing, but not fully abolishing TAU levels may be sufficient to abrogate TAU toxicity.

Keywords: Alzheimer's disease, *C. elegans*, frontotemporal dementia, knock-out, PP2A, PTL-1, TAU, transgenic

INTRODUCTION

TAU belongs to the family of microtubule-associated proteins (MAPs) (Dehmelt and Halpain, 2005). MAPs act in concert with heterodimers of α- and β-tubulin to assemble microtubules. They were named according to the three major size classes of polypeptides: MAP1 (>250 kDa), MAP2 (~200 kDa), and TAU (50–70 kDa) (Dehmelt and Halpain, 2005; Halpain and Dehmelt, 2006). MAP2 and TAU are expressed together in most neurons, where they localize to separate subcellular compartments. MAP2 is largely found in dendrites, whereas TAU is concentrated in axons (Matus, 1990). TAU has also been found in astrocytes and oligodendrocytes, although, under physiological conditions, its levels are relatively low (Tashiro et al., 1997). In concert with actin and intermediate filaments, microtubules establish and maintain the overall internal architecture of the cytoplasm and thereby comprise a major determinant of overall cell shape (Allen et al., 1985; Vale et al., 1985; Nangaku et al., 1994; Trinczek et al., 1999). Besides interacting with cytoskeletal proteins, MAPs also interact with proteins that have a range of cellular functions, suggesting that TAU is a scaffolding protein (Brandt and Leschik, 2004). Scaffolding proteins are defined as being able to bind at least two signaling proteins, and localizing signaling molecules and transduction pathways to defined cellular locations – for instance the cell body, dendrites or the axon, and to regulate signaling (Shaw and Filbert, 2009). We discuss here that TAU has these properties and that the toxicity of TAU is, at least in parts, because this scaffolding function is either altered or lost.

When TAU was discovered in 1975 (Weingarten et al., 1975), the subsequent years focused mainly on its tissue distribution and role in microtubule assembly and stabilization. The focus shifted radically when TAU was identified in Alzheimer's disease (AD) brains in a highly phosphorylated form as the filamentous core of the neurofibrillary tangles (NFTs) (Grundke-Iqbal et al., 1986; Goedert et al., 1988). Histopathologically, AD is also characterized by β-amyloid (Aβ)-containing plaques, reduced synaptic density, and neuronal loss in selected brain areas. Interestingly, although NFTs were initially correlated with dementia (Arriagada et al., 1992), it was also found that firstly, CA1 hippocampal neurons can survive with NFTs for about 20 years, and secondly that NFTs may not be obligatory for the death of CA1 hippocampal neurons in AD (Morsch et al., 1999). This does not, however, preclude that NFT-carrying neurons are functionally impaired. For a discussion of the role of NFTs in neurodegeneration, see e.g., Castillo-Carranza et al. (2013). In addition to AD, neurofibrillary lesions are also abundant in other neurodegenerative diseases such as Pick's disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD), and frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), where they occur in the absence of overt Aβ deposition. When we, for the first time, expressed human TAU in transgenic mice, we were able to reproduce key aspects of the human TAU pathology (Götz et al.,

1995). Specifically, overexpression of human TAU resulted in the accumulation of hyperphosphorylated forms of TAU not only in the axonal compartment, but also in the somatodendritic domain. NFT formation, however, was not achieved by this approach. It was the identification of pathogenic mutations in familial cases of frontotemporal lobar degeneration (FTLD) in the *MAPT* gene encoding TAU (Clark et al., 1998; Hutton et al., 1998; Poorkaj et al., 1998) that enabled us and others to express FTLD mutant forms of TAU in mice. Thus, we not only reproduced NFT formation, but in addition placed TAU downstream of Aβ in a patho-cascade – a central concept in the field (Götz et al., 2001a,b). Despite this, TAU is not simply an innocent bystander in Aβ toxicity, as discussed below. In fact, the dendritic localization of the "axonal" protein TAU not only has a role in physiological postsynaptic scaffolding, but it is also crucial for mediating Aβ's toxicity (Ittner et al., 2010). This and complementary studies in cell culture have put forth the argument that reducing or completely abolishing TAU expression might be beneficial in combating neurodegeneration in AD (Rapoport et al., 2002; Roberson et al., 2007; Ittner and Götz, 2011; Morris et al., 2011b).

In the following, we discuss what we have learned about TAU's toxicity in models that over-express TAU and as a consequence, develop TAU aggregates and NFTs. As this article is part of a series that investigates TAU toxicity from various angles, we will restrict our discussion to selected aspects and will specifically discuss TAU's role in non-axonal compartments, how it affects mitochondrial functions and what we have learned from invertebrate models. We draw the conclusion that TAU is a scaffolding protein and that its mislocalization disrupts cellular processes because it traps proteins with which it would otherwise not interact (or do so to a lesser degree) and thereby prevents them from executing their physiological functions. This does not rule out that the aggregates themselves are toxic, although the current experimental models do not allow for a dissociation of the effects elicited by the aggregates from those elicited by soluble TAU species. In the second part we discuss what we have learned from TAU knock-out models in mice and what the knockout of the worm homolog of TAU, PTL-1, has contributed to an understanding of TAU function. In the final paragraph, we attempt to integrate findings in both TAU over-expressing and knock-out models.

WHAT CAUSES TOXICITY WHEN THERE IS TOO MUCH TAU?

In an attempt to model TAU pathology in mice, the first TAU transgenic mouse model was established 20 years after TAU's discovery by over-expressing the longest of the six major human TAU isoforms, hTau40 (Götz et al., 1995) (For comparison, in the adult mouse brain, there are only three TAU isoforms expressed.). These htau40 transgenic mice reproduce aspects of the human pathology, such as somatodendritic localization and hyperphosphorylation of TAU. Subsequently, stronger promoters were used to drive expression, resulting in more pronounced phenotypes (Ishihara et al., 1999; Spittaels et al., 1999; Probst et al., 2000). Despite the formation of TAU aggregates and an age-dependent decrease in TAU solubility, NFTs did not form until a very old age in these mice (Ishihara et al., 2001). However, signs of Wallerian degeneration including axonal breakdown and segmentation of myelin into ellipsoids were observed. Furthermore, neurogenic muscle atrophy, with groups of small angular muscle fibers, was present in the hind leg musculature of the transgenic mice (Probst et al., 2000). Another characteristic was the presence of large numbers of axonal spheroids in brainstem and spinal cord (Ishihara et al., 1999; Spittaels et al., 1999; Probst et al., 2000). Similar swellings have also been described in transgenic mice with Aβ plaque formation, and in the human AD brain (Stokin et al., 2005).

Following the identification of pathogenic mutations in *MAPT* in FTDP-17, TAU filament formation was achieved in neurons and glia, both by constitutive and doxycycline-regulated expression of mutant human forms of TAU (Götz and Götz, 2009). The first published mouse strain with pronounced NFT formation, JNPL3, expressed P301L mutant human TAU under the control of the murine PrP promoter. This resulted in abnormal TAU filament formation in neurons as well as astrocytes and oligodendrocytes, with NFTs being present in brain and spinal cord (Lewis et al., 2000). Neuronal loss was found in the spinal cord, as evidenced by a twofold reduction in the numbers of motor neurons. Furthermore, the mice developed severe motor disturbances by 10 months of age. We established a second model with NFT formation, pR5 mice, by expressing the same P301L mutation, but using a different human TAU isoform and the neuron-specific mThy1.2 promoter for transgene expression (Götz et al., 2001a). While a motor phenotype was absent, reference memory was impaired and the mice displayed an increased exploratory behavior (Pennanen et al., 2006). Furthermore, owing to a pronounced expression of the transgene in the amygdala, the mice showed an accelerated extinction in the conditioned taste aversion paradigm (Pennanen et al., 2004). When experimental diabetes was induced in pR5 mice, this caused an earlier-onset and increased formation of NFTs, indicating that diabetes can accelerate the onset and increase the severity of disease in individuals with a predisposition to develop a tauopathy (Ke et al., 2009). To determine whether NFTs are integral components of the neurotoxic cascade in AD or whether they represent a protective neuronal response, transgenic mice were generated which allowed for the regulation of P301L TAU expression by adding doxycycline to the drinking water (Santacruz et al., 2005). Because the system resulted in a 15-fold over-expression, this caused a progressive formation of NFTs, a remarkable neuronal loss (70% in the CA1 region), gross atrophy of the brain, and behavioral impairment. Turning the system "off" caused a reduction of TAU levels to 2.5-fold over-expression. Nonetheless, this was sufficient to cause a recovery of memory functions and stabilization of neuron numbers, while NFTs continued to accumulate. These data imply that NFTs *per se* are not sufficient to cause cognitive decline or neuronal death (Santacruz et al., 2005).

While the pR5 mice display memory impairment as a major clinical feature of AD, another feature, Parkinsonism, that characterizes a significant subset of FTLD cases, has been modeled in K369I mutant TAU transgenic K3 mice. We established this strain based on the identification of the K369I mutation of TAU in a single patient with Pick's disease (Neumann et al., 2001), and reproduced the distinct characteristics of Pick's pathology in mice (Ittner et al., 2008). Memory functions were impaired as shown in the novel object recognition test. Owing to a unique expression pattern of the transgene that extends to the Substantia Nigra pars compacta (SNpc), the K3 mice also model early onset

Parkinsonism, i.e., resting tremor, bradykinesia, postural instability, and gait anomalies. They show an increased cataleptic response to haloperidol and an early, but not late, response to L-DOPA, indicating that the dopaminergic system is impaired. We found a selectively impaired axonal transport of distinct cargos including mitochondria and tyrosine hydroxylase (TH)-containing vesicles. An important finding is that at the molecular level, this transport impairment is the result of the accumulation of hyperphosphorylated TAU in the cell body, where it traps the adapter protein and a component of the kinesin motor machinery, JIP1, thereby preventing this molecule from executing its physiological function in the axon (Ittner et al., 2008). A pathological interaction between TAU and JIP1 was further revealed in AD and not control brain (Ittner et al., 2009). It seems therefore that for TAU to be able to exert toxicity, the subcellular compartments it is aberrantly targeted to are crucial, as this determines with which proteins it pathologically interacts and which cellular functions it impairs. In this process, phosphorylation of TAU has a critical role as reviewed by us recently (Götz et al., 2010). That phosphorylation of TAU is critical in toxicity is also evident from studies in protein phosphatase 2A (PP2A) dominant negative mutant strains. More specifically, to address the role of the TAU phosphatase PP2A in mice with a pre-existing TAU pathology, we crossed the PP2A dominant negative mutant strain Dom5 with pR5 mice that express P301L mutant TAU and found that this exacerbated the TAU pathology of pR5 mice significantly. The double-transgenic mice showed sevenfold increased numbers of hippocampal neurons that specifically phosphorylated the pathological Ser422 epitope of TAU (Deters et al., 2009). The mice showed eightfold increased numbers of NFTs compared with pR5 mice, in agreement with our previous finding that NFT formation is correlated with and preceded by phosphorylation of TAU at the Ser422 epitope (Götz et al., 2001b). We further used the Dom5 mice to show that a small compound, sodium selenate, improves TAU-dependent impairment and neurodegeneration in a PP2A-dependent manner (van Eersel et al., 2010). Together this demonstrates that phosphorylated forms of TAU have a critical role in TAU toxicity.

The tauopathies PSP and CBD are characterized by substantial glial TAU pathology. Aspects of this pathology have been reproduced by expressing G272V mutant TAU under the control of the PrP promoter that resulted in high transgene expression in a subset of both neurons and oligodendrocytes. Electron microscopy established that TAU filament formation was associated with hyperphosphorylation of TAU. Thioflavin S-positive fibrillary inclusions were identified in oligodendrocytes and motor neurons (Götz et al., 2001c), the clinical phenotype of these mice however was subtle. In contrast, when human wild-type TAU was overexpressed in neurons and glial cells under the control of the mouse Tα1 α-tubulin promoter, a glial pathology was obtained that closely resembled the astrocytic plaques of CBD and the coiled bodies of both CBD and PSP (Higuchi et al., 2002). A significant age-related neuronal loss was only found at 18 months of age, whereas oligodendrocytes were lost already at 6 months. The same team employed the 2′,3′-cyclic nucleotide 3′-phosphodiesterase promoter to express human P301L TAU exclusively in oligodendrocytes (Higuchi et al., 2005). Interestingly, the structural disruption of myelin and axons preceded the emergence of TAU inclusions in oligodendrocytes; also, impaired axonal transport was found to precede the motor deficits in these mice (Higuchi et al., 2005). Together, these studies highlight a role for glial TAU in disease.

While most studies on TAU use the mouse as a model organism, several wild-type and mutant human TAU transgenic models have been established in the nematode *C. elegans* (Kraemer et al., 2003; Miyasaka et al., 2005; Brandt et al., 2009; Fatouros et al., 2012). Some of these models have been reviewed in detail (Ewald and Li, 2010) and are not discussed here.

In the following, we will discuss two important aspects of TAU toxicity, one is the impairment of mitochondrial functions, and the second the role TAU has in the dendrite. Mitochondrial dysfunction has long been associated with the pathophysiology of AD (Blass and Gibson, 1991). The morphology of a cellular mitochondrial network is maintained by reciprocal rounds of fission and fusion, a process termed mitochondrial dynamics. Elevated fusion produces elongated, interconnected mitochondria, while enhanced fission results in mitochondrial fragmentation. Mitochondrial dynamics cannot be discussed in isolation, as fission (biogenesis), fusion, bioenergetics, motility/transport, and turnover by mitophagy are highly inter-dependent processes (Chen and Chan, 2009). How TAU impairs the fission and fusion of mitochondria, has been reviewed by us recently (Duboff et al., 2013), following the finding of a role of TAU in altering mitochondrial dynamics. Specifically we had found that in human TAU transgenic mice and flies, F-actin is increased, which disrupts the physical association of mitochondria and the fission protein DRP1, leading to mitochondrial elongation (Duboff et al., 2012). The resulting neurotoxicity can be rescued either by reducing mitochondrial fusion, or by enhancing fission, or by reversing actin stabilization.

Earlier, we had analyzed P301L TAU transgenic pR5 mice by MALDI TOF/TOF mass-spectrometric analysis, which revealed a deregulation of mainly metabolism-related proteins including mitochondrial respiratory chain complex components such as the complex V component ATP synthase D chain, antioxidant enzymes, and synaptic proteins (David et al., 2005). A subsequent functional analysis demonstrated a mitochondrial dysfunction in pR5 mice together with a reduced NADH-ubiquinone oxidoreductase activity and, with age, impaired mitochondrial respiration and ATP synthesis. Mitochondrial dysfunction was associated with higher levels of reactive oxygen species (ROS) in aged transgenic mice and an up-regulation of antioxidant enzymes. When complex V levels were analyzed in human FTDP-17 patient brains carrying the P301L TAU mutation, a significant decrease in complex V levels was found in all P301L human brain samples compared with controls, underscoring the validity of the proteomics findings in mice for the human disease (David et al., 2005). A serial analysis of gene expression (SAGE) of the same mouse strain looking specifically at the amygdala also revealed deregulated mitochondrial genes (Ke et al., 2012a).

Mitochondrial functions are also impaired by Aβ and as there are many findings that support the notion of a cross-talk of Aβ and TAU in affecting mitochondrial functions (Eckert et al., 2010), it was reasonable to cross pR5 mice with Aβ plaque-forming

$APP^{sw}PS2^{N141I}$ double-transgenic APP152 mice to generate triple-transgenic (^{triple}AD) mice that combine both pathologies in one model (Grueninger et al., 2010). Quantitative isobaric-tag labeling (iTRAQ) followed by mass spectrometry revealed a massive deregulation of 24 proteins, of which one third were mitochondrial proteins mainly related to complexes I and IV of the oxidative phosphorylation system (OXPHOS). When mitochondrial functions were addressed, this revealed that deregulation of complex I is TAU-dependent, whereas deregulation of complex IV is Aβ-dependent, both at the protein and activity level (Rhein et al., 2009). In addition, synergistic effects of Aβ and TAU were evident in the ^{triple}AD mice establishing a molecular link between Aβ and TAU protein in AD pathology *in vivo* (Rhein et al., 2009). In the P301L TAU transgenic pR5 mice, a transcriptomic analysis further revealed an up-regulation of glyoxalase I that detoxifies dicarbonyl compounds and thereby reduces the formation of advanced glycation end (AGE) products (Chen et al., 2004). Co-staining with a phospho-TAU antibody suggested glyoxalase I up-regulation as an early defense mechanism to combat elevated levels of aggregated TAU. To understand which processes are disrupted by Aβ in the presence of TAU aggregates, we applied comparative proteomics to Aβ-treated P301L TAU-expressing neuroblastoma cells and the amygdala of P301L TAU transgenic pR5 mice that had been stereotaxically injected with Aβ preparations. We found that a significant fraction of proteins that were altered in both systems belonged to the same functional categories, i.e., proteins involved in the stress-response associated with protein folding (David et al., 2006). Among the deregulated proteins was valosin containing protein (VCP), an essential component of the ER-associated degradation (ERAD) process, whereas members of the peroxiredoxin family were down-regulated. Together this indicates that TAU and Aβ exert both separate and synergistic toxic effects that are mediated by mitochondria and the stress-related unfolded protein response.

The amyloid cascade hypothesis in a patho-cascade places Aβ upstream of TAU (Hardy, 2006). This concept has been proven in P301L mutant TAU transgenic mice that develop an increased number of NFTs, either by crossing them with Aβ plaque-forming transgenic mice (Lewis et al., 2001), or by stereotaxically injecting Aβ into their brains (Götz et al., 2001b). That Aβ toxicity is dependent on TAU has first been shown *in vitro* because primary neuronal cultures derived from TAU knock-out mice were resistant to the toxic effects of Aβ (Rapoport et al., 2002). This finding was subsequently reproduced *in vivo*, by crossing Aβ plaque-forming mice that are characterized by premature mortality, high susceptibility to experimentally induced excitotoxic seizures and memory deficits, onto a TAU knock-out background (Roberson et al., 2007). Mechanistically, this protection appeared to be conferred by a reduced susceptibility to excitotoxicity when TAU was either absent or when its levels were reduced (Roberson et al., 2007). Excitotoxicity is the over-activation of NMDA receptors (NMDARs) that results in neuronal damage and death because of increased calcium influx and nitric oxide (NO) activation (Palop and Mucke, 2009). By using another APP plaque-forming mouse strain and a different TAU knock-out strain, we were able to reproduce the TAU-dependent protection from Aβ-induced premature mortality and memory deficits, and determined that this protection is conferred by a reduced susceptibility to excitotoxicity (Ittner et al., 2010). Likewise, overexpression of a truncated form of TAU (ΔTau) that lacks the microtubule-binding region MBR also rescued the phenotype of the Aβ plaque-forming mice (Ittner et al., 2010). In a more recent model, tau pathology was shown to develop independent of Aβ (Winton et al., 2011). This was shown by finding no differences in TAU lesion formation when crossing 3xTg-AD mice that co-express mutant forms of APP, presenilin and TAU and hence develop a plaque and TAU pathology, with a mouse strain that lacks the enzyme BACE1 required for Aβ generation. However, it has to be considered that the TAU pathology in the 3xTg-AD mice develops very late and is modest compared with that of other widely used TAU transgenic mouse models (Oddo et al., 2003a,b).

What is the mechanistic explanation for this protection conferred by reduction of TAU? We next showed that TAU is also present in the dendrite (although in low quantities compared with the axon), where it is critically involved in postsynaptic NMDAR downstream signaling by localizing the SRC kinase FYN to the dendrite. FYN phosphorylates the NMDAR that then recruits the postsynaptic scaffolding protein PSD-95 to form a complex. Because levels of postsynaptic FYN are massively reduced in TAU-deficient or ΔTau-expressing mice, this results in uncoupling of NMDARs from excitotoxic signaling and abrogation of Aβ-mediated toxicity (Ittner et al., 2010; Ittner and Götz, 2011). A subsequent study found that Aβ causes a range of deleterious effects on TAU including its localization to dendrites (Zempel et al., 2010). Aβ, Fyn, and TAU therefore seem to orchestrate neuronal damage (Haass and Mandelkow, 2010; Ittner et al., 2010; Roberson et al., 2011). An essential role for FYN in this process is supported by the fact that the pathology of Aβ plaque-forming mice is enhanced when FYN is overexpressed and ameliorated when FYN is absent (Chin et al., 2004, 2005).

TAU interacts with FYN at least in two ways. Firstly, although TAU is mainly phosphorylated on serine and threonine residues, it also contains three tyrosine residues. Tyr18 is phosphorylated by FYN, and the phosphorylated motif interacts with FYN via FYN's SH2 domain. Secondly, TAU contains seven PXXP motifs located in the amino-terminus (all of which are retained in the ΔTau construct). Of these, the seventh proline-rich RTPPKSP motif has been shown to be crucial for the interaction with the SH3 domain of FYN and other SRC non-receptor tyrosine kinases (Lee et al., 1998; Bhaskar et al., 2010; Ittner et al., 2010). Interestingly, this motif is also critical in the interaction of TAU with the phosphatase PP2A that exists as a heterotrimeric holoenzyme complex. It has been shown recently that the PP2A regulatory subunit Bα binds to and dephosphorylates TAU, and thereby regulates microtubule stability (Sontag et al., 2012). When FYN is bound to the (seventh) proline-rich RTPPKSP motif that is conserved in both TAU and MAP2, this inhibits the interaction of PP2A/Bα with either TAU or MAP2. The corresponding synthetic RTPPKSP peptide, but not the phosphorylated RpTPPKSP version, competes with TAU and MAP2 for binding to PP2A/Bα. This finding is remarkable because the down-regulation of PP2A/Bα and the deregulation of FYN/TAU interactions have been linked to enhanced TAU phosphorylation in AD (Sontag et al., 2012). MAP2 is mainly a dendritic protein as mentioned above. Why MAP2 cannot compensate for the absence of TAU in TAU knock-out mice and target FYN to the

dendritic spine is not understood, especially as both MAP2 and TAU have been shown to efficiently bind to FYN.

WHAT ARE THE EFFECTS OF REDUCED TAU LEVELS?

As reviewed in detail recently, a series of TAU knock-out mouse strains have been generated to gain insight into the physiological functions of TAU, and while initially reported to be overtly normal, behavioral changes and motor deficits were identified at least for some of these strains (Ke et al., 2012b).

Work in primary neuronal cultures laid the foundation: when TAU was down-regulated using antisense oligonucleotides, this caused a reduced neuronal outgrowth (Caceres and Kosik, 1990). However, the first TAU knock-out mice established by Harada et al. (1994) a few years later were surprisingly normal, with no evidence of either an altered axonal elongation or any macroscopic change. In a compensatory mechanism for the absence of TAU, MAP1A was up-regulated, and the axon caliber was altered to what is typically found in dendrites. Primary neuronal cultures established from these TAU knock-out mice did not display a reduced neuronal outgrowth phenotype, contrasting with earlier observations of neurons in which TAU levels had been reduced using antisense approaches (Harada et al., 1994; Takei et al., 2000). Nonetheless, the absence of TAU is not without consequences as the hypoplasticity of the commissural tract and the disorganization of the neuronal layering found in MAP1B knock-out mice (Takei et al., 1997) is exacerbated by cross-breeding these mice with TAU knock-out mice (Takei et al., 2000).

In 2001, two additional TAU knock-out lines became available. The first was established by Dawson et al. (2001), who used the same strategy as Harada and colleagues by replacing the first coding exon of TAU with a neomycin selection cassette, thereby abrogating TAU expression. The second "knock-in" strain was generated by Tucker et al. (2001), who inserted a GFP cassette in frame, resulting in a fusion protein that contained the first 31 amino acids of TAU. None of these two strains revealed any obvious impairment [at the time, the Tucker strain was only used as a "tool" to study neurotrophins; we have, however, since used this strain for behavioral studies (Ittner et al., 2010)]. Interestingly, in the Dawson strain, MAP1A levels were increased twofold at birth, while they were reduced back to normal levels as the mice became older suggesting that MAP1A may compensate for the loss of TAU during early brain development, but not in the mature brain (Dawson et al., 2001). In keeping with the very first *in vitro* studies (Caceres and Kosik, 1990), primary neurons obtained from this knock-out strain showed a slowed maturation with reduced neurite length throughout all developmental stages and a reduced axon length of stage 3 (onset of polarity) neurons (Dawson et al., 2001). A fourth TAU knockout was established in 2007, again by inserting a selection cassette into exon 1, but the cassette was flanked by FRT (flippase recognition target) recombination motifs to allow for subsequent manipulation of the targeted *MAPT* gene (Fujio et al., 2007). Not surprisingly, the mice looked quite normal, and MAP1A levels were found to be increased as previously reported in two of the three knock-out strains. Taken together, TAU is not essential in mice, although small differences were found between the four strains with regards to compensatory mechanisms and when analyzed *ex vivo*.

How do TAU knock-out mice fare in behavioral and motor studies? All four strains presented with no overt phenotype up to 8 months of age (Harada et al., 1994; Dawson et al., 2001; Fujio et al., 2007; Roberson et al., 2007; Ittner et al., 2010; Lei et al., 2012). At 10–12 months, the Dawson mice performed like wild-type in the radial arm and the Morris water maze (Dawson et al., 2010). Subtle motor deficits were detected at 3–3.5 months when the knock-out mice showed an increased latency to cross a beam and also made more slipped steps, but otherwise showed normal motor functions (Morris et al., 2011a). However, at 12 months of age, the Harada mice displayed signs of muscle weakness in the wire-hanging test, reduced balance in the rod-walking test, hyperactivity in a novel environment and impaired contextual fear conditioning (Ikegami et al., 2000). Interestingly, muscle weakness was also evident in heterozygous knock-out mice. Spatial learning, however, of this TAU knock-out strain was normal, when assessed in the eight-arm radial and the Morris water maze (Ikegami et al., 2000). The most pronounced phenotype was recently reported in the Dawson strain kept on a C57BL/6/SV129 background, with Parkinsonism evident at 12 months of age due to a massive loss of dopaminergic neurons in the SNpc, associated with motor impairments such as a reduced performance on the Rotarod and decreased locomotion in the open field (Lei et al., 2012). Because these findings contradict the earlier studies, Morris et al. (2013) performed a detailed behavioral and motor analysis of the Dawson strain that was kept on a C57BL/6 background. They found in their mice that the chronic lack of TAU did not impair learning and memory functions in the Morris water maze and the novel object recognition test, neither at 11–17 nor at 21–22 months of age (Morris et al., 2013). Interestingly, at 12–15 months the knock-out mice weighed more (with 21–22 month-old mice showing a trend) (Morris et al., 2013), while K369I TAU over-expressing mice weigh less (Ittner et al., 2008). The knockout did not alter rearing or activity in the open field, however, both aging and TAU ablation reduced the latency to fall off the Rotarod and this was correlated with body weight. At 12–15 months, TAU knock-out mice took longer to descend from the pole than heterozygous knock-out or wild-type mice, whereas for the older age group latencies did not differ. Together this suggests that complete TAU ablation causes subtle motor deficits that are related to an increased body weight. Finally, the newer study did find up to 16% reductions in dopamine levels but this was not sufficient to cause Parkinsonism nor did the administration of L-DOPA improve the performance of the mice in the pole test (Morris et al., 2013). In contrast to the work by Lei et al. (2012), no iron accumulation was found in brain areas such as the hippocampus, striatum or SN. A reason for these discrepancies may be the difference in the genetic background (C57BL/6/SV129 versus C57BL/6) as this has been reported to affect brain metal levels in both transgenic and normal mice (Maynard et al., 2006).

It is apparent that differences in genetic background and design of the targeting construct have an impact on the phenotype of transgenic animals. Another confounding factor for the study of TAU is the presence of other MAPs, which appear to share several biological roles (Dehmelt and Halpain, 2005; Sontag et al., 2012). Potential compensatory functions attributable to closely related MAPs can be excluded in the nematode *C. elegans*, where

protein with TAU-like repeats (PTL-1) is the sole homolog of TAU/MAP2/MAP4 in the worm (McDermott et al., 1996; Gordon et al., 2008), meaning that shared physiological functions of the different family members can be addressed.

PTL-1 contains a high level of sequence homology to TAU/MAP2/MAP4 within the microtubule-binding repeat (MBR) domain in the carboxy-terminus. Analysis of a *ptl-1* transcriptional reporter line demonstrates that PTL-1 has a neuronal expression pattern in adult worms, and in addition PTL-1 has been shown to regulate microtubule assembly *in vitro* (Goedert et al., 1996; McDermott et al., 1996). PTL-1 has been implicated in the regulation of microtubule-based motility in several neurons (Tien et al., 2011) and in the response to gentle touch (Gordon et al., 2008). Aging-related damage in *C. elegans* neurons is evident by neurons displaying abnormal structures such as branching or blebbing from the cell body or axon (**Figure 1**), and these changes progressively accumulate as the worms age (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). Using two *ptl-1* mutant strains, one a null knockout (Gordon et al., 2008) and the other putatively generating a protein product containing only the N-terminal region [i.e., similar to our ΔTau truncation construct (Ittner et al., 2010)], it was observed that *ptl-1* mutant strains showed an increased incidence of abnormal structures compared with wild-type animals (Chew et al., 2013). This suggests that the neurons of *ptl-1* mutant animals develop signs of aging at a faster rate than in wild-type animals. In addition, *ptl-1* mutant animals are also short-lived compared with wild-type controls (Chew et al., 2013). These phenotypes of accelerated neuronal aging and shortened organismal lifespan were rescued by re-expressing PTL-1 in a *ptl-1* null mutant. Interestingly, increasing the number of gene copies of *ptl-1* by incorporating the *ptl-1* transgene in a wild-type background was observed to mirror both the neuronal and lifespan phenotypes observed in the *ptl-1* mutant strains, indicating that gene dosage of PTL-1 is vital. This demonstrates a key role of PTL-1 in maintaining neuronal health with age and in regulating whole organism lifespan (Chew et al., 2013). These findings in *C. elegans* show that levels of PTL-1 need to be tightly regulated, suggesting that therapeutic strategies involving the reduction of TAU levels should not lead to a complete reduction of TAU.

CONCLUDING REMARKS

What causes sporadic forms of AD is not known, but it is tempting to speculate that Aβ and TAU act through a combination of excitotoxicity, inhibition of axonal transport and aberrant localization as well as combined effects on mitochondria. It is at the synapse where Aβ induces damage and impairs memory-related electrophysiological properties (Masliah, 1995; Arendt, 2009; Wu et al., 2010). Under basal conditions, mild activation of the NMDAR results in physiological ROS production, while under neurodegenerative conditions, triggered by Aβ, over-activation of NMDARs causes excessive calcium influx and generates NO (Nakamura and Lipton, 2011). These changes in calcium can affect mitochondria (Stanika et al., 2012). Furthermore, axonal transport of cargoes including mitochondria is impaired when TAU detaches from the microtubules and localizes to the somatodendritic domain (Ishihara et al., 1999; Ittner et al., 2008). It has been proposed that in disease conditions such as AD, rare species of toxic TAU exist that need to be removed in order to restore neuronal functions. Other possibilities include a role for non-coding RNAs including mi-RNAs and truncated forms of TAU in toxicity, as discussed elsewhere (Schonrock et al., 2010; Hebert et al., 2012; Zilka et al.,

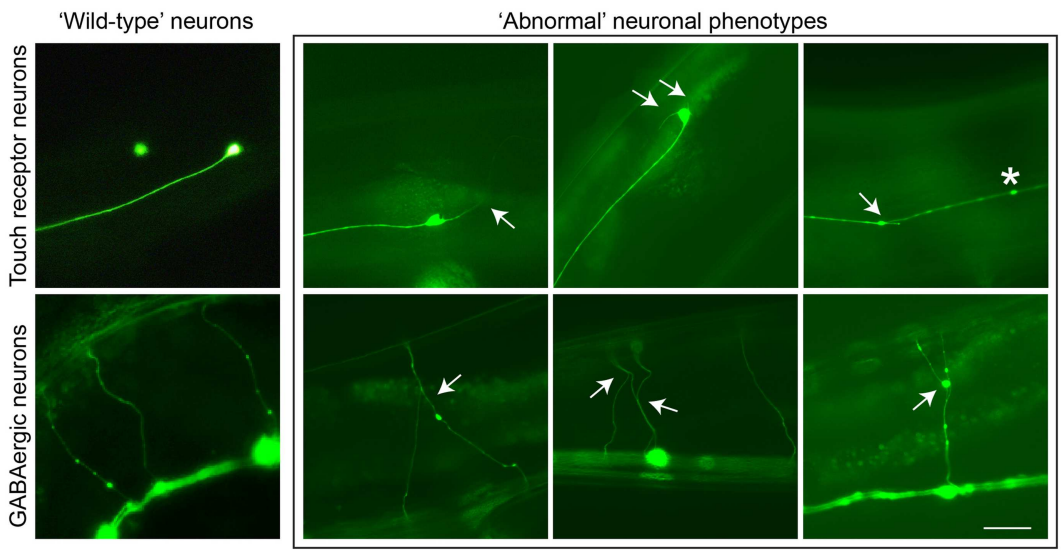

FIGURE 1 | Neuronal aging in C. elegans is demonstrated by the accumulation of abnormal neuronal structures. Reporter lines expressing GFP in particular neuronal subsets are used to track these phenotypes as animals age. In touch receptor neurons (top), branching from the cell body or axon, as well as beading along the axon, can be observed. In GABAergic motor neurons (bottom), branching from commissures extending dorsally can be visualized. These phenotypes usually accumulate in late adulthood in wild-type animals, but in *ptl-1* mutant strains these structures can be seen starting in early to mid-adulthood. Arrows indicate branching, asterisks indicate beading. Scale bar = 50 μ. Ventral is down.

2012). As discussed here, simply reducing TAU levels may be therapeutically beneficial. Work in TAU knock-out mice and PTL-1 knock-out worms suggests that especially in an aging brain, one would not aim for a full ablation of TAU expression. This is also because the TAU/FYN interaction is required for oligodendrocyte functions including myelination. Expression of the N-terminal domain of TAU (a construct similar to ΔTau) alone causes abnormal sorting of FYN, poor myelination and seizures (Klein et al., 2002). However, if one were to succeed in reducing TAU levels by any form of therapeutic intervention, in practical terms it is unlikely that this reduction will be complete. For a positive therapeutic outcome, it might even be sufficient to reduce TAU expression after a pathology has developed as suggested by studies in mice with inducible TAU expression, including those that express shorter variants of TAU (Van der Jeugd et al., 2012; Hochgrafe et al., 2013).

From a therapeutic point of view, besides manipulating TAU levels, its localization and the interaction with other proteins, a range of alternative strategies can be pursued. One is restoring neuronal functions by transplanting stem cells to provide neurotropic support (Yamasaki et al., 2007). As far as TAU is concerned, evidence is accumulating that its phosphorylation is critical for TAU to be toxic, and that reducing TAU phosphorylation is a promising strategy (Gong and Iqbal, 2008). What is not entirely clear is whether this requires distinct phosphorylation events or whether a generally elevated level of phosphorylation is sufficient. The latter possibility is suggested by work in Drosophila (Steinhilb et al., 2007). In principal, one would either like to inhibit TAU kinases such as GSK3 (Engel et al., 2006), or activate TAU phosphatases such as PP2A (Delobel et al., 2002), or both. In fact, the PP2A activator sodium selenate that dramatically reduced TAU pathology in several mouse models (van Eersel et al., 2010) is currently evaluated in a phase IIa clinical trial in mild to moderate AD (https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=83952).

Other suitable therapeutic strategies are the restoration of mitochondrial function, blocking the interaction of TAU with FYN or JIP1, or disrupting the excitotoxic complex of NMDAR and PSD-95. Other strategies are the activation of chaperones (Ward et al., 2012) and the use of aggregation blockers (Akoury et al., 2013). While it is clear that Aβ plays a key role in the pathogenesis of AD, it is becoming even clearer that there is a future of therapeutics for AD beyond amyloid (Lane et al., 2012; Yoshiyama et al., 2012). Whether in practical terms it will be necessary for the efficacy of emerging therapeutic strategies to exactly determine which TAU species is the most toxic form, remains to be determined.

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Tangles, toxicity, and tau secretion in AD – new approaches to a vexing problem

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When the microtubule (MT)-associated protein tau is not bound to axonal MTs, it becomes hyperphosphorylated and vulnerable to proteolytic cleavage and other changes typically seen in the hallmark tau deposits (neurofibrillary tangles) of tau-associated neurodegenerative diseases (tauopathies). Neurofibrillary tangle formation is preceded by tau oligomerization and accompanied by covalent crosslinking and cytotoxicity, making tangle cytopathogenesis a natural central focus of studies directed at understanding the role of tau in neurodegenerative disease. Recent studies suggest that the formation of tau oligomers may be more closely related to tau neurotoxicity than the presence of the tangles themselves. It has also become increasingly clear that tau pathobiology involves a wide variety of other cellular abnormalities including a disruption of autophagy, vesicle trafficking mechanisms, axoplasmic transport, neuronal polarity, and even the secretion of tau, which is normally a cytosolic protein, to the extracellular space. In this review, we discuss tau misprocessing, toxicity and secretion in the context of normal tau functions in developing and mature neurons. We also compare tau cytopathology to that of other aggregation-prone proteins involved in neurodegeneration (alpha synuclein, prion protein, and APP). Finally, we consider potential mechanisms of intra- and interneuronal tau lesion spreading, an area of particular recent interest.

Keywords: tau oligomerization, tau toxicity, tau secretion, interneuronal lesion spread, exosome

OVERVIEW

Cytotoxicity associated with the accumulation of abnormal protein aggregates has emerged as a central common mechanism underlying human neurodegenerative disease. Neurons are unique among differentiated cell types in that they do not re-enter the cell cycle and thus cannot use mitosis as a method for clearing abnormally aggregated proteins. As a result, they are inherently vulnerable to disruption of protein turnover mechanisms such as the ubiquitin/proteasome pathway and autophagy, especially in aged individuals. The abnormal turnover of aggregation-prone proteins such as alpha synuclein (SNCA), prion protein (PrP), amyloid beta (Ab) peptide, and tau are thus key factors in most (95%) of the neurodegenerative diseases that affect humans. Protein aggregation is typically accompanied and potentiated by abnormal phosphorylation, ubiquitination, covalent crosslinking, and the abnormal activation of autolytic proteases (1–6). A common feature of such proteins is an "intrinsically disordered" structure (4), in which the normal conformation can be readily changed into a beta-sheet rich structure with high aggregation propensity (6). Moreover, conditions in which SNCA (Parkinson's disease, Lewy Body Dementia), tau (Corticobasal Degeneration, Pick's disease, Frontotemporal Dementia), and PrP [Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker disease] form abnormal aggregates typically show overlapping neuropathology, suggesting that synergistic interactions may occur between these proteins in each of these conditions (7). The aggregation of the microtubule (MT)-associated protein tau, which is heat stable and normally exhibits a random coil conformation in aqueous solution, plays a central role in the neurodegeneration seen in Alzheimer's disease and non-Alzheimer's tauopathies that form the core of the paired helical filament (PHF) (8, 9). PHFs and related filamentous aggregates such as straight and ribbon-like filaments (10) in turn make up the hallmark tau lesions [neurodegenerative diseases (NFTs), Pick bodies, etc.] seen in Alzheimer's disease and other tauopathies. However it is likely that at least some of the characteristic features that distinguish tauopathies from other neurodegenerative syndromes have roots in specific normal cellular functions of tau.

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; CJD Creutzfeldt–Jacob disease; CNS, central nervous system; CSF, cerebrospinal fluid; ECF, extracellular fluid; ER, endoplasmic reticulum; FAD, familial AD; GA, Golgi apparatus; GO, gene ontology; HD, Huntington's disease; HSPG, heparan sulfate proteoglycan; LOAD, late onset AD; MAP, microtubule associated protein; MT, microtubule; MTBR, microtubule binding repeat domain; NDD, neurodegenerative disease; NFT, neurofibrillary tangle; NT, neuropil thread; PD, Parkinson's disease; PrP, prion protein; PrP^{Sc}, misfolded, pathogenic prion protein; SNCA, alpha synuclein; TGN, trans-Golgi network.

MT AND NON-MT ASSOCIATED FUNCTIONS OF TAU

Tau is expressed from a single gene on chromosome 17 and is alternatively spliced to yield six different isoforms in the adult central nervous system (CNS). Each of these contains a C-terminal microtubule binding domain (MTBR) consisting of three or four tandem repeat motifs. The best understood "normal" function of tau in mature neurons involves its binding to and stabilizing axonal

MTs via the MTBR. Tau belongs to a family of microtubule associated proteins (MAPs) that includes other neuronal proteins such as MAP1A, MAP1B, and MAP2, and also non-neuronally expressed members (MAP4). Each of these proteins contains a conserved region in and around the MTBR that suggests a common origin via gene duplication. The poor conservation of areas outside of the MTBR in both mammalian tau family molecules and in tau-like proteins in various vertebrate and invertebrate species suggests that they may play species-specific functions.

The regulation of tau-MT binding has been heavily studied and is now well established. Tau-MT binding associated with MT stabilization is mediated by the phosphorylation of serine and threonine residues at sites immediately adjacent to and within the MTBR by a wide variety of kinases [Ref. (11) for a good review]. Phosphorylation of the regions flanking the MTBR produce a stoichiometrically graded reduction in the affinity of tau for MTs, whereas phosphorylation at specific sites within the MTBR (Ser262 and Ser356) abolish virtually all tau-MT interactions (12). Subtleties of tau-MT binding appear to be particularly dependent on the phosphorylation pattern of proline-associated serine and threonine resides on the N-terminal side of the MTBR by proline-directed kinases (e.g., CDK5, MAP kinase 1, GSK3b).

It has become clear that tau has functions in addition to axonal MT stabilization in both mature and developing neurons that involve alternative binding partners for the MTBR (e.g., actin- and actin-associated proteins, heparin sulfate proteoglycan) and/or other parts of the tau molecule, such as the amino terminal projection domain (13). These functions include the integration of cellular cytoskeletal functions with interneuronal signaling pathways. Important developmental functions of tau include various aspects of axonogenesis, such as the establishment of axonal identity (i.e., neuronal polarization) (14) and the subsequent outgrowth (15) and myelination of developing axons. Each of these developmental functions involves MTBR interactions with the subcortical actin network and plasma membrane (16, 17), via either the MTBR itself [actin – Ref. (18)] or via the N-terminal projection domain, which interacts with Src family non-receptor tyrosine kinases such as fyn (13, 19, 20).

DISEASE-ASSOCIATED TAU MODIFICATIONS ARE CORRELATED WITH THEIR DISSOCIATION FROM MTs

Tau hyperphosphorylation

A key alteration that is associated with NFT formation is the phosphorylation of multiple serine and threonine residues in and around the MTBR that normally regulate tau binding to MTs (hyperphosphorylation). Hyperphosphorylation reversibly decreases the affinity of tau for MTs (21, 22) and is consistently seen in PHFs isolated from AD and non-AD tauopathy brains even at early stages in the development of disease (23–27). Hyperphosphorylated tau defined as containing 10 or more moles of phosphate per tau molecule (28) isolated from AD brains has been shown to be capable of self-assembly *in vitro* (29), suggesting that tau hyperphosphorylation may directly induce aggregate formation as well as altering the normal functions of tau. Tau mutations associated with frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) increase the rate and amount of tau phosphorylation and decrease the number of phosphate groups required for aggregation (11, 28). Although clearly associated with PHF formation, hyperphosphorylation may not be a prerequisite for tau aggregation, since tau constructs containing only the MTBRs are able to form filaments in the unphosphorylated state (29, 30). However, dephosphorylation prevents the self-assembly of full-length isoforms, suggesting that the N- and extreme C-terminal regions of tau inhibit this (31, 32). Curiously, phosphorylation of tau at certain residues (Ser262 and Ser 214) that decrease tau-MT affinity act to prevent formation of PHFs (33). This, together with multiple reports of phosphorylation state-contingent kinase specificity for individual sites in the flanking domains, suggests that regulation of both tau:tubulin and tau:tau affinity by phosphorylation is highly subtle and remains incompletely understood.

Tau truncation

The evolution of tangle-intrinsic tau from full-length isoforms to MTBR-only fragments (34) suggests that proteolysis at both the C and N termini of tau may play a significant role in NFT formation. This is directly supported by a number of studies of tau filament formation. Temporal analysis of filament formation in AD brains shows that tau misfolding [recognized by the Alz50 antibody (35)] precedes initial truncation at D421, with subsequent truncation at E391 appearing at later stages of the disease (34). Truncated C-terminal tau fragments can act as nucleation seeds *in vitro*, sequestering full-length tau in both mutant and wild-type forms (11). Such nucleation may be related to conformation specific tau:tau interactions that have recently been proposed to mediate the intercellular propagation of neurofibrillary lesions (36, 37). Truncation of tau at E391 has been identified within the core of PHFs (38) and at D421 in the brains of AD patients (39). The presence of the C terminal has an inhibitory effect on polymerization of full-length tau (31) and C-terminal truncation accelerates tau filament formation *in vitro* (40–42). A study using cells expressing the MTBR of tau containing an FTDP-17 mutation demonstrated that proteolysis of the N and C regions flanking the MTBR produced aggregation-prone fragments capable of seeding further aggregation, while blocking N-terminal truncation of this fragment prevented C-terminal proteolysis, suggesting that this region (near K257) may act to shield downstream residues (11). It should be noted that although C-terminal truncation is sufficient to cause tau aggregation in cellular models, additional mechanisms might drive filament formation in diseased neurons. Tangle-bearing neurons in a transgenic mouse model contained little D421-cleaved tau (43), suggesting that multiple pathways to aggregate formation are likely active in neurons affected by tauopathy. This is consistent with the presence of multiple types of lesions in the various forms of non-AD tauopathy and in AD brain, where neuropil threads (NTs) occur with or perhaps even before the onset of NFT formation (44) and with the characteristic differences in neurofilament content and tau conformation seen in NTs (45) and Pick bodies (10, 46) versus NFTs.

Other variables affecting tau aggregation

In addition to phosphorylation and truncation, there are a number of factors that appear to modulate tau aggregate formation,

some of which are associated with tauopathy cytopathogenesis. Foremost among these are the intronic and exonic point and deletion mutations in and around the MTBR that cause familial non-AD tauopathies [reviewed in Ref. (47)]. The restriction of such mutations to the MTBR and their ability to drive the neurofibrillary pathogenesis of presenile tauopathies constitutes some of the strongest evidence for the importance of tau aggregation in human disease. Polyanions such as heparan sulfate proteoglycan (HSPG) are abnormally distributed in pre-tangle and tangle-bearing neurons and can catalyze the formation of straight filaments and PHFs *in vitro* (48, 49) regardless of the phosphorylation state of tau (30, 50, 51). Specific isoforms of tau also differ in their ability to form filaments *in vitro*. Different ratios of three and four repeat (3R and 4R) tau isoforms are characteristic of tau aggregates found in specific tauopathies (26, 52) and the presence of intronic mutations interfering with the splicing of exon 10 in patients with FTDP-17 (30, 53) confirms that the resulting change in the ratio of 3R:4R tau is sufficient to drive tau filamentation and neurodegeneration (54). Three and four repeat isoforms differ in characteristics that may affect their participation in NFT formation, including the ability to form disulfide bridges (55) and their relative affinity for MTs and fyn kinase (56). The presence of 3R tau may reduce the tendency of 4R tau to form filaments (57), possibly by interfering with disulfide bond formation. Finally, the larger cellular and genetic context is likely to have a significant bearing on the tendency of tau to aggregate into NFTs. Early cellular changes involving lysosomal and autophagy pathway abnormalities may either result from or modulate tau aggregate formation (58, 59). Genetic factors such as the H1 haplotype, which affects the overall expression levels of tau and the splicing of exon 10 and possibly exons 2 and 3 (60), and the presence of ApoE4 allele have been reported to be associated with high disease incidence (61). Additional genetic factors that affect tau aggregation and thus disease propensity include interactions with elements on chromosome 21 that affect tau splicing and the function of non-APP proteins such as DYRK1A (53, 62) as well as APP itself (63). Overall, tau interactions with non-MT elements via its MTBR and in particular, tau:tau interactions associated with aggregation and their involvement in tau-mediated neurodegeneration have been a subject of intensive investigation. However, while a great deal is now known about both the mechanisms of tau aggregation and the circumstances of tau modifications associated with aggregate formation, it remains unclear exactly how each of these elements contributes to tau cytopathogenesis. This uncertainty has been exacerbated by the identification of tau toxicity mechanisms that are not associated with the MTBR but which may also play important roles in neurodegeneration.

REACTIVATION OF DEVELOPMENTAL TAU FUNCTIONS IN NEURODEGENERATION

While tau aggregation is clearly a central factor in tauopathy pathogenesis, the role tau plays in the development of axonal identity and other aspects of axonogenesis has increasingly been linked to neurodegenerative disease mechanisms. These include the appearance of cell cycle markers (64) and ectopically sprouting axonlike processes (NTs) emerging from the dendrites (45, 65–67) during the development of neurofibrillary lesions in neurodegenerative tauopathies. Dendritic NTs in particular suggest that tau functions in the development of axonal identity may play a role in the early stages of AD where they may reflect damage to mechanisms that maintain the terminally differentiated neuronal state (68, 69). The "fetal" (3R0N) tau isoform is the shortest three repeat tau isoform with the lowest binding affinity for MTs (30), possibly reflecting the far greater importance of tau N-terminal interactions and functions during development relative to the role tau plays in the mature CNS. A key tau interactor during development is the non-receptor tyrosine kinase fyn, which plays a critical role in axonal outgrowth and myelination (15, 70). Interactions between the tau N terminus and signaling molecules such as fyn typically occur in the actin-rich cortical cytoskeleton largely in the absence of MTs and are necessary for functions such as growth cone motility (16). Fyn, like tau, localizes to NFTs (20, 71) and is essential to the development and possibly the propagation of Abeta-mediated toxicity in mouse models of AD (72). Fyn-mediated interactions with tau play a role in the localization of a small amount of tau to the plasma membrane (73), particularly at dendritic loci (74) where it is involved in synaptic functions associated with learning and memory (75–77). Abnormal interactions of tau with fyn kinase increasingly appear to play a critical role in membrane-associate tau dysfunction especially via the generation of synaptotoxic species of Abeta from APP. This occurs in the context of fyn-mediated phosphorylation of APP in early endosomes (78) and in turn exacerbates both tau localization to rafts and tau phosphorylation by fyn (79). Such correlations suggest that tau mislocalization to dendrites and the generation of abnormal amounts of Abeta may interact synergistically to produce both cytotoxicity and abnormal developmental events such as NT growth and cell cycle re-entry (80) in AD neuropathogenesis.

INTERNEURONAL ASPECTS OF TAUOPATHY

Tau interactions with lipid raft proteins such as fyn and (possibly) APP may be centrally important in two recently emerging interneuronal aspects of tau pathobiology: (a) tau secretion and interneuronal transfer via unconventional mechanisms and (b) tau:tau interactions involved in templated misfolding and "prion-like" lesion spreading mechanisms. Membrane localization favors tau oligomerization, which increasingly appears to be a key event in at least some forms of tau toxicity. Membrane-associated tau functions mediated by its N terminus appear to be linked to the diversion of tau from the cytosol to membrane-bound vesicles, in particular those associated with the trans-Golgi network and the autophagosome-lysosome pathway (81). As a consequence, it is likely that both cellular and molecular aspects of interneuronal lesion spreading have their roots in membrane-associated tau misprocessing mechanisms.

Selective neuronal vulnerability to tau toxicity

Ever since it became apparent in the mid-1980s that Alzheimer's disease is a common neurodegenerative condition of the elderly as opposed to a relatively rare familial syndrome, it has been clear that the number and distribution of NFTs is strongly correlated with progressive cognitive loss (82, 83). There are two possible mechanisms that might account for disease-associated

stereotyped patterns of lesion development with increasing disease severity. One possibility is that loci that are affected early in the disease sequence are more vulnerable to the mechanisms underlying tau-induced degeneration at the cellular level. A hierarchy of vulnerability to tau toxicity might then result in a stereotyped sequence of lesion development over time. One characteristic shared by brain regions that develop early tau lesions in AD is synaptic plasticity; highly plastic cortical pyramidal neurons such as those of the hippocampus are inherently vulnerable to excitotoxic insults by virtue of their glutamatergic pharmacology and the prevalence of LTP-associated plasticity mechanisms (84). Selective vulnerability may also be acquired via injury, as with the greatly increased risk posed by antecedent head trauma (85–87). It seems increasingly likely that both intrinsic and extrinsic selective vulnerability factors interact with lesion spreading mechanisms associated with synaptic connectivity patterns (discussed at length below) to produce the variety of clinical syndromes associated with neurofibrillary degeneration.

Interneuronal movement of misprocessed tau

For many years, the interconnectedness of affected regions and the highly stereotyped, disease-specific pattern of neurofibrillary lesion spread within the brain in AD and non-AD tauopathies has suggested that the actual interneuronal transfer of a toxic factor must be involved in the progression of neurodegenerative tauopathies (88–91). The actual movement of toxic tau species between neurons is now thought by many to be the primary mechanism mediating the progressive appearance of tau pathology and clinical dysfunction in tauopathy, including those associated with repeated head injury (87). In particular, the templated misfolding mechanism that mediates the infectivity of prion diseases such as CJD is increasingly invoked as a model for the interneuronal transfer of tau-mediated toxicity (36, 92–97), especially in murine transgenic models of tauopathy (98–100). Evidence that different abnormal conformations of PrP may in fact define different prion-mediated diseases (101, 102) raises the possibility that very different clinical presentations and neuronal vulnerabilities could be attributable to molecular level differences in a single protein. However, the study of tau as a "tauon" [a term for a tau species that spreads toxicity via templated misfolding coined by Novak et al. (93)] has been conducted largely in the absence of a cellular context for the actual transfer of tau between neurons, making it difficult to connect the molecular mechanisms involved in protein templating to specific cellular events and mechanisms associated with tau cytopathology. In particular, while the secretion (103–105) and uptake (92) of tau itself in cellular models has been demonstrated and linked to the elevated tau levels in early AD (106), it is unclear how secretion is related to the spreading of neurofibrillary lesions and whether that occurs via an oligomer-associated mechanism, such as templated misfolding or ionophore formation (107, 108), or via some mechanism that does not require the tau MTBR at all (109–112). The subtle but important questions that remain about the relationships between the development of NFTs, toxic oligomer formation, templated misfolding, N-terminal tau toxicity, and the actual cellular mechanisms responsible for secretion and uptake of misprocessed tau species will be explored at greater length below.

CURRENT FOCI OF TAUOPATHY RESEARCH

THE BIOGENESIS OF NEUROFIBRILLARY TANGLES

The development of NFTs, the characteristic tau lesions that develop in cortical pyramidal neurons during the course of AD, has been relatively well characterized but is still incompletely understood. NFT development begins with the abnormal phosphorylation of tau at multiple sites in and around the MTBR, accompanied by abnormal somatodendritic tau accumulation resulting in the appearance of pre-fibrillary phosphorylated punctate deposits in the cell body and dendrites of affected neurons (24, 113). These eventually form condensed fibrillar deposits near the nucleus and near dendritic branch points, displacing normal cytoskeletal elements such as MTs (113, 114). Over time, these fill most of the cell and take on a characteristic flame-shaped appearance. Eventually, the neuron containing the NFT dies and the NFT remains as a "tombstone" lesion or "ghost" tangle.

While all tau isomers and cleavage fragments that contain the MTBR appear to be capable of forming filaments, their precise morphology may vary considerably; they can appear as straight, ribbon-like, or any of a variety of PHF-like structures, all of which are reproducible under *in vitro* conditions (10, 46, 115). Moreover, the wide variety of tau modifications that affect aggregation suggest that considerable subtlety exists in the mechanisms responsible for tau assembly. Such factors include the presence of tauopathy-inducing point mutations (116, 117) the presence/absence of the C-terminal (31) or N-terminal (10, 32) domains, and variations in the experimental conditions used (118). The fact that the range of filaments and aggregate types seen with human tauopathies can be reproduced in sporadic tauopathy syndromes without benefit of tau mutations indicate that additional relevant variables to tau aggregate form come from cellular factors, such as the inclusion of NFs (Pick bodies) or internal membrane elements (granulovacuolar degeneration) or even the shape of the host cell. Understanding how molecular and cellular factors interact to produce a specific type of aggregate can thus be seen as an index of our overall grasp of tauopathy pathogenesis.

Direct formation of NFTs from cytosolic tau

A key unresolved issue in NFT formation is the cellular context in which NFTs form during the course of neurodegenerative disease. In particular, it is unclear whether NFTs form as a result of cytosolic or even MT-templated interactions between tau species or whether NFT formation requires additional cellular elements, such as membrane-bound vesicles, to occur. The most straightforward hypothesis of NFT generation and growth is direct assembly from free tau monomers and/or oligomers in the cytosol. The ability of tau filaments to form *in vitro* by MT-templated mechanisms (119), and the ability of tauopathy mutations to actively disrupt MT networks (116) as well as favor filament formation (117, 120) are all consistent with the idea that tau aggregate formation, eventually leading to NFTs, begins immediately upon dissociation from MTs or even by conformational changes that occur in MT-bound tau. For instance, tau oligomerization has been observed to occur on the surface of MTs (119), which might then generate cytosolic tau oligomers capable of directly seeding NFT growth. This seems to account directly for the granular pre-fibrillary deposits that constitute the earliest visible stage of NFT formation (121, 122) and

is consistent with *in vitro* experiments demonstrating that tau filament assembly resulting in NFT formation is potentiated by tau interactions with fatty acids and/or polyanionic molecules such as heparin sulfate proteoglycans and RNA (30, 50, 123, 124). Moreover, electron and atomic force microscopy studies have revealed numerous intermediates in tau fibril formation, including abnormally folded monomers capable of aggregation (125) and granular tau aggregates that may be formed from these monomers (121, 122). All of these observations are consistent with the direct formation of NFTs from cytosolic tau oligomers and this mechanism is therefore widely assumed to account for the biogenesis of most, if not all, NFTs.

Does vesicle-associated tau contribute to NFT formation?

Studies of tau biology raise the possibility of an alternative (or perhaps additional) mechanism to direct oligomerization in the cytosol. The catalysis of tau oligomerization by fatty acids is as consistent with a membrane-mediated assembly mechanism as it would be with a cytosol-only mechanism. More direct support for membrane-mediated NFT formation includes electron microscopic observations of membrane-associated PHFs in AD brain (126) and the experimental generation of tau filaments *in vitro* on the surfaces of anionic micelles (127). This occurs via tau intermediates containing β structures whose conformations are dependent on the presence of the anion and which are capable of seeding tau fibril formation. Tau that is not associated with MTs interacts with actin (18) and/or actin-associated proteins (128, 129) causing misprocessed tau to accumulate under the plasma membrane and in membrane-bound vesicles in cellular tauopathy models (19, 81, 105, 130, 131). This could plausibly result in tau endocytosis leading to vesicle-associated tau being returned to the perinuclear region via the retromer pathway, followed by NFT formation via membrane-templated oligomerization (125, 127). The Golgi apparatus and the autophagy-lysosome pathway are additional possible sources of tau-bearing vesicles that could contribute to NFTs. Abnormalities in Golgi structure have been identified both in association with tau overexpression (132) and with NFTs in AD brain (133). A recent study of PHF tau uptake in neuronal cultures suggested that endocytosed tau aggregates eventually form perinuclear aggresome-like deposits, which also suggests involvement of trans-Golgi "retromer" pathways in NFT formation (134). Similarly, the disruption of proteasome/macroautophagy (autophagy) mediated tau turnover is a prominent early element in pre-fibrillar changes in AD cytopathology (59), as evidenced by the polyubiquitination of tau in NFTs (135). Experimental disruption of lysosomal function via chloroquine administration induced lysosomal accumulation of tau aggregates (136), suggesting that disease-associated changes might contribute to NFT formation in AD via a similar mechanism. Similar patterns of somatodendritic tau distribution and associated signs of toxicity are seen in cortical pyramidal neurons with pre-fibrillar tau deposits or nascent NFTs (24, 113, 114, 137). These patterns suggest a role for vesicle-associated tau in both local tau cytotoxicity and in the generation of NFTs (81, 138). Examples are shown in **Figure 2**. In both cases, tau accumulates first in distal, membrane-rich dendritic structures (113, 139) and then at branch points and along dendritic shafts, where it is correlated with local MT loss, causing varicosities to appear in dendrites (113, 131). In the lamprey model, which is the only *in situ* tauopathy model from which high resolution localization information is available, these are associated with the accumulation of tau-bearing vesicles (and membrane-bound organelles such as mitochondria) at either end of the varicosity, suggesting that they are due to the failure of bidirectional MT mediated transport (**Figure 1B**). The accumulation of tau-containing vesicles at dendritic branch points is likely due to the shift in MT polarity patterns (140) typically found there, which induces the accumulation of vesicular organelles such as mitochondria. This vesicular build-up may be either the cause or consequence of MT-mediated transport failure (141–143).

TAU TOXICITY MECHANISMS – ARE NFTs TOXIC?

The widely observed correlation between NFT distribution and neurodegeneration in nearly all tauopathies including AD (82, 83, 113, 114) has led to the widespread assumption that NFTs are an integral feature of tau neurotoxicity. Although toxic tau aggregates are notoriously difficult to generate in cell culture from wild-type tau isoforms (144), studies with hyperaggregating tau mutants have demonstrated that cleavage products are toxic when expressed in culture, with aggregate formation and apoptotic cell death occurring within 24–48 h of tau expression (145, 146). That said, it is becoming increasingly evident that NFTs may not be the agent driving neurotoxicity in whole animal tauopathy models. In *Drosophila*, expression of both mutant and wild-type human tau leads to AD-like pathology (late onset neurodegeneration, selective toxicity of cholinergic neurons) in the absence of NFTs (147). In addition, the long time courses (up to 20 years) proposed for NFT formation based on imaging data from AD brain (148–150) are not consistent with direct causality between NFTs and tau toxicity. While NFT growth is largely irreversible in inducible mouse tauopathy models, more dynamic aspects of tau toxicity are clearly reversible (151–154), suggesting that the mature NFT itself is much less toxic than the events associated with building it. Dendritic and axonal changes associated with tau accumulation and NFT formation in both transgenic mice (155) and AD brains (114, 156) appear to be correlated with abnormal mitochondrial distribution, which in turn recruits low ATP and Ca++ mediated toxicity mechanisms (144). Ca++ mediated tau toxicity is also suggested by the effects of specific tauopathy mutations on Ca++ channel properties (55) and high resolution correlations between localized secretion, MT loss, and accumulations of vesicular tau (81, 131) as illustrated in **Figure 1**. These findings and others have complicated our understanding of the relationship between NFT distribution and abundance and the actual toxicity mechanisms driving human neurodegenerative disease. Recent studies have also tended to dissociate degenerative cellular changes from NFT formation. It has even been suggested that large tau aggregates such as NFTs may serve a neuroprotective role (157, 158), preventing hyperphosphorylated tau from sequestering normal, MT-bound tau. Studies in other tauopathy models have also called into question the role played by hyperphosphorylation in the chain of events leading to degeneration; recent fly model studies (159) have suggested that hyperphosphorylation can be neuroprotective by blocking other aspects of tau toxicity, such as apoptotic changes associated with cell cycle re-entry (80, 160).

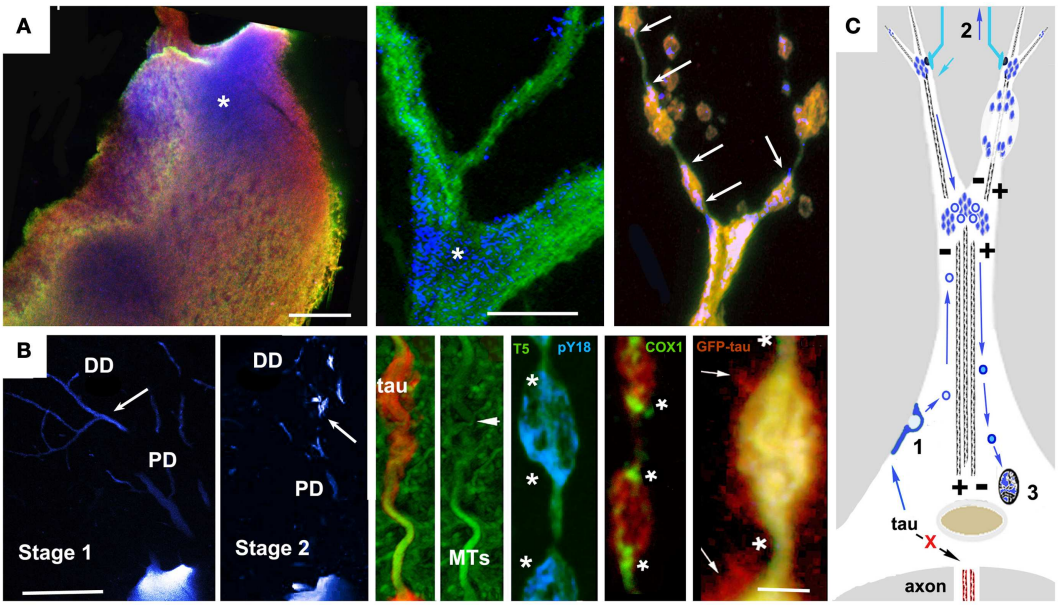

FIGURE 1 | Accumulation of vesicular tau in ABC dendrites and at dendritic branch points causes local transport failure, MT loss, and localized secretion from dendrites. High resolution confocal imaging of somatodendritic tau accumulation in the lamprey tauopathy model suggests a cellular mechanism to account for the relationship between localized tau toxicity, somatodendritic MT loss, and the pattern of NFT evolution in pyramidal neurons as described by Braak et al. (113), Braak and Braak (137), and Blazquez-Llorca et al. (139). **(A)** Cell body (left) and dendritic branch point (center) of a lamprey ABC expressing full-length 4R0N human tau bearing the P301L tauopathy mutation before the onset of tau-induced degeneration. Tau is triple labeled: MT associated (Tau5, green channel), MT dissociated (9G3, blue channel), and total tau (GFP epitope tag red channel). Tau phosphorylated at Y18 (pY18 tau or 9G3 positive MT dissociated tau) is accumulating at the base of large dendrites and at branch points, a pattern typical of MT-transported vesicles (asterisks). The rightmost panel shows pY18 tau accumulating at either end of dendritic varicosities (arrows). **(B)** Left panels show pY18 distribution in non-degenerating (Stage 1) and degenerating (Stage 2) ABC dendrites. Distally transported tau is distributed throughout distal (but not proximal) dendrites in non-degenerating cells, but becomes localized to dendrite branch points and varicosities with the onset of degeneration [arrows – see Refs (81, 165)]. Center and right: Dendritic beading is caused by the localized failure of MT mediated transport, resulting in the accumulation of pY18 (fyn phosphorylated) tau associated with vesicles and membrane-bound organelles. The accumulation of mitochondria (COX2 label) is particularly well marked. With the onset of dendritic degeneration, total and pY18+ tau accumulates at each end and eventually in the center of dendritic varicosities in what appear to be MT-transported vesicles. The localized secretion occurring in the vicinity of such deposits suggests that tau-bearing vesicles first destabilize the MTs responsible for their transport, accumulate in the resulting varicosities and are then secreted. While the mechanism responsible for this has not been demonstrated directly, the concomitant loss of MTs and localized secretion suggests a Ca++ flux mediated mechanism. **(C)** A model for vesicle-associated tau in NFT formation and cytodegeneration. Failure of tau to become axonally localized and bind axonal MTs results in actin association and endocytosis (1). Tau-bearing vesicles are transported both distally and proximally on dendritic MTs, accumulating at dendritic branch points (where MT polarity patterns favor localized cargo accumulation Aronov 01) and near synaptic terminals (2), where it may become locally toxic possibly via interacting with Abeta in synapse-associated endosomes, resulting in structural failure of dendrites (top right) and uptake by afferents resulting in retrograde trans-synaptic movement. Synaptic activity may also result in the centripetal transport of tau-bearing vesicles to the Golgi apparatus (3) where it may modulate NFT formation. Scale bars: **(A)**: 20 μ, **(B)** 100 μ (left), 5 μ (right).

Oligomer-associated toxicity mechanisms

Tau oligomers have been the most widely proposed candidate for the toxic intermediate species in NFT biogenesis responsible for the correlation between neurofibrillary lesions and neurodegeneration in AD and non AD tauopathies (83). The toxicity of oligomeric tau is suggested by numerous correlative studies (161) and in particular with respect to the dynamic effects of tau aggregation; for instance, the concentration of tau multimers (162) but not large aggregates or monomers (95) in the brains of tauopathy mice are correlated with memory and cognitive deficits.

Oligomer-mediated membrane permeability changes. The structural similarity between amyloid proteins associated with neurodegeneration supports the existence of a common toxicity mechanism based on common properties of such proteins such as their propensity for oligomerization and close association with membrane. The toxicity of amyloid oligomers unrelated to neurodegenerative diseases suggests that a specific, shared conformation may be responsible, with toxicity being mediated by mitochondrial dysfunction associated with an increase in reactive oxygen species (141, 163). Oligomers of several different amyloids cause an increase in ion conductance across lipid bilayers (164) raising the possibility that they might alter or the permeability of the plasma membrane, resulting in increased internal [Ca++] and associated toxic changes. Both Ab and SNCA can form oligomeric structures that increase ion permeability of synthetic vesicular membranes and which may allow them to create pores within cell membranes (107). Leakage of cellular contents across the plasma membrane in SH-SY5Y cells was observed after the external application of tau oligomers (108), suggesting a similar toxicity

mechanism for tau. These results collectively suggest that tau oligomers may mediate any of several toxicity mechanisms associated with Ca++ dysregulation or abnormal generation of reactive oxygen species. Such changes are typically seen with excitotoxicity and mitochondrial dysfunction, both of which appear to be important elements of AD-associated neurotoxicity (141). Studies

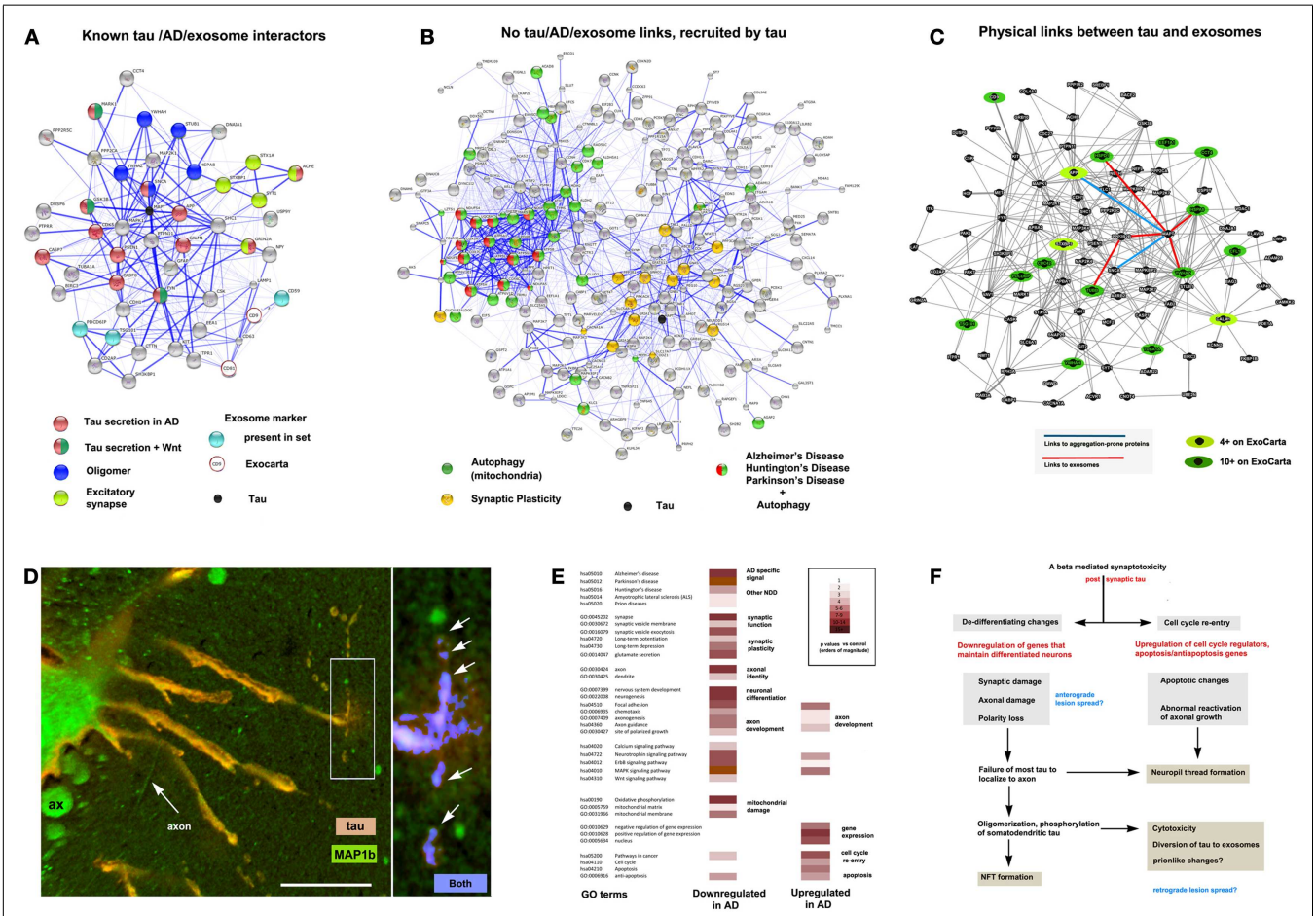

FIGURE 2 | The context of exosomal tau secretion and transneuronal neurodegeneration. **(A)** Connectivity diagrams summarizing proteomic analysis of the exosomal proteome associated with tau overexpression from neuroblastoma cell cultures (106) using the String online dataset (104). GO term analysis shows that exosomal tau secretion involves factors with known links to tau misprocessing (APP, oligomerization, Wnt pathway) and also suggests the involvement of mechanisms with less established and no apparent links to tau, AD, and exosomes. Of the ∼660 proteins identified, 50 were both present on the AlzGene list (http://www.alzgene.org/) of 616 AD related proteins and had clear links (0.4 confidence or more (104)) to tau and/or exosomal markers (CD9 and CD81 were not present in our set, but were part of the probe query) – see ExoCarta (239) via the String 9.05 connectivity algorithm. Note the strong linkage to tau (MAPT – shown as a black circle for reference). **(B)** Another group (right diagram) consisted of internally connected proteins that did not have clear functional links to tau on String (confidence <0.4 or not detected). These nonetheless had significant signals for AD, PD, and HD that were tightly linked to mitochondrial markers, suggesting the association of abnormal autophagy with tau secretion, and with synaptic plasticity (LTP, LDP). All terms shown reflect significant enrichment ($p < 0.001$) by the String algorithm. **(C)** Connectivity diagram from GeneMANIA (http://www.genemania.org) showing physical interactions between proteins isolated from exosomes using the same set as shown in **(A)**. In order to identify proteins typically localized to exosomes, we scored each protein for the number of times it appeared in the nearly 70 different mammalian exosomal datasets at ExoCarta. Links between tau and highly exosome associate proteins (dark green icons), mildly exosome associated (light green icons), and aggregation-prone proteins such as SNCA and APP (Abeta) are shown as well to illustrate the plausibility of tau diversion to exosomes in the context of these co-purified proteins. **(D)** Expression of 4R0N tau in lamprey ABCs produces dendritic localization of tau (red channel), dendritic degeneration, and localized focal secretion (left box, orange label) as shown in a confocal micrograph after 10 days of expression (81). Immunolabel for LC3/MAP1b (green channel) shows cytoskeletal localization in non-transfected giant axons (seen in cross section – ax), and in circumferential axons (arrow axon). One of the latter has taken up secreted tau shown at higher magnification (right) and is exhibiting toxicity in the form of varicosities (arrows) similar to those shown in ABC dendrites (dendrite, center). Highly co-localized tau (blue channel) shows cleaved, autophagosome-associated LC3II associated with tau (arrows) in a pattern similar to that seen for dendritic mitochondria in **Figure 1B**. Scale bar: 50 μ (left), 10 μ (right). **(E)** Consensus sets of 1575 downregulated and 1383 upregulated proteins in both LOAD (238) and early onset FAD (237). **(F)** Schematic outlining a hypothesis of AD cytopathogenesis that accounts for tau associated exosomal secretion and downregulation of synaptic and axonal proteins in AD (red) in the context of current knowledge. Likely triggers for tau secretion and trans-synaptic lesion spreading (blue) may occur either directly from the axon due to loss of axonal MT integrity and secretion regulation, or indirectly from dendrites as the results of toxicity caused by somatodendritic tau accumulation.

in cellular tauopathy models showing a correlation between localized cytotoxicity, tau membrane association, and localized MT loss (81, 130, 165) are consistent with this (see examples in **Figure 2**), as is the presence at the plasma membrane of polyanionic molecules known to catalyze tau oligomer/filament formation such as HSPGs (30, 166) and RNA (123).

Oligomers as prions. The "prionlike" toxicity and propagation mechanisms recently proposed for tau may be considered as a special category of oligomer-mediated toxicity, if one makes the assumption that a prionlike misfolded tau conformation would be toxic and would propagate in a manner similar to that of the PrP itself. However, despite the considerable amount of research effort devoted to understanding the relationship between propagation and toxicity mechanisms of the PrP, this relationship remains quite unclear. Mutant forms of PrP that do not create large amounts of misfolded, pathogenic prion protein (PrP^{Sc}) with high beta-sheet content in plaques have still been shown to generate CJD-like syndromes in mice (167). Moreover, interference with the normal GPI linkage that anchors PrP to the membrane can affect the ability of the mutant form to propagate interneuronally and generate a clinical syndrome but without affecting local cytotoxicity (168). Tau misprocessing appears to have a number of parallels to PrP toxicity in this regard; like PrP, tau oligomerization, and post-translational modifications that favor tau oligomer formation (e.g., hyperphosphorylation, truncation) are closely associated with toxicity. In the case of tau, it remains unclear whether higher-level oligomers and/or polymers propagate interneuronally in human tauopathies. Recent studies using murine tauopathy models have suggested that they are capable of this (98, 99, 169).

Disruption of protein turnover pathways. The dysfunction of proteasomal, autophagosomal, and lysosomal pathways with protein aggregate formation in tauopathy is a candidate for mediating tau toxicity as well as NFT formation, and may play an important role in the development of other tau containing cellular lesions, such as granulovacuolar degeneration [Ref. (170), reviewed in Ref. (171)] in which tau accumulations appear to be membrane associated. The polyubiquitinated state of NFT-tau suggests involvement of the ubiquitin-proteasome pathway (135). Additionally, the colocalization of tau aggregates with high concentrations of acid hydrolases in granulovascular degeneration (GVD) bodies in the hippocampal neurons of AD patients suggests that they may be the result of incomplete autophagy (172, 173). Lysosomal activation is also observed in cultured cells transfected with mutant tau and in mice expressing mutant tau transgenes (174). Inhibition of lysosomal proteases by tau misprocessing causes the accumulation of amphisome-resembling vacuoles in cultured neurons that are morphologically similar to those seen in AD brains. Inhibition of vesicular transport by tau, especially N-terminal tau fragments (175) may also prevent the fusion of autophagosomes and lysosomes (176), causing the retention of tau-bearing autosomes in axons and dendrites, where their accumulation may result in localized degeneration and even tau secretion [**Figure 2**, also see Ref. (81)]. One way that dysfunction of the autophagy-lysosomal machinery caused by tau aggregates may induce toxicity is enhancing tau oligomerization by the generation of hyperaggregating tau cleavage fragments due to the incomplete activity of lysosomal proteases. Cathepsin L has been shown to generate aggregation-prone fragments mutant but not wild-type tau via association with Lamp2A and Hsc70 on the cytosolic face of the lysosomal membrane (177). Tau-induced disruption of autophagy may also recruit synergistic effects related to the production of Abeta from APP, since PS1 is also necessary for autophagosome-lysosome fusion and lysosomal proteolysis (178). It should also be noted that the vesicle trafficking pathways associated with aggresome formation may also become abnormally involved in tauopathy as demonstrated by a recent study in which tau aggregates were endocytosed and then localized to perinuclear deposits (134).

Non-oligomer-mediated tau toxicity mechanisms

Receptor-mediated toxicity. An alternative to oligomer/aggregate associated mechanisms of tau toxicity to explain the selective vulnerability of neurons and spread of NFTs through synaptically connected regions in Alzheimer's disease is toxicity in response to tau binding to extracellular receptors, especially those for synaptic transmitters such as glutamate and acetylcholine. Tau is toxic to neurons in culture when applied extracellularly (179), apparently via the generation of Ca++ fluxes via the activation of M1 and M3 muscarinic acetylcholine receptors (180), which bind tau with a greater affinity than acetylcholine. This is consistent with the preferential distribution of muscarinic acetylcholine receptors on entorhinal and hippocampal pyramidal neurons, and accounts for their vulnerability in early stages of Alzheimer's disease (181). Interestingly, the dephosphorylation of secreted tau by tissue-non-specific alkaline phosphatase could potentiate its high-affinity binding to muscarinic receptors of nearby neurons (182), thus accounting for the "clustering" lesion spread patterns characteristically seen in tauopathies (90, 91, 183).

The generation of Ca++ fluxes via receptor-mediated toxicity need not directly involve aggregate or oligomer formation as a variety of MTBR− tau species appear to form multiple toxic fragments via Ca++ mediated activation of calpains and caspases in neuroblastoma cultures (109, 184) in at least some cases by NMDA receptor activation (109). A well studied fragment generated by calpain activity is a 17-kDa N-terminal fragment consisting of tau residues 45–230; this is toxic when applied to aged primary hippocampus cells and when expressed in the brains of transgenic *Drosophila* and in ABCs in the lamprey tauopathy model (142, 185, 186). The 17-kDa fragment is elevated in cortical neurons of AD and tauopathy patients that exhibit increased calpain activity (112) and is unusual among toxic tau species in its ability to produce neuritic pathology in neuroblastoma lines (185). However, it remains unclear if the 17-kDa fragment produced by calpain cleavage is a cause of neuronal death in tauopathy (187). Another N-terminal tau fragment with a molar mass of 20–22 kDa was found enriched specifically in the synaptosomes of AD brains in comparison to control brains (184). When overexpressed in primary rat neurons, an N-terminal tau fragment containing residues 26–44 impaired mitochondrial function and caused neuronal death (188). Down-regulation of calpains in a tauopathy model of *Drosophila* was associated with decreased neurodegeneration (186). Both the tau N-terminal domain and fyn-tau interactions are associated with the mechanism of Ab toxicity that is mediated by the presence of

tau (63, 74, 111), as are many fragments generated by similarly activated caspases (189, 190).

Can "prionic" and receptor-mediated N-terminal tau toxicity coexist? The ability of tau to mediate Ab toxicity in the absence of the MTBR (111, 190) and the potential for NFTs to act as neuroprotective agents (157, 158, 191) raise questions about how large a role tau aggregate toxicity plays as a direct agent of neurodegeneration, at least in AD (112, 192). The links between toxic N-terminal tau fragments and excitotoxicity are consistent with the known vulnerability of cortical neurons with glutamatergic inputs and high levels of synaptic plasticity to AD (84) and the strong association between Ab mediated toxicity in early stage disease with synaptic dysfunction (193), suggesting that oligomer toxicity is not necessarily required for neurodegenerative disease pathogenesis. We recently pointed out that receptor-mediated toxicity mechanisms could account for neurofibrillary lesion propagation via Ca++ mediated activation of calpains and caspases, which could (at least in theory) generate both NFTs and secretable toxic fragments in downstream neurons, which could then repeat the cycle. Ironically, such a mechanism would technically fulfill the requirements of the original Prion Hypothesis, which makes no mention of templated misfolding (194). It thus seems safe to say that coexistence is not only possible but necessary given what we know (and don't know) about how tau toxicity actually operates in neurodegenerative disease.

TAU SECRETION

Cellular mechanisms of tau secretion

Tau secretion by neurons via multiple biologically distinct pathways has been demonstrated both in culture and *in situ* (105, 106, 195), despite its lack of a signal peptide and of lipidation or GPI anchor sites that would permit its secretion via the conventional ER/Golgi route. Tau resembles other aggregation-prone proteins with key roles in neurodegenerative disease (i.e., SNCA, PrP, and Ab) in that it is secreted at least in part via the exosome pathway (106, 196–198); however the full range of secretion routes and their significance to tauopathy pathogenesis remain unclear. Human tau phosphorylated at Thr181 (epitope AT270) is secreted in exosomes in culture and is also found in exosomes in the cerebrospinal fluid (CSF) of early stage AD patients (106). The early appearance of exosomal CSF tau in AD argues strongly in favor of CSF-tau biogenesis by active secretion rather than passive postmortem release, since it occurs at a stage (Braak Stage 3) when neurofibrillary degeneration is restricted to a small proportion of the brain (199). The N terminal of tau, which interacts with the plasma membrane and membrane-associated proteins, is required for secretion in culture and in an *in situ* lamprey model (195), where tau secretion occurred in two distinct patterns depending on the presence of the MTBR, a pattern consistent with the CSF-tau species observed in AD (200). In the lamprey model, N-terminal tau species lacking the MTBR become distributed in a diffuse, gradient-like pattern with secretion occurring from the soma, while full-length tau was secreted from the dendrites in discrete foci (195), where it set up much steeper tissue gradients, suggesting a role for interactions between the tau MTBR and extracellular matrix elements in the distribution of extracellular tau that could have relevance to interneuronal tau toxicity patterns and lesion spreading mechanisms (81). Interestingly, the secreted tau in the lamprey model is largely dephosphorylated, which is consistent with the phosphatase activity described by Diaz-Hernandez and co-workers (182), although no overt toxicity to non-expressing cells was observed. Secreted tau cleaved at the C terminal has been observed in cell culture (105), and in transgenic mouse models expressing human tau as well (201, 202). One of these studies suggested that cleavage at D421 as well as phosphorylation increased the rate of tau secretion (203), which is consistent with the low level oligomerization observed in exosomal tau purified from CSF samples (106).

The method(s) by which tau undergoes secretion remain elusive, although most evidence supports an unconventional secretion pathway resulting in the release of tau in membrane-bound vesicles (81, 106, 195, 203), where it is favored by the absence of the MTBR and/or the E2/E3 inserts in the secreted tau species (195). The failure of 4R2N tau to localize to and be secreted via exosomes is consistent with this as well (134), as are recent demonstrations that minute amounts of full-length, non-vesicle-associated tau can be released by induced forebrain iPS cells in culture (204). Such findings are consistent with a specific, non-universal release mechanism for tau that is associated with exosomes, as is the failure of an earlier study to detect exosomal tau secretion from healthy cortical neurons (205). Vesicle-free tau secretion has also been reported from neuroblastomas (106, 204) and may also occur in the lamprey tau secretion model (195). It thus remains possible that the presence of non-vesicular tau in the extracellular space in any model is due to post-secretion release of tau from exosomes or other microvesicles (105, 106, 195, 201, 202). Conversely, it could be that the presence of tau in exosomes may be due to the passive adherence of extracellular tau to exosomes that have already been released. This seems unlikely, given: (a) the restriction to E2− tau seen for exosomal tau and (b) the degree of interconnectivity of exosomal proteins associated with E2− overexpression via both functionally (**Figure 2C**) and via observed physical interactions (**Figure 2C**). Overexpression of tau in culture causes cells to secrete exosomes containing tau that has been phosphorylated at several proline-directed sites (106), possibly as a protective response to high concentrations of membrane-associated tau (206). Overall, it appears that tau may be secreted via multiple mechanisms from neurons in tauopathy, including microvesicle shedding, exosomal secretion via endocytosis and fusion with multivesicular bodies, and exophagy, a pathway involving the diversion of autophagosomes to exosomes (69, 81, 134, 207). Uptake mechanisms into trans-synaptic and adjacent cells have been characterized in even less detail that have tau secretion pathways. In the lamprey system, uptake has largely mirrored the "focal" and "diffuse" secretion mechanisms, with specificities for MTBR+ and tauopathy mutations and MTBR−, E2− tau respectively. A recent study of PHF tau uptake found evidence for perinuclear localization of tau in aggresome-like bodies (134), which is consistent with the scheme suggested in **Figure 1**. While the uptake of tau aggregates has been addressed by a number of recent studies directed at "prion-like" interneuronal transmission mechanisms via oligomeric tau species, the general mechanisms that might mediate tau uptake have not yet been characterized in detail in cell culture.

Interneuronal propagation of tau lesions and prions

Injection of brain extract from mutant human tau P301S transgenic mice into the brains of mice expressing wild-type human tau caused aggregation of wild-type tau in anatomically connected regions of the brain at a distance from the inoculation site (169). Sequential progression of tau aggregation (but not the transfer of individual tau molecules) between synaptically connected regions of the brain was later confirmed in mouse models in which human tau expression was spatially limited to synaptically connected areas of known vulnerability to neurofibrillary degeneration such as the entorhinal (98) and cingulate cortices (99). Further studies using antibodies specific to oligomerized tau have demonstrated that pre-fibrillar tau in AD brain is in fact oligomeric (208) and that tau oligomers derived from the brains of AD patients can recruit endogenous tau to oligomers both *in vitro* (97) and *in vivo* (96) and that intracerebral injection of oligomeric but not fibrillar wild-type tau caused aggregate formation from endogenous tau in synaptically connected but distant areas (96). Interestingly, regions receiving propagated tau in this study do not exhibit signs of tau toxicity, unlike SNCA, which did mediate toxicity as well as protein propagation via oligomer inoculation (100), a finding that illustrates the current ambiguous status of the "tauon" both as a toxicity agent and as a lesion spreading mechanism. In cell culture models, intercellular movement of misprocessed tau between cells and the apparent transfer of conformational alterations to endogenous tau has been more directly demonstrated. Endocytic tau uptake (verified using dextran co-localization) followed by fibril formation was observed in the proximity of labeled aggregates, suggesting recruitment of endogenous tau (92). Double immunolabeling (36) and fluorescence resonance energy transfer (37) has confirmed association of internalized tau with newly formed aggregates composed of endogenous tau. The uptake and axonal transport (retrograde and anterograde) of exogenous full-length tau aggregates has since been reported in differentiated primary neurons grown in Campenot chambers (209). Uptake was described as occurring via a process resembling bulk endocytosis, with the internalized tau associated with general lysosomal and endosomal markers (209). Common features of both cellular and mouse model observations of prionlike tau transfer include a specificity for oligomeric tau species and a requirement for the tau MTBR. The successful use of the 4R2N tau isoform for oligomer propagation (36) and the uptake of MTBR-only tau species (92) are obviously very different from the N-terminal specific E2− favored pattern observed for tau secretion in the lamprey model and in NB2A and M1C neuroblastoma cells (195). It should be noted that these differences reflect the tightly focused nature of the question being posed in the "prion" studies, i.e., whether oligomeric tau could be shown to propagate its oligomeric conformation between cells by recruiting endogenous tau in the recipient cell in the manner now established for misfolded PrP. This is very likely relevant to the one point of agreement between these approaches – that tauopathy mutations favor trans-synaptic tau transfer – since this type of movement may well be associated with oligomerization.

Recent investigations into interneuronal aspects of tauopathy pathogenesis have suffered from a bifurcated focus on either: (1) "prionlike" mechanisms of lesion spreading in terms of templated misfolding mechanisms derived from our understanding of prion diseases at either the whole animal or molecular level or (2) the morphological changes associated with tau secretion in cell autonomous models. These disparate approaches have (inevitably) been limited by: (a) their strict focus on molecular mechanism at the expense of cellular context and (b) exclusion of biochemical methodology in favor of spatial co-localization (respectively). These differences have dictated the choice of experimental models suited to the experimental approach taken (e.g., transgenic mice or lampreys), but have resulted in largely non-overlapping datasets devoid of a common context with which to consider them. This problem is exacerbated by a notable peculiarity of tau biology – its sensitivity to the terminally differentiated neuronal state. The inability of experimenters to induce tau-specific toxicity responses in cell lines (144) is likely due to the greater dependence of tau toxicity on axonal and synaptic functions relative to other "disease" proteins that are readily toxic in culture (210). While this has until now been a formidable obstruction to finding a broad-based approach to the characterization of mechanisms underlying tau toxicity and lesion spreading, it offers important hints for the direction of future studies on the topic – i.e., to focus on synaptic functions and developmental axon identity and guidance mechanisms to identify tauopathy-specific mechanisms, and to focus on aggregation-mediated mechanisms for identifying common themes of neurodegenerative disease pathogenesis.

IS SYNAPTIC DYSFUNCTION THE POINT AT WHICH TAU OLIGOMERIZATION, AUTOPHAGY DISRUPTION, TAU SECRETION, AND TAU TOXICITY MEET?

While the means by which tau becomes membrane associated and then diverted into unconventional secretion pathways is not yet clear, the interaction between tau and fyn kinase and the effects of that interaction on synaptic function is emerging as a key feature that might provide clarity. Fyn is rapidly localized to membrane raft domains by virtue of its double lipidation anchor sites (211). Fyn is capable of inducing exocytosis and/or endocytosis of membrane localized proteins (212, 213) and binds tau strongly via its proline rich region (19). Interactions between tau, fyn, and actin potentiate fyn-driven endocytosis of lipid raft markers flotillin-1 and flotillin-2 (129, 214). As shown in **Figure 1**, the Y18 residue of tau, a fyn substrate, is phosphorylated in distal dendritic vesicle accumulations localized to sites of focal tau secretion via microvesicles that contain both pY18 tau and endogenous fyn (81). This offers direct support for a mechanism by which fyn-activated endocytosis of raft domains containing tau results in localized tau secretion via unconventional pathways, one of which is exosomes. The phosphorylation of membrane and vesicle-associated tau by tauopathy-associated kinases (e.g., GSK3b, MARK kinases, and fyn) and their association with the exosome pathway is consistent with both receptor-mediated and oligomer-associated spreading and toxicity mechanisms, especially in the context of synaptic dysfunction.

Mislocalized tau and synaptotoxicity at glutamatergic synapses

Toxicity resulting from overexpression or extracellular application of N-terminal tau fragments in primary neurons appears to be caused at least in part by NMDA receptor-mediated Ca++ dysregulation (109), suggesting a potential link between Ca++ mediated

transmitter release, tau secretion and plasticity-associated excitotoxicity and the dendritic accumulation of misprocessed tau at postsynaptic densities. A central role in this interaction may be mediated by fyn, particularly at highly plastic, glutamatergic synapses. NMDA receptor activity at glutamatergic synapses may mediate the tau-induced dendritic degeneration seen in lamprey ABCs, where fyn associates with and phosphorylates dendritically localized tau, resulting in both localized MT loss and tau secretion (81). Fyn-mediated activation of NMDA receptors normally appears to prevent excitotoxicity at glutamatergic synapses that exhibit LTP and LTD (77), but this mechanism may be vulnerable to disruption by abnormal perisynaptic tau accumulations (74, 215) and may account for the increased resistance to Ab-induced excitotoxicity observed in tau-reduced mice (216). Both the P301S (217) and P301L tauopathy mutations (75) in mice cause changes in hippocampal synaptic transmission and plasticity well before the onset of neuronal loss. This is consistent with the dependence of Ab toxicity on tau at synapses that exhibit plasticity (218) and suggests a modulatory role for both tau and Ab in synaptic plasticity that could increase sensitivity to excitotoxicity with the onset of neurodegenerative disease (216), accounting for the selective vulnerability of the hippocampus in AD. Similarly, mislocalized tau may also disrupt the AMPA receptor recycling associated with NMDA receptor activation via an increase in fyn-tau association and as a resulting increase in the rate of internalization of AMPA receptors on dendritic spines (77).

Chemical induction of LTD under conditions that prevent autophagosome/lysosome fusion causes LC3II-positive puncta to build up in dendritic spines, suggesting a link between AMPA recycling and autophagy (219). This could provide another point of access for misprocessed tau to toxicity and secretion mechanisms in AD, since inhibition of lysosomal acidification and/or the increased generation of Ab associated with *PSEN1* mutations associated with AD can increase Wnt signaling (220) as well as induce the collection of tau aggregates in lysosomes (221). Tau overexpression in both cell culture (136) and *in situ* tauopathy models (81) causes this as well, leading to the recruitment of both GSK3b, MARK and fyn kinases, which are all key mediators of Wnt pathway activity and tauopathy-associated tau kinases (222). This is consistent with the aberrant Wnt activity seen in AD (223) and the tau-dependent nature of Ab-induced toxicity via LTP in hippocampal synapses (218). Moreover, inhibition of GSK3b decreases the number of GR1 AMPA receptor subunits on the cell surface and increases their intracellular concentration (224). This pathway requires endocytosis of GSK3b into multivesicular bodies (225, 226). This provides potential links between the NMDA/fyn-mediated endocytosis of AMPA receptors seen with LTD (227–229) and the recruitment of tau into unconventional secretion pathways.

A unifying hypothesis

The tau accumulation at or near synapses that accompanies its mislocalization to dendrites and phosphorylation by GSK3b in tauopathy could easily potentiate the sequestration of both GSK3b and tau to late endosomes. This could result in increased sensitivity to LTP associated excitotoxicity (216) and the diversion of GSK3b (and tau) to exosomes, resulting in exosome-mediated tau secretion [(106) – also see **Figure 2**]. This mechanism is consistent with the increased exosome release (230) and tau secretion (231) seen with glutamate-induced AMPA and NMDA receptor activity and the decreased presence of postsynaptic AMPA receptors in the dendritic spines of P301 tauopathy mice (232). It is also consistent with the disruption of axonogenesis mechanisms in AD (such as those involving Wnt activity) and tau oligomerization, which may help recruit endosomal tau into exosomes (106, 233), as well as the observed distribution to and effects of tau in the dendrites in various model systems (78, 81, 234–236). Interestingly, we recently found that all of the major players in the above hypothetical scenario (Wnt markers, GSK3b, tau, fyn, MARK1, autophagy markers and NMDA, AMPA, and cholinergic receptor subunits) are enriched in exosomes released by neuroblastoma cells overexpressing full-length exon 2− (4R0N) human tau isoform (**Figure 2A**). Moreover, somatodendritic tau accumulation in the lamprey model appears to induce retrograde trans-synaptic localization toxicity accompanied by the cleavage and localization of LC3II to autophagosomes (**Figure 2D**). Such observations are consistent with a hypothesis in which misprocessed tau that has been dissociated from MTs and mislocalized to dendrites accumulates via fyn-dependent localization at postsynaptic densities and is then endocytosed and diverted to exosomes. The key element in this hypothesis is an endocytosis-associated mechanism that depends on tau-fyn interaction, induces tau oligomerization and is potentiated by both the activity of excitotoxicity-prone synapses and the progressive failure of tau to segregate normally to the axon in tauopathy pathogenesis, which increases the exposure of dendritic elements to tau, thereby amplifying perisynaptic tau toxicity [Ref. (69), also see **Figure 2F**]. The potential importance of changes in neuronal differentiation state to tau toxicity was recently underscored by a study of changes in gene regulation in two separate cohorts of early onset AD patients (237). They found that downregulated genes were preferentially associated with synaptic function and axonal identity, whereas upregulated genes were preferentially involved in the control of the cell cycle and in gene expression. We compared their results with those of an earlier study (238), which reported similar findings in LOAD patients. GO term analysis of a consensus set of down and upregulated proteins common to these studies (**Figure 2E**) are highly consistent with a failure of axonal and synaptic functions in the context of de-differentiation (possibly driven by cell cycle re-entry) producing a synergistic amplification of dendritic tau toxicity, a hypothesis outlined in more detail elsewhere (69). We therefore propose that tau secretion and toxicity via synapse-associated disruption of the TGN by mislocalized tau in dendrites may serve as a unifying idea that links multiple elements of tau pathobiology and that provides a context for better understanding the role of oligomerization in tau toxicity and lesion spreading.

CONCLUSION

The increased appreciation of the significance of tau oligomerization in mediating tau neurotoxicity over the past decade must be counted as a major advance in our understanding of neurodegenerative disease. Increased understanding of the inherent toxicity of oligomers and the ability of at least some of them to induce the oligomerization of monomeric tau species, raises the possibility

that the prionlike propagation of abnormal tau conformations may play a role in the spreading of tau lesions in the brain and that the complex process of oligomer and fibril formation may generate multiple toxic tau conformations that may have different mechanisms of action. However, it is not known if all oligomers are toxic and the toxicity mechanism of oligomeric tau remains obscure. Moreover, we have also learned that N-terminal tau fragments that are incapable of oligomerization can indeed be toxic and can mediate Ab-induced toxicity in the absence of the tau MTBR. Ironically, NFTs themselves do not appear to be toxic in many circumstances and may even be the result of a neuroprotective cell response that permits the long-term survival of neurons that bear them. While significant progress has been made on understanding disease-associated tau oligomerization and NFT biogenesis, it seems clear that not enough is yet known about the cellular mechanisms responsible for either tau secretion or tau toxicity for us to understand the relative significance of conformation specific or receptor-mediated tau cytopathology in models to the spreading of neurofibrillary tau lesions and neurodegeneration in human disease. Indeed, it remains possible that both N- and C-terminal fragments generated by incomplete tau proteolysis might act as synergistic prionlike vectors in a chain-reacting "Sorcerer's Apprentice" scenario that could potentially account for sporadic tauopathy lesion patterns that are poorly explained by an oligomer mediated mechanism alone. Obviously, the operation of any mechanism generating multiple toxic N- and C-terminal tau fragments from a single molecule remains to be elucidated and cannot be evaluated seriously without far more knowledge of tau cell biology that we currently possess. Learning how phenomena such as exosomal (and non-exosomal) tau secretion and tau lesion propagation fit with tau-specific disease features linked to axonal identity and synaptic plasticity in more detail will mark a major step toward understanding the roles tau oligomers and other misprocessed tau species play in human disease.

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Is it all about contact? Neurodegeneration as a "protein freeze tag game" inside the central nervous system

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A commentary on

Trans-synaptic spread of tau pathology *in vivo*
by Liu, L., Drouet, V., Wu, J. W., Witter, M. P., Small, S. A., Clelland, C., Duff, K. (2012). PLoS ONE 7:e31302. doi: 10.1371/journal.pone.0031302

The so-called "prion hypothesis" for explaining spongiform encephalopathies is classically attributed to Prusiner, who in 1982 suggested that the scrapie agent was a proteinaceous infectious particle which would be resistant to known methods of nucleic acids inactivation (Prusiner, 1982).

Nevertheless such idea was not completely novel, once it has been already previously suggested (Gibbons and Hunter, 1967; Levine, 1972) that the scrapie agent might be devoid of disease-specific nucleic acid and, therefore, would have a different form of dissemination than known viral particles.

Earlier in 1968 the mathematician Griffith (1968) had proposed three distinct ways through which proteins might induce their own replication without the DNA/RNA machinery for nucleotide synthesis. Interestingly one of the explanations involved an analogy from the known necessity of the presence of initial atomic nuclei for gas condensation. Similarly, according to Griffith, in the protein level the "condensation nuclei" of a pre-existent polymer might (at least theoretically) be able to induce polymerization of other sub-units.

As protein polymerization with subsequent formation of deposit aggregates (such as beta-amyloid and neurofibrillary tangles) have been implied in the pathogenesis of several degenerative processes in the central nervous system (CNS), it was logical to suppose that the underlying pathogenesis of these diseases might have some similarity with the aforementioned "polymer hypothesis", which has been postulated as the cause of propagation of misfolded proteins in spongiform encephalopathies.

In fact, several recent studies (Jucker and Walker, 2011; Hall and Patuto, 2012; Kanouchi et al., 2012) have suggested that the basic proteins implied in a variety of neurodegenerative diseases [like beta-amyloid and tau proteins in Alzheimer's disease (AD), α-synuclein in Parkinson Disease and dementia with Lewy bodies, polyglutamine proteins in Huntington's disease and spinocerebellar ataxia, and superoxide dismutase 1 in amyotrophic lateral sclerosis] may share important similarities with the mammalian prion protein (PrP^{C}) involved in spongiform encephalopathies, such as the ability to translocate between neurons and further recruit normal proteins to aggregate.

The first suggestion of such possibility came from studies that demonstrated that a prion-like propagation mechanism of systemic amyloidoses occurred in animals through fecal transmission (Zhang et al., 2008). As several similarities exist between the pathophysiology of systemic and CNS amyloidoses, there has been a growing interest in the experimental evaluation of a possible protein-to-protein contact-induced transmission as the pathophysiological explanation for the progression of neurodegenerative diseases.

In a recent report Liu et al. (2012) described a new experimental protocol for the study of AD which involves a transgenic mouse that differentially expresses pathological human tau protein. In such animal model the authors demonstrated propagation of the pathological tau protein from the mesial portion of the entorhinal cortex into the CA1 region of the hippocampus and the dentate gyrus granule cells. Such findings strongly support a trans-synaptic mechanism of tau protein spreading between neurons along anatomically connected networks.

Actually, early experimental studies which investigated the mechanisms of propagation of AD had already shown that the injection of brain extracts from patients with AD into the brain of transgenic mice promoted the aggregation and deposition of β-amyloid in the injected brain (Kane et al., 2000).

Regarding the question about how would these initial abnormal proteins be able to spread the degenerative process to distant regions, it has been postulated that such cellular proteins could be released from neurons via vesicle mediated exocytosis or direct leakage through damaged cell membranes. The spatial propagation of these misfolded proteins would, therefore, explain the sequential symptomatic progression observed in the majority of the neurodegenerative diseases (Walker et al., 2002).

Although in some experiments involving artificial injection of brain extracts from patients with AD into the brains of mice, the induction of β-amyloid deposits was initially most evident within the injected area, recent cross-sectional autopsy studies have demonstrated that the accumulation of misfolded proteins follows a characteristic and predictable pattern of spatial progression in the brain of patients affected by AD (Jucker and Walker, 2011; **Figure 1**). These findings confirm the results of earlier studies which have shown sequential progression of neurofibrillary degeneration from the phylogenetically older mesial temporal regions to temporal cortical regions and finally to several other neocortical areas (Delacourte et al., 1999). Such dissemination was observed to occur first between non-contiguous (but axonally interconnected) regions, suggesting migration along already established neuronal pathways (Weller et al., 2008). Additionally a so-called "perivascular drainage pathway"

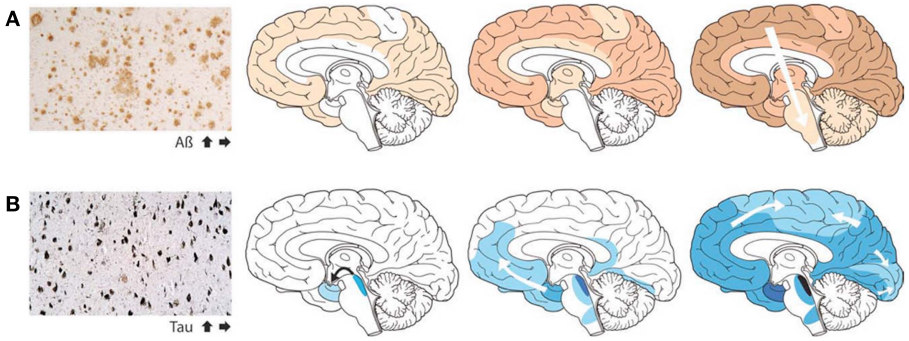

FIGURE 1 | The accumulation of misfolded proteins in Alzheimer disease follows a very characteristic and predictable pattern. Cross-sectional autopsy studies indicate that β-amyloid plaques **(A)** first appear in the neocortex, followed by the allocortex and finally subcortical regions. In the brain, neurofibrillary tangles **(B)** occur first in the locus coeruleus and transentorhinal area, and then spread to the amygdala and interconnected neocortical brain regions. These relatively stereotyped patterns of expansion strongly suggest the involvement of neuronal transport mechanisms in the spread of such proteopathic seeds (Re-published with authorization from Jucker and Walker, 2011).

has also been shown to possibly contribute to the observed dissemination (Klinge et al., 2006).

The experimental studies on such "prion-like" characteristics of the abnormal proteins involved in other neurodegenerative diseases is still their very initial phase, and the exact mechanism and routes through which such spreading might occur is still unknown. One important consequence which arises from the growing evidence for an infective role of the abnormal proteins related to neurodegeneration is an increasing attention to the possible role of the cerebrospinal fluid (CSF) circulation in the propagation (or alternatively in the clearance) of such abnormal proteins, rendering it as a possible valuable target for future therapies (Serot et al., 2011).

In summary, in the last decade several experimental and post-mortem autopsy studies have suggested that the abnormal proteins involved in several neurodegenerative diseases might present a "prion-like" behavior, in which the protein-to-protein contact would induce the further propagation of such abnormalities.

As already, mentioned such progression might also involve the active transport of such abnormal proteins to distant regions through axonal flow, perivascular spread, and, maybe, even through natural CSF circulation pathways. Despite the fact that, differently from the prionic proteins involved in spongiform encephalopathies, the inter-individual transmissibility of neurodegenerative diseases has never been reported, such new concept of disease progression by direct transmission through protein-to-protein contact present major implications for the current understanding of the pathophysiology of neurodegeneration. By emphasizing the likely relation between inter-cellular transmissibility and disease progression, such discoveries provide a new framework for experimental research in neurodegenerative diseases, as it promises to open further therapeutic avenues directed to inhibiting and eliminating such natural propagation processes.

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The involvement of cholinergic neurons in the spreading of tau pathology

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Long time ago, it was described the selective loss of cholinergic neurons during the development of Alzheimer disease (AD). Recently, it has been suggested that tau protein may play a role in that loss of cholinergic neurons through a mechanism involving the interaction of extracellular tau with M1/M3 muscarinic receptors present in the cholinergic neurons. This interaction between tau and muscarinic receptors may be a way, although not the only one, to explain the spreading of tau pathology occurring in AD.

Keywords: tau, muscarinic receptors, cholinergic neurons

INTRODUCTION

Alzheimer disease (AD) is characterized by the presence of two aberrant structures in the brain of the patients, senile plaques and neurofibrillary tangles, together with a clear loss of neurons that results, with the development of the disease, in a decrease in brain volume. Senile plaques are extracellular deposits of beta amyloid peptide (Masters et al., 1985) whereas tangles are composed of intracellular filamentous (paired helical filaments, PHFs) aggregates of tau protein in phosphorylated form (Grundke-Iqbal et al., 1986). Thus, in AD there are amyloid and tau pathologies. We will focus on tau pathology, but first we will comment on tau protein.

TAU PROTEIN

Tau protein was first described as a brain microtubule associated protein (Weingarten et al., 1975). cDNA tau was isolated later on from a mouse brain library, cloned, and sequenced (Lee et al., 1988). Studies in human brain samples showed that six different tau isoforms are expressed in the central nervous system (CNS) (Goedert et al., 1989, 1992a) whereas in peripheral nervous system a characteristic big tau isoform can be found (Goedert et al., 1992a,b).

The presence or absence of exons 2, 3, and 10 (Himmler, 1989) determines the presence of CNS tau isoforms. Exon 2 can appear alone in a tau isoform but exon 3 never appears independently of exon 2 (Andreadis et al., 1995). On the other hand there are tau isoforms with or without exon 10. The combination of all of these features results in the appearance of six tau isoforms. Exons 2 and 3 are located at the N-terminal region whereas exon 10 is presented close to the C-terminal end.

By comparing tau proteins from different organisms (Nelson et al., 1996), several variations were found at the N-terminal half of the protein whereas the C-terminal half of the molecule is well conserved among the different tau proteins (Nelson et al., 1996; Leon-Espinosa et al., 2013). The previous structural characteristics indicated for tau proteins could be related to their functions. These functions may be related to its subcellular localization and their binding to other proteins. The best tau-binding protein is tubulin, the main component of microtubules. This binding takes place through the conserved C-terminal half of tau molecule (Lee et al., 1988). On the other hand, tau can bind to other proteins through its N-terminal half. Among those proteins may be those containing SH3 domains (for a review, see Avila et al., 2004). More recently, it has been indicated that tau sequence RTPPKSP could bind to the SH3 domain of protein FYN (Bhaskar et al., 2010; Ittner et al., 2010). This tau sequence could also be involved in the interaction of tau with the protein phosphatase PP2A/B alpha (Sontag et al., 2012).

SUBCELLULAR LOCALIZATION OF TAU PROTEIN

Tau protein is mainly located at the cytoplasm of neurons where it binds to microtubules. The binding of tau to microtubules results in the stabilization of the polymers (Drubin and Kirschner, 1986), suppression of microtubule dynamics, and promotion of the formation of cytoplasmic extensions (Caceres and Kosik, 1990). At the cytoplasm, tau can bind to other proteins like kinases, phosphatases, acetylases, or deacetylases resulting, after those interactions, in a modified protein, which determines the subsequently binding of tau to other proteins. Other tau-binding protein is calmodulin, a protein that could be located at the cytoplasm or at the nucleus. Recently, it was suggested that tau could do a partial trapping of calmodulin at the cytoplasm decreasing the presence of calmodulin on nucleus and thus regulating, in this way, its activity as a co-transcription factor (Barreda and Avila, 2011).

Tau protein can also be present in the cell nucleus, although it has not yet been identified a nuclear transport signal on tau protein. Sometime ago, it was described that tau phosphorylation could be required for its transport to the nucleus (Greenwood and Johnson, 1995). Little is known about the function of nuclear tau but we know that tau binds to DNA (Corces et al., 1980). Tau could interact with nucleolar organizer regions of acrocentric chromosomes in some non-neuronal cells (Thurston et al., 1996). *In vitro*, tau prevents DNA replication but not transcription (Li et al., 2005) and it may behave like a histone-like protein. A role in neuronal DNA protection has also been proposed (Sultan et al., 2011).

Tau has also been found associated with some membrane components, like those involved in the formation of dendritic spines (Ittner et al., 2010) or at the presynaptic density (Moreno et al., 2011). The region of tau involved in the binding to the neuronal plasma membrane is the aminoterminal projection domain (Brandt et al., 1995). This tau region contains a proline rich sequence and it was described that phosphorylation of this sequence prevents the association of tau with plasma membrane (Arrasate et al., 2000). In the proline rich region there is a motif, PPXXP, that could bind to the SH3 domains present in some membrane associated proteins (Avila et al., 2004) and it may explain, at least in part, the interaction of tau protein with membrane.

TAU MODIFICATIONS

Two modifications, phosphorylation and aggregation, can regulate the interaction of tau with cytoplasmic, nuclear, or membrane components and it may result toxic for a cell. The largest CNS human tau isoform (Goedert et al., 1989) contains 79 potential serine/threonine sites that could be phosphorylated. Only few of those sites could be modified in normal conditions but in pathologies, like AD, this number could grow significantly (Hanger et al., 2009). Tau hyperphosphorylation could be toxic for a neuron as indicated by using cell culture and animal models (Brandt et al., 2005; Yoshiyama et al., 2007; Gomez de Barreda et al., 2010).

On the other hand, hyperphosphorylated tau can induce tau aggregation (Trojanowski and Lee, 1994; Alonso et al., 2001; Sato et al., 2002; Perez et al., 2003). The consequences of tau aggregation are a topic that remains in the field. It is discussed whether the presence of large tau aggregates could be toxic or beneficial for neurons (Bretteville and Planel, 2008). It has been shown that the number of extracellular tau aggregates (extracellular ghost tangles) is inversely proportional to the number of surviving neurons in the brain of AD patients. This observation is suggesting that at least some of the neurons that degenerate in the disease have previously developed tau aggregates (Bondareff et al., 1989). On the other hand, it has been proposed that the presence of tau aggregates could prevent the activation of cell promoting death molecules like caspase 3 (de Calignon et al., 2010). A possible explanation for those discrepancies could be found in the suggestion that the size of tau aggregates could be important for their toxic effect and that may be the small tau oligomers, and not large aggregates, the toxic agents (Maeda et al., 2007).

Also, overexpression of intracellular tau could be toxic for a cell (Andorfer et al., 2005). Since the levels of an intracellular protein are the consequence of its synthesis, degradation, and secretion, it was tested if an overexpression of intracellular tau could result in its secretion into microvesicles (Simon et al., 2012).

TAU PATHOLOGY SPREADING IN THE PRESENCE OR ABSENCE OF NEURON DEATH

Tau pathology usually starts at the entorhinal cortex and hippocampal region (Braak and Braak, 1991) and it may correlate with the loss of episodic memory occurring in the patients at the first stages of the disease. From the hippocampal region, tau pathology spreads to other brain areas and during the progression of the disease neurodegeneration and neuron death take place allowing that intracellular tau could be released to the extracellular space. Thus, intracellular and extracellular tau is present in neurodegenerative disorders like AD. Intracellular tau could be toxic due to its hyperphosphorylation level (Avila et al., 2004) or due to its aggregation (Bondareff et al., 1989; Gomez-Isla et al., 1997). However, it is discussed if larger aggregates like PHF, could be toxic (Cras et al., 1995; Avila, 2010; de Calignon et al., 2010).

EXTRACELLULAR TAU AND MUSCARINIC RECEPTORS

About extracellular tau, it has been suggested that once it is at the extracellular space it could become toxic for the surrounding neurons (Gomez-Ramos et al., 2006). Which is the mechanism for that toxicity will be commented below. However, an alternative way for tau pathology spreading, involving tau, has been reported. Thus, tau transmission from cell to cell could occur by exocytosis and endocytosis being not necessary neuron death (Clavaguera et al., 2009; Frost et al., 2009; de Calignon et al., 2012; Liu et al., 2012; Wu et al., 2012; Iba et al., 2013). On the other hand, to explain that the transmission could occur only in neurodegenerative disorders and not in a normal situation it has been proposed that aggregated tau is the toxic form for that spreading (Clavaguera et al., 2009; Frost et al., 2009; Iba et al., 2013). It is not clear if the endocytosis could take place in any cell type or if a specific cell receptor component is required. In this way, a specific transmission through synaptic connections has been proposed (de Calignon et al., 2012; Liu et al., 2012).

In the case of neuron death, intracellular tau is released to the extracellular space, and this extracellular tau could interact with surrounding neuronal cells and, as consequence of that, an increase in intracellular calcium can take place in those neurons (Gomez-Ramos et al., 2006). This increase in calcium could be due to calcium-permeable channels, to the activation of cell surface receptors coupled to calcium-influx or to calcium liberation from intracellular stores, induced by the activation of metabotropic receptors like muscarinic receptors (Gomez-Ramos et al., 2006). The published data have indicated that are, indeed, the muscarinic receptors, the ones involved in the interaction with extracellular tau and the responsible factors for raising intracellular calcium (Gomez-Ramos et al., 2008).

Muscarinic receptors subtypes have been classified in two groups: M1, M3, and M5, in one group, and M2 and M4 in the other group (Felder, 1995). The activation of M1 receptor group could activate phospholipase C, the release of inositol 1,4,5 triphosphate, and the subsequent mobilization of intracellular calcium (Felder, 1995). On the other hand, activation of M2 receptor

group results in an inhibition of the intracellular levels of cAMP (Felder, 1995).

By using specific antagonists of either muscarinic receptors it was found that extracellular tau binds to M1 and M3 receptors and that it may explain the increase of intracellular calcium found in neuronal cells upon tau-binding (Gomez-Ramos et al., 2006, 2008, 2009). The region of human tau molecule involved in the binding to muscarinic receptors was described like that comprising residues 390–423 of the largest CNS human tau isoform (Gomez-Ramos et al., 2008). As consequence of that binding, tau protein could be or not endocytosed in a vesicle as M1 receptor does (Lameh et al., 1992).

BINDING OF MODIFIED TAU TO MUSCARINIC RECEPTORS

It was indicated that the toxicity of intracellular tau could be a consequence of its phosphorylation, or its aggregation. Thus, we have tested the consequences of phosphorylation or aggregation of extracellular tau on its interaction with muscarinic M1/M3 receptors. It was found that tau phosphorylation prevents the interaction of tau with muscarinic receptors. Also, it was described that extracellular phosphorylated tau is dephosphorylated by tissue-non-specific alkaline phosphatase (TNAP) and that this phosphatase, promotes the neurotoxic effect of extracellular tau (Diaz-Hernandez et al., 2010). The level of this phosphatase is increased in the brain of AD patients (Diaz-Hernandez et al., 2010).

It should be indicated that the level of both unphosphorylated and phosphotau, that could arise from dead neurons, are increased in the cerebrospinal fluid (CSF) of AD patients (Olsson et al., 2011).

Different levels of tau aggregation have been analyzed to study their interaction to muscarinic receptors. Thus, soluble tau containing monomers and small oligomers of tau, as well as purified larger tau aggregates (PHFs) have been tested. These studies have demonstrated that soluble tau but not PHFs interacted with muscarinic receptors (Gomez-Ramos et al., 2006).

CONSEQUENCES OF THE INTERACTION OF TAU WITH MUSCARINIC RECEPTORS

A consequence of that interaction is an increase in the level of intracellular calcium as previously described. A secondary effect of tau upon its binding to neuronal cells is to increase TNAP gene expression (Diaz-Hernandez et al., 2010). This effect could be the consequence of activation of DREAM, a transcription factor regulated by calcium (Carrion et al., 1999). However, this transcription factor probably is not involved in TNAP gene expression (Naranjo et al., unpublished results) and further analysis should be done to clarify the connection between calcium increase and TNAP gene expression.

OTHER CONSEQUENCES OF TAU-BINDING TO MUSCARINIC RECEPTORS

As previously indicated, upon interaction of tau with muscarinic M1/M3 receptors an increase in intracellular calcium takes place and some consequences of an increase in intracellular calcium could be an increase in secreted compounds. Preliminary experiments suggest an increase in the secretion of vesicles (containing flotilin) upon activation of M1/M3 receptors induced by tau protein, being that increase prevented by calcium chelators (Simon et al., unpublished results). Also, it has been described different affinities of tau and acetylcholine for M1/M3 receptors (Gomez-Ramos et al., 2009) and differences in the increase of intracellular calcium induced by ACh or tau protein through M1/M3 muscarinic receptors. Thus, for cell expressing M3 receptor, a minimum tau concentration of 50 pM was needed to find an increase in intracellular calcium while 5 nM ACh was required to have a similar effect (Gomez-Ramos et al., 2009). It was also found that a continuous increase in calcium level due to the presence of tau may result in cell death (Gomez-Ramos et al., 2009). Thus, it can be proposed that extracellular tau may promote cell death and it will result in the release of intracellular tau to the extracellular space and this new extracellular tau could again interact with other cells and, in this way, propagate neuron degeneration. This manner to propagate tau pathology may occur in AD or in other related pathologies (tauopathies). Little is known about how an increase of calcium mediated by the interaction of tau with muscarinic receptors could result in cell death. M1 and M3 receptors are coupled with G_q/G_{11} proteins leading to activation of phospholipase C and an increase in the level of intracellular calcium. This calcium increase could activate some protein kinases, and these kinases could modify tau protein doing the protein toxic. In any case, further studies focused in the consequences of tau phosphorylation on neuron degeneration should be done.

TAU PATHOLOGY PROGRESSION IN ALZHEIMER DISEASE

Tau pathology spreading could involve (Gomez-Ramos et al., 2006), or not (Frost et al., 2009), neuronal death. It has been described that neuronal death and the presence of extracellular tau could be linked in some cases (Gomez-Ramos et al., 2006). In this way, an inverse correlation can be found between the number of extracellular tangles and the number of living neurons in the hippocampus (Bondareff et al., 1989). Also, extracellular tau is present in CSF of AD patients, suggesting neuronal death. As previously indicated, extracellular tau can have two different origins; one raised by exocytosis without cell death being this tau present, at least in part, in membrane vesicles and the other one, from neuronal death present in a naked form. The presence of both tau populations has been found in the CSF of AD patients. Tau present in vesicle particles was mainly found at the first stages of the disease whereas the amount of uncoated tau, in CSF, increases with the development of the disease (Saman et al., 2012).

We suggest that tau pathology spreading in cell culture, or *in vivo*, has a first step in which, probably, small tau oligomers specifically interact with neuron specific receptors. These receptors could be the M1/M3 muscarinic receptors although we cannot exclude other possibilities as an unspecific endocytosis pathway for tau internalization (Wu et al., 2012). Once tau is bound to the cell receptor, it could be endocytosed in a vesicle, a mechanism that occurs with ligands of M1/M3 receptors (Lameh et al., 1992), or simply, tau could promote the increase of intracellular calcium level and the appearance of second messengers or toxic compounds that will result in neuron death without being internalized. In the first case, the endocyted tau may interact with some cellular components, including tau itself, and it could

be secreted uncoated or in a membrane vesicle (Lameh et al., 1992; Saman et al., 2012; Simon et al., 2012). These secreted vesicles could interact with other cells and be endocytosed in an unspecific way. In an alternative way, during the secretion, vesicles and cell membrane can be fused and uncoated tau protein (in aggregated or unaggregated form) could be released to the extracellular space where it can be toxic, upon interaction with muscarinic cell receptor. On the other hand, in other works have been reported that extracellular tau can induce intracellular tau aggregation and afterward the spreading of aggregated tau may occur in a prion-like manner (Clavaguera et al., 2009; Iba et al., 2013).

In summary, there are, at the present, different alternatives to explain tau pathology spreading in tauopathies like AD, a disease that long time ago was associated with severe loss of cholinergic markers in the brain (Davies and Maloney, 1976), and that such loss may be due to the toxic interaction of tau with muscarinic receptors.

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Tau clearance mechanisms and their possible role in the pathogenesis of Alzheimer disease

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One of the defining pathological features of Alzheimer disease (AD) is the intraneuronal accumulation of tau. The tau that forms these accumulations is altered both posttranslationally and conformationally, and there is now significant evidence that soluble forms of these modified tau species are the toxic entities rather than the insoluble neurofibrillary tangles. However there is still noteworthy debate concerning which specific pathological forms of tau are the contributors to neuronal dysfunction and death in AD. Given that increases in aberrant forms of tau play a role in the neurodegeneration process in AD, there is growing interest in understanding the degradative pathways that remove tau from the cell, and the selectivity of these different pathways for various forms of tau. Indeed, one can speculate that deficits in a pathway that selectively removes certain pathological forms of tau could play a pivotal role in AD. In this review we will discuss the different proteolytic and degradative machineries that may be involved in removing tau from the cell. How deficits in these different degradative pathways may contribute to abnormal accumulation of tau in AD will also be considered. In addition, the issue of the selective targeting of specific tau species to a given degradative pathway for clearance from the cell will be addressed.

Keywords: tau, proteasome, autophagy, proteolysis, degradation

INTRODUCTION

Insoluble, fibrillar intraneuronal accumulations of pathological forms of the tau protein called neurofibrillary tangles (NFTs) are important and defining hallmarks of the Alzheimer disease (AD) brain. Indeed, the progression of AD can be neuropathologically staged based on the location and extent of tau pathology (1). The predominant post-translational modification of tau in the NFTs is phosphorylation; however numerous modifications have been noted including truncation, acetylation, nitration, and several others (2–4). Historically the NFTs were considered to be the toxic entities, however over the past decade a new conceptual framework has developed in which pathologically modified monomeric and/or soluble oligomeric forms of tau are considered to be the harmful species (5, 6). Nevertheless, determining exactly which forms of tau compromise neuronal function is still an area of significant investigation. Even though the modifications of tau that are the primary contributors to toxicity have not been conclusively determined, it is clear that tau plays an essential role in the pathogenesis of AD. Given that in animal models of AD reducing tau levels attenuates neuronal dysfunction (7, 8), and in humans the extent of tau pathology correlates with cognitive decline (9), there is a growing interest in defining the degradative pathways that remove tau from the cell. Also of importance is understanding the role of non-degradative cleavage in influencing the eventual clearance of tau. Numerous proteases have been shown to proteolyze tau including aminopeptidases (10–12), thrombin (13–15), human high temperature requirement serine protease A1 (HTRA1) (16), calpain (17–20), and caspases (21–24). Overall, however, most of these enzymes do not appear to be principally responsible for tau clearance. Instead, they are able to generate modified tau species which may then contribute to developing tau pathology, enhanced tau clearance, or both. The bulk of clearance of both physiological and pathological forms of tau is instead mediated by the proteasomal and autophagic degradative systems (25). The contribution of each of these pathways in the turnover of tau, and which forms of tau – including various proteolytic forms – are degraded by each pathway, is an area of significant interest. Our understanding of this issue to date will be reviewed below, and the role of tau proteolysis on subsequent degradation will be discussed. Delineating how these pathways may be compromised in AD and how this contributes to tau pathology is of great importance and could have significance for informing new therapeutic approaches.

TAU PROTEOLYSIS

Tau is a cytosolic, dynamically regulated protein. In differentiated PC12 cells, a pulse-chase experiment showed that ∼90% of the tau was degraded in 18 h (26). Normal, monomeric tau is likely a proteasomal substrate. However, there is evidence that tau is also a substrate for a wide range of proteases as indicated above. This is significant as tau proteolysis could be beneficial in disease by helping to enhance removal of abnormal tau from the cell. Alternately, it could be detrimental by generating toxic fragments. Below we will discuss the different proteases that have been shown to act on tau, at least *in vitro*, and the possible involvement of these proteolytic events in AD.

AMINOPEPTIDASES

Aminopeptidases are a group of enzymes that cleave from the N-terminal end of a protein. The family includes alanyl, arginyl, and glutamyl peptidases. Puromycin sensitive aminopeptidase (PSA) is an alanyl peptidase that is responsible for ∼90% of the aminopeptidase activity in the brain (10). PSA was identified as a potential player in tau pathology through a microarray analysis of gene expression in disease-vulnerable vs. disease-resistant brain regions in JNPL3 mice that overexpress a mutant form of tau (P301L) found in the disease frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). These mice develop neurodegeneration in the cortex while the cerebellum is relatively spared [although in the original description of these animals pathology was found in the deep cerebellar nuclei (27)]. Interestingly, PSA was found to be elevated in the cerebellum of these TAU^{P301L} mice (10). The levels of PSA are also higher in human cerebellum compared to cortex in both controls and FTD cases. A slight elevation in PSA was also observed in FTD cortices compared to controls. In addition, a non-functional PSA mutant exacerbated tau pathology in a *Drosophila* model of tauopathy, while overexpressing PSA ameliorated the tau phenotype and diminished tau levels (10). Overexpressing PSA had a similar effect in the TAU^{P301L} mice, reducing the pathologic phenotype (delaying paralysis, increasing motor neuron density in the spinal cord, decreasing gliosis) and decreasing tau levels (12). PSA was able to cleave recombinant tau *in vitro*, as well as tau from control human brain (11). However, the data presented in this study suggest that PSA is cleaving tau from both the C- and N-terminal ends, which is not expected from an aminopeptidase. Additionally, other studies failed to demonstrate tau cleavage by PSA (28, 29). One explanation for these discrepancies may be the limitations of *in vitro* assays and experimental techniques. For example, the FTDP-17 mutant tau used in many studies, while relevant for human tauopathy, is not found in AD. Additionally, this form of tau may be processed differently than tau without this mutation. For example, it has been shown that the isomerase Pin1, which has been implicated in AD (30), had opposite effects on P301L and wild-type tau degradation (31). An alternative explanation for the effects of PSA may be that PSA is indirectly regulating tau degradation. PSA has been shown to be involved in the induction of autophagy and specifically the formation of autophagosomes, in a model of overexpressed mutant huntingtin (32). Thus, the *in vivo* effects of PSA on promoting tau clearance may relate to its ability to modulate the key clearance pathway for abnormal and aggregated proteins (to be described in more detail below).

THROMBIN

Thrombin is a serine protease that is a well characterized component of the coagulation cascade. It is typically produced and secreted by endothelial cells, including those in the brain in response to hemodynamic injury. Thrombin may be inappropriately expressed in AD brain. A recent study showed that thrombin is elevated in microvessels isolated from AD brain compared to microvessels from control brain (33). Additionally, thrombin was present in the CSF of AD patients but not in that of controls (33). This is important, as thrombin can act as a neurotoxin by activating intracellular signaling cascades causing neurite retraction and stimulating apoptosis (34–36). Thrombin may also be influencing tau pathology, as treatment of immortalized hippocampal neuronal cells (HT22 cells) with thrombin resulted in the formation of thioflavin-S positive tau aggregates within 24 h, followed by an increase in cell death at 72 h (37). It is unclear how this exogenously applied thrombin may be altering tau within the cells. There are also data to suggest that thrombin may act intracellularly to mediate tau pathology. Thrombin is expressed within neurons and astrocytes in both normal and AD brain (38). In AD brain the staining pattern for thrombin and prothrombin was characteristic of the pattern of NFTs, although these structures were not colabeled with antibodies for tau (38). Evidence supporting a role for thrombin in tau proteolysis came initially from an *in vitro* study showing that thrombin degraded recombinant full-length tau from the N-terminus yielding a 25-kDa fragment, while preserving the microtubule binding repeat domain (13). A later study, however, showed that in N2a neuroblastoma cells expressing a construct of only the tau repeat domain, thrombin cleavage could still occur, indicating additional cleavage sites (15). Similar results were observed in an *in vitro* assay (15).

The products of thrombin proteolysis are potentially pathogenic. Thrombin cleavage of the repeat domain construct yielded fragments that rapidly aggregated, which closely correlated with toxicity in cell culture (15). These fragments can also induce the aggregation of full-length tau (39). A final point of interest relates to potential upstream modifications of tau. Endogenous tau is phosphorylated, and in AD, tau phosphorylation becomes dysregulated. This may interfere with subsequent processes including cleavage and degradation. For example, tau that is in the *cis*-conformation at T231 appears resistant to degradation, as *cis*-tau is found in dystrophic neurites while *trans*-tau is not. Additionally *cis*-tau partitions to the insoluble fraction (30). Phosphorylation at T231 prevents the isomerase Pin1 from converting *cis*-tau to *trans*-tau (30). Interestingly, phosphorylation of tau also appears to disrupt some thrombin cleavage sites, changing the pattern of cleavage without impeding the thrombin-mediated proteolysis (14, 28). It has yet to be determined whether there is a difference in toxicity potential between fragments generated from phosphorylated vs. unphosphorylated tau. Nonetheless, thrombin is a potential candidate for contributing to tau proteolysis and pathology.

HUMAN HIGH TEMPERATURE REQUIREMENT SERINE PROTEASE A1

Another serine protease recently implicated in tau processing is HTRA1. This is a ubiquitously expressed, ATP-independent intracellular protease. Expression is detectable in many tissues, including the nervous system, although expression is low (40). Nonetheless, this enzyme was initially implicated in AD because it may play a role in amyloid processing (41). Tubulin was later identified as a substrate for HTRA1, suggesting HTRA1 may be involved in mediating microtubule function (42, 43). A more recent study showed that HTRA1 can cleave recombinant tau *in vitro* into multiple fragments of varying sizes, and furthermore can degrade insoluble and fibrillarized tau (16). This ability to degrade aggregates is particularly intriguing, especially in light of the fact that HTRA1 has potential chaperone activity due to its C-terminal PDZ domains and has a preference for misfolded substrates (44). While more work needs to be done on the role this enzyme plays in

tau proteolysis, these findings further indicate the complexity and likely involvement of multiple players in this process.

CALPAINS

Calpains are calcium-activated cytosolic cysteine proteases. Two isoforms differentiated and named by their sensitivities to calcium (i.e., μ-calpain and m-calpain, also called calpain-1 and calpain-2) are abundant in the central nervous system, and respond to micromolar and millimolar concentrations of calcium, respectively (45). Calpain has been implicated in a number of neurodegenerative diseases [for a review, see (46)]. The active form of calpain-2 is found in 50–75% of NFTs in tauopathies including AD, but not in protein aggregates found in other diseases (47). This is consistent with another study that found equivalent calpain levels between control and AD cases, but the activity level of the enzyme isolated from AD brain tissue was increased (48). Excitotoxicity leading to elevated intracellular calcium is a common feature of neurodegenerative diseases, and is implicated in AD (49, 50). This process may lead to enhanced activation of calpains (51). This in turn could influence a number of pathologic processes, including tau proteolysis. Indeed, tau has a number of putative calpain cleavage sites, and incubation of recombinant tau with calpain generates specific fragments, including one that is ∼35 kDa and one that is ∼17 kDa (19, 20). Increasing intracellular calcium levels in PC12 cells leads to calpain-induced cleavage of tau (18). This may reflect a potential effect of excitotoxicity in AD. Inducing apoptosis in cerebellar granule cells yields calpain-mediated tau fragments, including a dominant ∼17 kDa fragment (17). Also, treating primary hippocampal neurons with pre-aggregated amyloid β (Aβ) led to the generation of tau fragments of ∼35, ∼24, and ∼17 kDa, which was blocked by addition of a calpain inhibitor (52, 53). Tau fragments of the same size were also found in AD brain tissue (19).

The pathological role of this calpain-cleaved tau is unclear. While some studies demonstrate toxicity resulting from calpain proteolysis of tau, other studies do not support this conclusion. On the one hand, expressing a 17-kDa fragment of tau based on calpain cleavage site mapping in hippocampal neurons led to neurite retraction and the appearance of varicosities after 48 h (52). Additionally, suppressing calpain activity in a fly model of tauopathy prevented neurodegeneration, as did expressing a calpain-resistant form of tau (54). In contrast, another study used mass spectroscopy and sequencing to identify the "17 kDa" tau cleavage product and found it did not correspond to the recombinant fragment utilized in the above studies (19). Expression of a recombinant form of the mass spectroscopy-identified fragment in hippocampal neurons was not toxic (19). Further studies are needed to clarify the contribution of calpain-mediated proteolysis of tau to AD pathology.

CASPASES

There is significant evidence that tau is a caspase substrate and that caspase-mediated tau cleavage may play a role in AD pathology. Early *in vitro* studies demonstrated that tau is cleaved in the C-terminus by several caspases including caspase-3 and caspase-6 (21–23). Caspase-6 was also shown to cleave the N-terminus of tau *in vitro* (24). Caspase-3, which is a key effector in the apoptotic cascade, cleaves tau predominantly at the C-terminal D421 site generating a fragment often referred to as tauC3 (22, 23). There may be reciprocity with the apoptosis pathway as activating caspase-3 by inducing apoptosis in cortical neuronal culture led to tau cleavage (22), and selectively expressing tauC3 led to apoptosis in NT2 and COS cells (21). This might represent a feed-forward loop of neurotoxicity. Furthermore, expressing a cleavage resistant form of tau (D421E) protects cells from apoptotic cell death (22). Another potential mechanism of inducing caspase-3 cleavage of tau is the presence of Aβ peptides. TauC3 is formed in primary cortical neurons after treatment with Aβ (23).

Caspase cleavage of tau may play a role in stimulating the tau aggregation seen in AD. Indeed, *in vitro* polymerization assays demonstrate that caspase-cleaved tau has a greater propensity to aggregate compared to full-length tau (23, 55). Intriguingly, caspase activation was shown to immediately and consistently precede the formation of tangles (56). This group used *in vivo* multiphoton imaging in Tg4510 TAU^{P301L} mice to simultaneously image activated caspases and Thioflavin-S positive tau tangles. There was a strong correlation between active caspases and the presence of tangles within viable neurons. In the few cells found that were caspase-positive and tangle-negative, 88% had tangles within 24 h (56). This seems to further support a role for caspase cleavage in the evolution of tau pathology.

In order for caspase to cleave tau in the AD brain, it needs to be present in its active form. The active forms of both caspase-3 and caspase-6 are elevated in AD-specific brain regions (temporal and frontal lobes) compared to unaffected regions (cerebellum) and control brains (57, 58). Furthermore, active caspase co-localizes to NFTs (58), and caspase-cleaved tau is found in AD-affected brain regions, particularly in neurons displaying tangle pathology (59, 60). This includes tau cleaved by caspase-6 in the C-terminus (58–60) as well as in the N-terminus (24). TauC3 is present in AD brain – in neurons and co-localized with NFTs – and inversely correlates with cognitive function (55, 60, 61).

The activation of caspases and the subsequent cleavage of tau is likely to occur independent of apoptotic cell death (56). The processes that may result in the activation of caspases in an apoptosis independent manner have not been clearly delineated; however several possibilities have been suggested. First, inflammation, which is a common feature of AD, may contribute to tau pathology by activating caspases. Treating cells with the prostaglandin cyclopentenone byproduct PGJ2 increased caspase activity and increased cleaved tau (62). Thrombin signaling can also activate caspases (36). Proteasomal impairment appears to be upstream of caspase activation, as inhibiting the proteasome with epoxomicin (EPX) led to activation of caspase-3 in primary neurons (63) and in a neuroblastoma cell line expressing wild-type tau (64). In both studies caspase activation correlated with the appearance and increase over time of caspase-cleaved tau species, which appeared to subsequently form aggregates in the neurons (63). While the mechanism is unclear, a possibility is that accumulating proteins might be a factor in initiating caspase activation.

PROTEOLYSIS vs. DEGRADATION

As discussed above, a number of enzymes have been shown to act on tau, under potentially pathological, as well as physiological conditions. Many of these enzymes cleave tau at discrete sites,

generating specific fragments. Some of these fragments, such as those generated by thrombin, calpain, and caspase, are potentially toxic to the cell if they accumulate due to inefficient clearance mechanisms. **Figure 1** illustrates the potential contribution of these different proteases to the processing of tau. These proteolytically generated tau fragments can show an increased propensity for self-association, prior to the formation of overt aggregates. Thus, in the context of enhanced proteolysis (for example by caspases) there may be increased low-order oligomers formed by cleaved tau species. These oligomers may be unable to be cleared as effectively by the cell and contribute to neuronal dysfunction. Therefore coordination between proteolytic processing of tau and clearance by degradative pathways is essential for maintaining the appropriate levels of tau in a functional state. Below we will discuss the main degradative pathways of the cell-the proteasome and autophagy-which likely clear full-length tau as well as proteolytically generated tau fragments.

THE PROTEASOME

The proteasome is a multimeric barrel-shaped structure that is a key complex for clearing soluble cytosolic proteins. The 26S proteasome has a regulatory cap (19S, or alternatively the 11S regulatory particle) on either end of its catalytic core (20S), which contains the proteolytic activities and degrades substrates tagged with poly-ubiquitin chains as the targeting sequence. The regulatory cap in conjunction with chaperone proteins unfolds the protein substrate and removes the ubiquitin tag in an ATP-dependent process prior to feeding the protein into the catalytic core, where it is systematically degraded by the enzymatic properties of the proteasome. The 20S proteasome, which is the catalytic core without its regulatory caps, is also able to degrade natively unfolded substrates directly through an ATP- and ubiquitin-independent process. As shown in **Figure 2**, tau is an ideal proteasomal substrate for either form of the proteasome because it is a relatively small, unfolded, short-lived cytosolic protein (64–67).

The accumulation of proteins in AD patients' brains generated interest in the role of proteasomal function. There is evidence suggesting that proteasomal activity, but not protein level, is decreased in AD-sensitive brain regions specifically compared to unaffected regions (68, 69). Additionally, tau appears to be physically associated with the proteasome in brain tissue from AD cases. When tau was immunoprecipitated it pulled down both the 26S and 20S proteasomes, while immunoprecipitating for the 20S catalytic core pulled down tau (69). This suggests tau is being targeted to the proteasome, but may also indicate impaired ability to complete degradation; hence it is remaining associated with the proteasome. Further, there was an inverse correlation between proteasomal activity and high molecular weight forms of tau (69). This may suggest that abnormal proteins themselves may interfere with proteasomal degradative processes. Indeed, *in vitro* aggregated paired helical filament tau could inhibit proteasome activity (69).

EVIDENCE THAT TAU IS DEGRADED BY THE PROTEASOME

A number of studies have used various *in vitro* techniques to analyze proteasomal degradation of tau. These include cell culture and cell free studies. Not surprisingly, if recombinant tau is incubated with isolated 20S proteasomal complexes, degradation occurs (65). In this system proteolysis is bidirectional. Also, if tau is first ubiquitylated in an *in vitro* reaction and then incubated with isolated 26S proteasomes supplemented with $MgCl_2$ and ATP, degradation proceeds (66). These data indicate tau can be a substrate for both forms of the proteasome. Similar data has been obtained from studies using various cell culture systems as well as animal tissue and primary cultures with a variety of proteasomal inhibitors. When HEK cells are co-transfected with tau and ubiquitin, tau accumulates in the insoluble fraction. Its accumulation in the insoluble fraction is enhanced by proteasomal inhibition (using ALLN or MG-132) suggesting that tau is degraded by the proteasome (66). In SH-SY5Y neuroblastoma cells, treatment with lactacystin, a selective inhibitor of the 20S catalytic

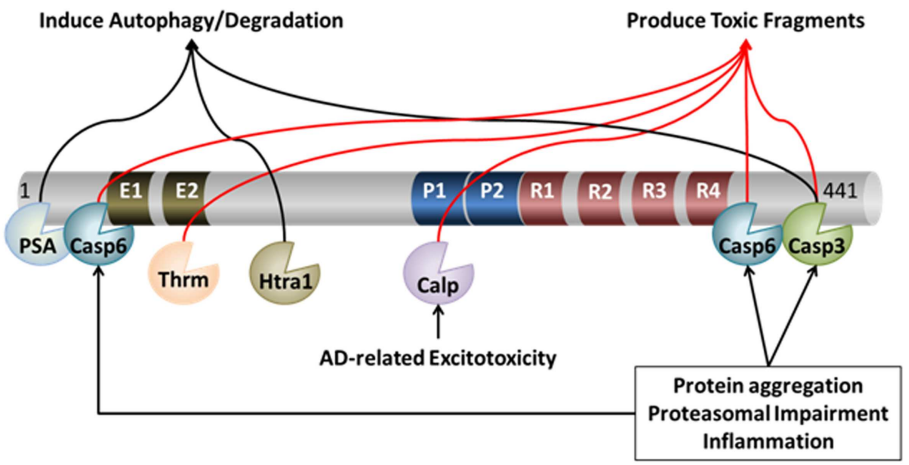

FIGURE 1 | Proteolytic processing of tau. Under pathological and physiological conditions, tau undergoes cleavage at many distinct proteolytic sites by a myriad of proteases. The action of these proteases can lead to both protection and/or exacerbation of pathology. For example, cleavage of tau by caspase (Casp) 3, caspase-6, calpain (Calp), and thrombin (Thrm) leads to the production of toxic fragments of tau that exacerbate pathology. On the other hand, cleavage of tau by PSA, Htra1, and – in some circumstances – caspase-3, may facilitate its degradation, which may protect neurons from AD-related neuronal death.

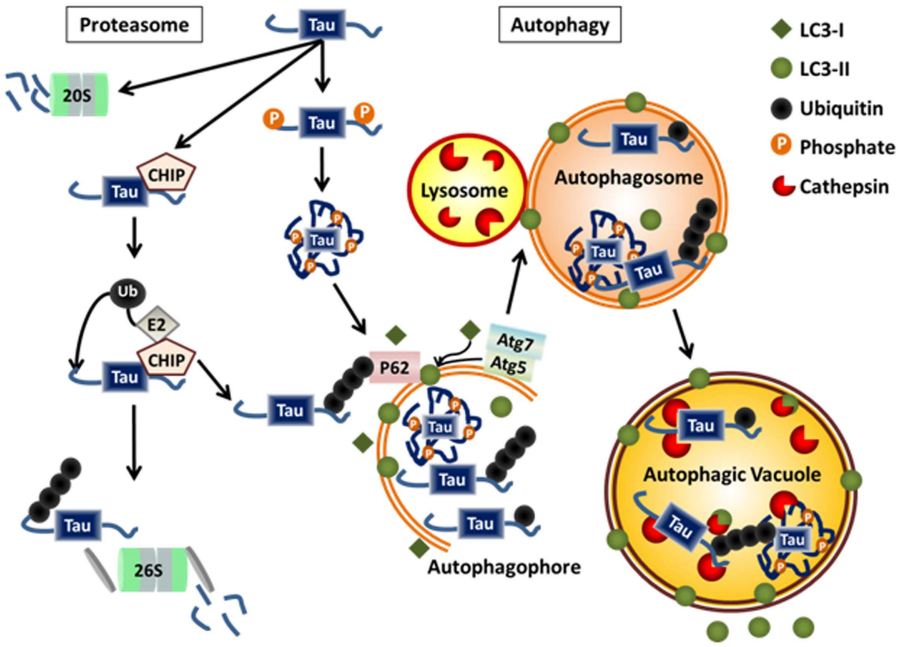

FIGURE 2 | Physiological degradation of tau. Tau is degraded by both the proteasome and autophagy systems. Targeting of tau to either system may be determined by the extent and nature of post-translational modifications, the folding state, the level of aggregation, and its interaction with chaperone proteins or ubiquitin ligases. Monomeric tau is natively unfolded making it a likely target for the 20S proteasome. Monomeric tau also interacts with the E3 ligase, CHIP, which can lead to its ubiquitylation and degradation via the 26S proteasome or autophagy. Certain cleavage products and phosphorylated forms of tau, as well as, monoubiquitylated tau and tau aggregates are selectively degraded by autophagy.

core, maintained levels of transfected wild-type full-length tau (4R0N) after cycloheximide treatment halted protein synthesis (65). Similarly, overexpressing the FTDP-17 mutant P301L tau in SH-SY5Y cells and then treating with lactacystin led to significantly increased tau levels (70). Lactacystin also caused accumulation of endogenous tau in the HT22 murine neuronal cell line (71). In immortalized mouse cortical neuronal cells inducibly expressing full-length wild-type tau, EPX slowed the degradation of full-length tau (72). In M1C neuroblastoma cells that inducibly express wild-type full-length tau (4R0N), EPX, and MG-132 induced accumulation of full-length tau but there was a concomitant loss of C-terminus immunoreactivity (64). This was attributed to caspase cleavage, as activated caspase-3 was detected, and a caspase inhibitor preserved C-terminal immunoreactivity (64). Additionally, incubation of rat brain extract (containing endogenous tau and proteasomal enzymes) with the proteasome activators Mg^{2+} and ATP resulted in lower total tau levels with an increase in smaller forms, compared to extract not supplemented with Mg^{2+} and ATP (73). The loss of tau was blocked by lactacystin giving further evidence that the proteasome was degrading tau (73). The story is more complex, however, as proteasomal inhibition under physiological conditions does not consistently lead to tau accumulation. For example, treatment of primary neurons with an Hsp90 inhibitor to interrupt the proper chaperoning of tau leads to decreased levels of tau. Adding MG-132 to block the proteasome prevented the Hsp90 inhibitor-induced reduction in total tau. MG-132 alone had no effect on tau levels (67). This might suggest that under normal circumstances, if proteasomal impairment occurs, tau levels are maintained by autophagic degradation. But when the system is pushed to promote proteasomal degradation over autophagy – such as by inhibiting Hsp90 – then the homeostatic maintenance of tau levels is disrupted and tau degradation does not occur when the proteasome is inhibited.

AUTOPHAGY

Autophagy is the process of "self-eating." Under starvation conditions, bulk autophagy can be induced to catabolize cellular substrates to generate energy. However it is now evident that autophagy is an ongoing clearance mechanism for larger, longer-lived proteins and aggregates, as well as organelles such as mitochondria and peroxisomes (74) and pathogenic bacteria (75–77). There are three forms of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy. The most common and well understood is macroautophagy, hereafter referred to simply as autophagy. For a more complete review of autophagy, see (78). Briefly, a double membrane autophagophore is initiated and subsequently expanded to engulf a region of cytoplasm containing the substrate/substrates to be degraded, such as tau (see **Figure 2**). Once fully formed into an enclosed vesicle called an autophagosome, it is trafficked to a lysosome where it undergoes fusion to become an autophagic vacuole (AV). The lysosomal enzymes degrade the inner membrane of the autophagosome as well as the delivered contents. The enzymes responsible for degrading protein substrates of autophagy are the cathepsins. Once the contents are fully degraded the lysosome is regenerated via acidification through vacuolar ATPases. There are 15 core autophagy related

genes (Atgs) that are involved in the process of autophagy. Many of these have E1, E2, or E3 ligase activity to catalyze the reactions necessary for the initiation and expansion of the autophagosomal membrane. Critical early steps in the formation of the autophagopore require a complex of Atg proteins that conjugate phosphatidylethanolamine onto Atg8 family members (including LC3), a process that is critical for allowing expansion of the autophagosomal membrane. Conjugated LC3, called LC3-II, is the canonical marker of autophagosomes. Atg7 is a critical E1 ligase for several of the reactions necessary for autophagy (74).

EVIDENCE THAT AUTOPHAGY IS IMPAIRED IN AD

There is significant support for the possibility of defective autophagy in AD. Electron microscopic analysis of brain tissue from confirmed AD cases revealed that AVs accumulated in dystrophic neurites and correlated with the presence of filamentous tau (79). However, this correlation was not quantified (79). Similar results were observed in mouse models of AD. For example, in a presenilin 1 (PS1)/Amyloid Precursor Protein (APP) double transgenic mouse, AVs were prevalent in dystrophic neurites at as early as 4.5 months without a similar accumulation of other structures such as lysosomes (80, 81). In these transgenic mice LC3-positive bodies were particularly apparent in neurites surrounding amyloid plaques, and immunoblotting of hippocampi from 6 month old transgenic PS1/APP mice revealed increased levels of LC3-II compared to wild-type mice (81). It is well established that mutations in PS1 result in familial AD, and until recently it was thought that this was only due to alterations in APP processing. However PS1 has a number of non-secretase functions, including acting as the chaperone for the vacuolar-ATPase used to acidify the lysosomal lumen (82, 83). Mutations in PS1 were shown to impair the acidification of lysosomes, which is necessary for activating the proteolytic enzymes in this compartment. Improper acidification and impaired proteolysis of substrates would compromise the autophagy system and result in the accumulation of AVs as described above. However, another mouse model, the TgCRND8 mouse, which expresses mutant APP only, also has increased staining for LC3-II, as well as an increase in cathepsin D-positive lysosomes (84). This demonstrates that in the absence of mutant PS1, AD-associated impairment in autophagy occurs and thus is due to other factors. Treatment of *ex vivo* hippocampal slice cultures with lysosomal disruptors causes the formation of enlarged, dystrophic neurites filled with AVs and lysosomes, similar to what is seen in mouse AD models and human AD tissue (85, 86). It has also been suggested that specific cathepsins may become extralysosomal in certain diseases, including AD (87, 88). Together these observations implicate a possible failure of autophagy as part of AD pathogenesis.

EVIDENCE TAU CAN BE DEGRADED BY AUTOPHAGY

As indicated above, a functioning lysosomal compartment is critical for the completion of autophagy. Given the possibility of a defect at this level of autophagy, numerous studies have directly assessed the effects of impairing lysosomal function on tau turnover, including specifically targeting the cathepsins. In an early study the direct cleavage of tau by cathepsin D was investigated in an *in vitro* assay using tau partially purified from rat brain in combination with cathepsin D from human liver. Incubation of tau with cathepsin D at pH 4.0 resulted in a decrease in full-length tau and a concomitant increase in cleaved fragments of varying sizes (89). Similarly, adding exogenous cathepsin D to homogenates of rat cortex at a neutral pH also generated tau fragments. Intriguingly, if a cysteine protease inhibitor was added to the assay, tau cleavage stopped at the 29-kDa fragment, suggesting that cathepsin D (an aspartyl protease) could cleave tau to a 29-kDa fragment after which other proteases may act to further degrade the protein. This also suggests if cathepsin D was able to escape from the lysosome, for example in the context of an AD-related stressor, it could still function in the neutral environment of the cytosol. However, the activity of cathepsin D at the more neutral pH may be more impeded than appears, as a previous study found cathepsin D's proteolytic activity was significantly reduced above pH 6.0 (90). Treating hippocampal slices with chloroquine (CQ), which raises the pH of lysosomes to impair enzymatic function, was associated with increased levels of full-length tau (89, 91). This was in conjunction with an accumulation of intracellular PHF1 immunopositive tau (91). In M1C neuroblastoma cells that inducibly express full-length wild-type tau (4R0N), treatment with CQ also significantly slowed down tau degradation, and caused its accumulation (92). Treatment of hippocampal slices with the cathepsin modulator ZPAD (which stimulates cathepsin D very strongly) appears to increase the proteolysis of full-length tau resulting in the production of smaller fragments, including a phosphorylated 29 kDa fragment (86, 89). This partial degradation of tau was inhibited by inclusion of a selective cathepsin D inhibitor (86). Cathepsin D seems particularly important for degrading tau, as its expression was neuroprotective in a *Drosophila* tauopathy model. Levels of cathepsin D are elevated in flies expressing mutant human tau. If cathepsin D is genetically ablated, these tau flies exhibit enhanced neurotoxicity and a shorter lifespan (93).

Modulating autophagy through other approaches also indicates that tau can be degraded through this pathway. Overexpressing only the repeat domain of tau containing an FTDP-17 mutation in neuroblastoma cells leads to tau aggregation as well as the appearance of smaller proteolytic fragments. Using the autophagy inhibitor 3-methyladenine (3-MA) to block the formation of autophagosomes led to an increase in both soluble and insoluble tau (94). Directly activating autophagy through a variety of mechanisms leads consistently to enhanced tau clearance – either pathological forms or total tau. In a hippocampal slice preparation methylene blue was used to induce autophagy, which resulted in a decrease in phosphorylated tau and insoluble tau, specifically (95). In a cell line expressing the repeat domain of tau containing the FTDP-17ΔK280 mutant, treatment with the disaccharide trehalose, an mTor-independent autophagy activator, significantly reduced aggregated tau as measured by Thioflavin-S staining, as well as total tau levels both soluble and insoluble as detected by western blotting (96). Stimulating autophagy either through serum withdrawal or rapamycin treatment in SH-SY5Y cells overexpressing P301L tau that had been induced to aggregate led to substantial reduction in aggregates that was prevented by 3-MA (70). In a mouse model expressing the FTDP-17 mutant P301S, promoting autophagy with trehalose treatment beginning at weaning significantly reduced insoluble tau, as well as tau

phosphorylated at T212/S214 (AT100) (97). However, no other phosphorylation sites were assessed. This effect was correlated with improved neuronal survival in cortical layers I–III (97). Stimulating autophagy via genetic manipulation of the mTor pathway decreased total and phosphorylated tau in the same mouse model (98). Conversely, inhibiting autophagy (also via mTor) lead to increased total and AT8-positive phosphorylated tau (98). Mice in which the critical autophagy gene Atg7 is knocked out in forebrain neurons develop age-dependent neurodegeneration with accumulation of phosphorylated tau within intracellular inclusions (99). These inclusions specifically contained tau phosphorylated at AT8, AT100, and TG3 epitopes, but not PHF1. Significantly, if tau was also knocked out in these autophagy-deficient mice, neurodegeneration was reduced (99).

Interestingly, other evidence for the role of autophagy in clearing tau was the result of attempting to elucidate the role of the proteasome in tau degradation. Treating rat primary neurons with the proteasomal inhibitor MG-132 actually led to a reduction in total tau. This effect was likely due to a compensatory upregulation of autophagy, as evidenced by increased LC3-II protein and an increased number of autophagosomes in treated cells (96). This will be discussed in more detail below, as it has important ramifications for the intersection of these two degradative pathways.

INTERPLAY BETWEEN AUTOPHAGY AND THE PROTEASOME

There is compelling evidence for significant and extensive interplay between the autophagy and proteasomal systems. This has intriguing implications for disease processes and specifically tau degradation in AD. First, while each system preferentially degrades specific substrates, there are many substrates that can be degraded by both systems, tau being a prime example (25). For instance, a particular substrate may be degraded by the proteasome under normal conditions, but if that system is impaired and/or there is an excess of that substrate it may be degraded in a compensatory manner by autophagy. Another possibility is that particular forms of a substrate may be shuttled to one pathway or another. In the case of tau, as a monomer it is natively unfolded and hence a likely proteasomal substrate, as discussed above. However, any of the numerous modifications tau undergoes during AD pathogenesis may render it less able to do so, for example, by inducing conformational changes to a more ordered structure as suggested by several conformation-specific antibodies that label tau in AD brain (Alz-50, MC-1, etc.). Additionally, oligomerized or aggregated tau may not be a preferred proteasomal target. It has been suggested that this change in state is part of the signal for tau to be degraded by autophagy. This is supported by evidence that full-length tau, which has a lower propensity for aggregating, is cleared by the proteasome while caspase-cleaved tau, which is more aggregate prone, goes through autophagy (72). Also, aggregated tau can be cleared by inducing autophagy (70, 96).

Ubiquitin is implicated in targeting substrates to both pathways. Historically, poly-ubiquitin chains generated through lysine 48 (K48) linkages were viewed as the prototypical proteasomal targeting sequence, while K63 chains appeared more specific for autophagy. However, the experimental evidence indicates a more complex picture. For example, if HEK cells are transfected with tau and ubiquitin, tau is readily ubiquitylated and degraded by the proteasome (66). However, if a ubiquitin construct containing a site mutation at K63 is transfected in, no ubiquitylation of tau occurs. Other mutated forms of ubiquitin, including ubiquitin unable to form linkages at K48, still resulted in tau ubiquitylation. This indicates that in this experimental overexpression paradigm, K63 linkages are the primary ubiquitin linkage for tau, and they target tau to the proteasome rather than to autophagy (66). However, another study, also using HEK cells and ubiquitin K48 and K63 mutants, demonstrated that in the presence of the E3 ligase CHIP, tau could be ubiquitylated by both K48 and K63 linkages (100). The likelihood that *in vivo* tau can be ubiquitylated in multiple ways is supported by studies showing tau isolated from NFTs in human brain has several forms of ubiquitin linkages as well as mono-ubiquitylation (101, 102). These data suggest that the physical structure of the ubiquitin chain is unlikely to be a sufficient signal for selectively targeting tau to either the proteasome or autophagy. An alternate mechanism for specifically targeting substrates is the involvement of chaperone proteins. The chaperones involved in proteasomal targeting are not well characterized, although it is known that ubiquitin-tagged substrates are trafficked to the organelle. Currently identified chaperones include p62 and Hsp70 (66, 100). Slightly more is known about autophagy adaptors, and there is significant overlap, as both p62 and Hsp70 are adaptors for this pathway as well (103, 104). This further complicates the understanding of how a substrate is selectively targeted to one path or the other. For example, a ubiquitylated substrate can be bound by p62 and either delivered to the proteasome (66) or engulfed by an autophagosome via p62 binding to LC3 (105). These findings suggest the involvement of a currently unidentified chaperones and/or targeting signals, or undetermined additional factors.

Other characteristics of the substrate are likely to also play a role in successfully targeting the protein either to the proteasome or to autophagy. In the case of tau, two modifications seem to be critical for this process: phosphorylation and truncation. For example, in the study where rat brain extract was incubated with Mg^{2+} and ATP, there was an overall decrease in tau due to proteasomal activity; however tau phosphorylated at the PHF1 and Tau-1 epitopes seemed to be preferentially degraded as they were non-detectable within 3 h (73). The preferential degradation of specific phospho-forms of tau by a particular pathway has been reported in other studies as well. In CHO cells overexpressing P301L mutant tau, treatment with the Hsp90 inhibitor geldanamycin led to a more pronounced proteasome-mediated reduction in tau phosphorylated at proline-directed S/T sites compared to total tau (67). However, the levels of tau phosphorylated at KXGS sites within the repeat domain were not reduced by geldanamycin treatment. In agreement with those findings, inhibiting autophagy in primary rat cortical neurons with 3-MA resulted in the selective accumulation of tau phosphorylated at the KXGS motif S262 (recognized by the 12E8 antibody) (106). Additionally, in a hippocampal slice preparation, induction of autophagy by treatment with methylene blue led to a decrease in phosphorylated tau and insoluble tau without an effect on total tau (95). Activating autophagy with trehalose in rat cortical neurons demonstrated certain phospho-epitopes (AT8, PHF1, and 12E8) were reduced more significantly

than total tau – up to 80% compared to the 20% reduction in total tau (96). Finally, caspase-3 cleaved tau has a shorter half-life than full-length tau and is preferentially degraded by autophagy (72). Additional modified forms of tau have yet to be fully examined for their preferred route of degradation.

As specific substrates are targeted to one degradative pathway or the other, the function of each system can also directly impact the functioning of the other. It is well documented that blocking the proteasome with small molecule inhibitors causes an increase in autophagic flux (107). This can be seen both as increased autophagosome formation and maturation as well as enhanced degradation of autophagy substrates (96). However the converse is not true; autophagy impairment does not elevate proteasomal function and, in fact, rather strikingly inhibits it. There are several possible mechanisms for this inhibition. The accumulation of large aggregated substrates might impair the proteasome, as seen for PHF tau (69). Also, reduced recycling of p62 by impairing autophagy (causing its accumulation) will impair proteasomal processing, potentially by p62 competing with other chaperones for proteasomal targets and impeding their delivery (107). The degradation of tau is thus a complex process mediated by multiple factors. While much is known about how tau can be cleared, additional studies are needed to clarify what actually happens in both the normal brain and in the context of AD. This information will yield critical insights into potential therapeutics.

ROLE OF OLIGOMERS IN AFFECTING TAU DEGRADATION DECISIONS

Given the data indicating tau can be processed by both autophagy and the proteasome, and furthermore that the signaling mechanisms directing substrates to either path are shared, it is unclear how decisions regarding which way tau is degraded are made. One possibility could be tau's physical state of oligomerization. Soluble, monomeric tau is an ideal proteasomal substrate. Indeed, it has been clearly demonstrated that tau can be degraded by the proteasome (65–67, 73). It thus can be suggested that under physiologic circumstances much of tau is degraded in this manner, with select modified forms being cleared by autophagy. However, within the context of the AD milieu, additional tau modifications and degradative impairments may cause the balance to shift away from proteasomal degradation toward autophagy. For example, as discussed above, certain modified forms of tau, such as caspase-cleaved tau, have a stronger tendency to aggregate. As tau begins to assemble into oligomers, it may become increasingly undesirable as a proteasomal substrate. These low-order, soluble oligomers may be preferentially degraded by autophagy. However,

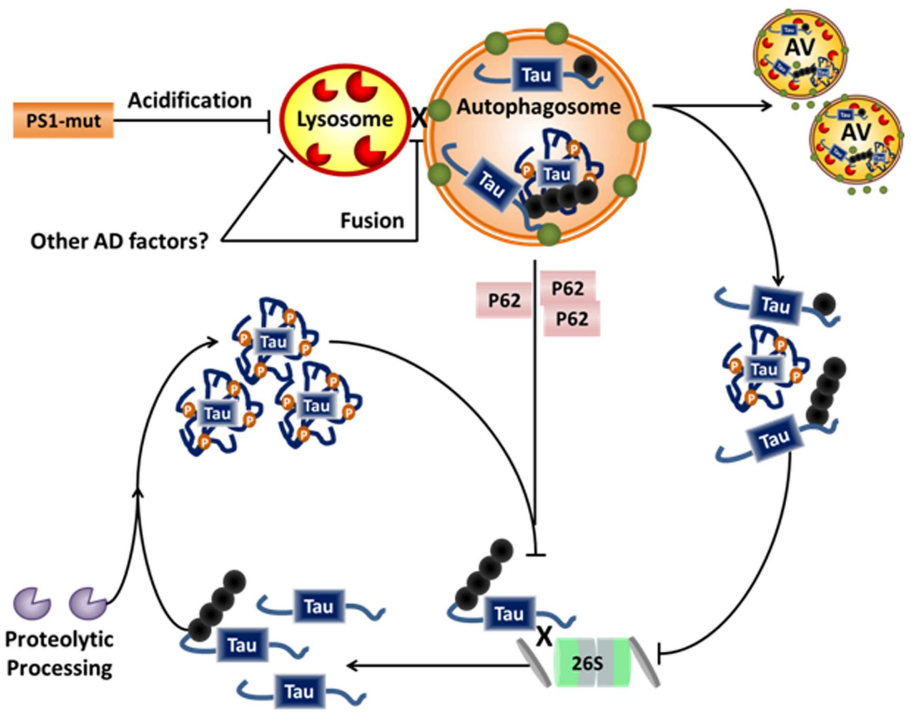

FIGURE 3 | Alzheimer disease-related disruption of tau degradation. Impairment of protein degradation is a known component of both familial and sporadic AD. Familial AD-related mutations of PS1 are linked to impairment of lysosomal acidification and/or fusion to the autophagosome, while sporadic factors leading to similar impairments have not yet been elucidated. Impairment of the lysosome/autophagosome leads to accumulation of dysfunctional autophagic vacuoles (AVs), cytosolic p62, and aggregates of tau. Dysfunction of autophagy further inhibits the proteasome, possibly via accumulation of p62, which can be a chaperone for both systems. P62 is usually recycled via autophagy and accumulates in the cytosol when autophagy is impaired. Hypothetically, this allows p62 to compete with other proteasome chaperones thus inhibiting proteasomal degradation. Further, accumulation of aggregated proteins has also been shown to inhibit the proteasome. Taken together, this could lead to accumulation of soluble tau, and thus more proteolytic processing and further aggregate. Hence, a vicious cycle of degradative pathway impairment and tau accumulation/aggregation may contribute to the neurodegenerative processes in AD.

as previously discussed, autophagy is likely impaired in AD. The tendency for certain phospho-epitopes to show preferential clearance by certain pathways may also relate to their propensity for aggregation. As tau oligomers increase in size, density, and modifications during the development of filaments and tangles, not only will they be unable to undergo proteasomal degradation, they may directly impair proteasomal function (69). This proteasomal impairment could have multiple effects. For example, autophagy may initially be activated as a compensatory response. Caspase-3 and possibly other proteases may also be activated as well. However, this may result in an accumulation of potentially toxic cleaved forms of tau. Additionally, given the significant evidence that autophagy is impaired in AD, possibly at the level of the lysosomes, proteasomally mediated activation may serve to further obstruct the autophagy system (see **Figure 3**).

CONCLUSION

It is clear that tau plays a significant role in AD pathology, although the mechanisms involved have not been clearly delineated. Tau is a normal neuronal protein that modulates microtubule-based functions, and becomes increasingly hyperphosphorylated, truncated, and otherwise modified in AD. These modifications not only impair tau's normal function, but also appear to promote its oligomerization. These oligomers eventually accumulate to form the NFTs which are pathognomonic for AD. While the NFTs may be harmful to the cell in some ways, it is now believed that the principal toxicity results from pre-aggregated, soluble tau oligomers. Thus, understanding how these tau species can be cleared may allow for the development of effective therapeutic approaches. It is clear from the data that certain species of tau are preferentially degraded by the proteasome and others by autophagy. There is evidence that both of these degradative systems are likely impaired at some level in AD. Additionally, there is a complex interplay between the proteolytic and degradative pathways that suggests a cycle of pathology may develop in AD whereby alterations in tau processing, including by cytosolic proteases, pushes more tau toward the autophagy system. Decreased autophagic function would result in accumulation of these autophagy-cleared tau species. The combination of impaired autophagy and accumulating substrates has the potential to lead to proteasomal inhibition, in addition to other factors (such as Aβ) that may impair the proteasome in AD (108). This then further promotes cytosolic accumulation of tau leading to its aggregation. Additionally, modifications including caspase-3 cleavage and hyperphosphorylation promote aggregation even of full-length tau, reducing the pool of functional tau.

Understanding how tau is cleared may enable us to identify potential mechanisms for enhancing clearance of pathological forms of tau. Ameliorating the deficit in autophagy is a likely target for this process, and initial results of stimulating autophagy show promise for clearing tau. Indeed, several studies aimed at stimulating autophagy have demonstrated efficacy in reducing phosphorylated and aggregated tau in both *in vitro* and *in vivo* models (95–98). These studies are an important initial step toward elucidating the exact role of tau degradation in modulating neurodegeneration in AD. Further studies that better gauge the contribution of each degradative pathway will be necessary. Due to the complexity of the cellular environment, *in vitro* studies that can tightly control for variables including tau modifications and proteolytic pathway function will likely be instrumental. Ultimately, a more complete understanding of the differential contribution of various proteolytic and degradative pathways will provide critical opportunities for therapeutically addressing the tau pathology associated with neurodegeneration in AD.

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The importance of tau phosphorylation for neurodegenerative diseases

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Fibrillar deposits of highly phosphorylated tau are a key pathological feature of several neurodegenerative tauopathies including Alzheimer's disease (AD) and some frontotemporal dementias. Increasing evidence suggests that the presence of these end-stage neurofibrillary lesions do not cause neuronal loss, but rather that alterations to soluble tau proteins induce neurodegeneration. In particular, aberrant tau phosphorylation is acknowledged to be a key disease process, influencing tau structure, distribution, and function in neurons. Although typically described as a cytosolic protein that associates with microtubules and regulates axonal transport, several additional functions of tau have recently been demonstrated, including roles in DNA stabilization, and synaptic function. Most recently, studies examining the trans-synaptic spread of tau pathology in disease models have suggested a potential role for extracellular tau in cell signaling pathways intrinsic to neurodegeneration. Here we review the evidence showing that tau phosphorylation plays a key role in neurodegenerative tauopathies. We also comment on the tractability of altering phosphorylation-dependent tau functions for therapeutic intervention in AD and related disorders.

Keywords: tau, phosphorylation, oligomers, Alzheimer's disease, function, extracellular

INTRODUCTION

Characteristic accumulations of highly phosphorylated tau protein aggregates are found in several neurodegenerative tauopathies including Alzheimer's disease (AD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and some forms of frontotemporal lobar dementia (FTLD-tau). It was assumed that these pathological tau aggregates are the toxic form of tau. However, recent studies indicate that soluble and highly phosphorylated tau species are more closely associated with synaptic dysfunction and cell loss (1–4).

Tau is normally a highly soluble protein found predominantly in neurons. A total of six different isoforms of tau are expressed in the adult human CNS via alternative splicing of the MAPT gene, which comprises 16 exons and is found on chromosome 17q21.3. Regulated inclusion of exons 2 and 3 gives rise to tau isoforms with 0, 1, or 2 N-terminal inserts, whereas exclusion or inclusion of exon 10 leads to expression of tau isoforms with three (3R) or four (4R) microtubule-binding repeats (**Figure 1A**). In normal human brain the ratio of 4R–3R tau is approximately one, whereas in many tauopathies, this ratio is altered; PSP, corticobasal degeneration (CBD), and argyrophilic grain disease all exhibit over-expression of 4R tau isoforms, whereas Pick's disease is mainly characterized by tau inclusions rich in 3R tau isoforms (5–9).

Tau is a phosphorylated protein, containing 85 potential serine (S), threonine (T), and tyrosine (Y) phosphorylation sites. Many of the phosphorylated residues on tau are found in the proline-rich domain of tau, flanking the microtubule-binding domain (**Figure 1B**). Both the phosphorylation status and isoform expression of tau are developmentally regulated and both are important factors for cytoskeletal plasticity during embryogenesis and early development. In early developmental stages a single tau isoform, 0N3R, is expressed and tau phosphorylation is elevated relative to adult brain. In contrast, all six tau isoforms are present in normal mature human brain, and at this stage tau phosphorylation is relatively reduced (8, 10).

Despite the significant heterogeneity that exists between and within the various tauopathies, the deposited tau in pathological lesions is invariably highly phosphorylated. Mass spectrometric analysis, combined with Edman sequencing and specific antibody reactivity, shows that approximately ten phosphorylation sites can be detected on soluble tau purified from normal brain (10). In contrast, when insoluble aggregated tau is extracted from tauopathy brain, at least 16 phosphorylated residues have been found in PSP (11–13), and approximately 45 different serine, threonine, and tyrosine phosphorylation sites, representing more than 50% of all phosphorylatable residues, have been found in AD brain (10, 14–17).

A large number of different kinases and phosphatases have been shown to regulate tau phosphorylation, and an imbalance in tau kinase and phosphatase activity is believed to result in tau hyperphosphorylation in disease. Tau kinases include:

- The proline-directed kinases glycogen synthase kinase-3 (GSK-3) (18–22), cyclin-dependent kinase 5 (cdk5) (23–25), and 5′ adenosine monophosphate-activated protein kinase (AMPK) (26, 27).

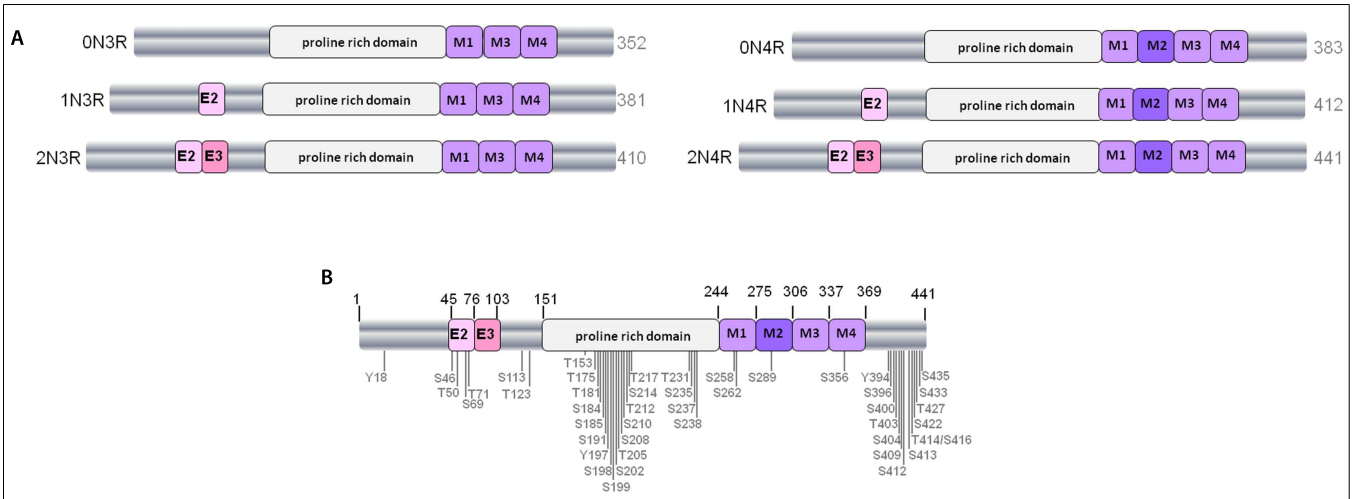

FIGURE 1 | The human tau gene and six protein isoforms. (A) The six isoforms of human CNS tau. Exons 2 and 3 (E2 and E3) encode two different inserts of 28 amino acids near the N-terminus of tau. Absence of E2 and E3 gives rise to 0N tau isoforms, whereas inclusion of E2 produces 1N and inclusion of both E2 and E3 results in 2N tau isoforms. M1–M4 represent the four imperfect-repeat microtubule-binding domains, M2 being encoded by exon 10. Lack of M2 results in the formation of 3R tau and M2 inclusion results in 4R tau isoforms. The proline-rich domain in the center of the tau polypeptide is indicated. Alternative-splicing produces tau isoforms of 352–441 amino acids, as indicated. **(B)** Positioning of phosphorylation sites on tau from Alzheimer brain. Approximately 45 phosphorylation sites have been identified, these are found predominantly in the proline-rich domain and the regions flanking the microtubule-binding domain.

- Non-proline-directed kinases, such as casein kinase 1 (CK1) (10), microtubule affinity-regulating kinases (MARKs) (28–30), cyclic AMP-dependent protein kinase A (PKA) (31, 32), and dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK-1A) (33, 34).
- Tyrosine kinases including Fyn (35, 36), Abl (37, 38), and Syk (39).

In addition, several phosphatases dephosphorylate tau, including protein phosphatase-1, -2A, and -5 (PP1, PP2A, and PP5) (reviewed by (40).

Importantly, many of these enzymes have been implicated in pathways affected by amyloid-beta (Aβ) in models of AD (27, 41–43). It remains to be established if the overall phosphorylation state of tau or phosphorylation at specific residues is important in disease pathogenesis, as suggested by studies in flies (44). However, there is evidence that phosphorylation of individual residues on tau can significantly impact its function, and this is discussed below.

THE RELATIONSHIP BETWEEN PHOSPHORYLATION AND TAU STRUCTURE

In addition to abnormal phosphorylation, tau protein in neurodegenerative disease brain can be modified in a number of ways, including N- and C-terminal proteolytic cleavage, altered conformation, nitration, glycosylation, acetylation, glycation, ubiquitylation, *O*-GlcNAcylation, aggregation, and filament formation (45, 46). Much research has focused on elucidating the relationship between phosphorylation and the changes in tau structure that are common in neurodegenerative disease brain. Evidence from this research suggests that phosphorylation occurs either prior to, or at the same time as, these other post-translational modifications and before aggregation occurs. It remains to be seen whether this temporal precendence indicates a causative relationship.

PROTEOLYTIC TAU CLEAVAGE

Tau is subject to proteolytic cleavage by caspase-3 at aspartate (D) residue 421 (47), and N-terminal cleavage by calpain-1 (48) and caspase-6 (49). The tau fragments that are generated have been detected in affected regions of human tauopathy brain (47, 50). Caspase-cleaved tau fragments show an increased propensity to aggregate, and these may form a seeding nidus that promotes the aggregation and fibrillization of full-length tau species (51). In contrast, cleavage of tau by calpain may partially inhibit tau aggregation (50). The temporal relationship between tau cleavage and phosphorylation is unclear, with data showing that phosphorylation of different tau residues precedes (52), follows (47), and inhibits (53) the proteolytic cleavage of tau by caspase-3. However, substantial evidence shows that caspase-3-cleaved tau species are particularly prone to phosphorylation in both primary neuronal cells (54) and human tauopathy brain (47), and that phosphorylated and caspase-3-cleaved tau species readily form aggregates in cells (55). These results therefore suggest that phosphorylation and caspase-mediated cleavage of tau are important events during the development of the characteristic tau aggregates that accumulate in AD and other tauopathies.

ALTERED TAU CONFORMATION

Tau is a natively unfolded protein that adopts abnormal conformations in tauopathy brain. For example, tau cleavage by caspase-3 at D421 occurs early in disease development, following an alteration in tau conformation detected by the Alz50 antibody, and prior to the formation of the conformational Tau-66 epitope (tau residues 155–244 and 305–331) which is detected in late-stage

AD (56). Altered tau conformation is suggested to be a major determinant in inducing tauopathy development *in vivo* (57), and abnormal tau conformers are detected in mouse models of tauopathy where elevated tau phosphorylation is apparent, but prior to the appearance of substantial tau aggregation (22, 58). Thus, caspase-3-induced tau cleavage appears to occur relatively early during the development of tauopathies, contemporaneous with increased phosphorylation and altered conformation of tau.

THE DEVELOPMENT OF TAU OLIGOMERS

A number of soluble and insoluble tau oligomers have been detected in AD and FTLD brain (2). Tau oligomers display altered conformation (59), are formed during the early stages of tau aggregation (59), and are closely associated with neurodegenerative phenotypes (2, 60). For example, transgenic mice that conditionally express a proline to leucine mutation at residue 301 (P301L) in human tau (1) exhibit high molecular weight tau oligomers, prior to the presence of neurofibrillary tangles (NFTs), that correlate with the development of cognitive deficits (2). Similarly, in a *Drosophila* model of tauopathy, the suppression of tau-induced neurodegeneration is associated with clearance of ubiquitinated and phosphorylated low molecular weight (<250 kDa) tau oligomers, concomitant with increases in ubiquitinated tau monomers and high molecular weight (>250 kDa) tau oligomers (61). It should be noted that protection from tau-associated toxicity in this latter study was also accompanied by reduced phosphorylation of soluble monomeric tau. Phosphorylation of tau by GSK-3 promotes the formation of insoluble oligomeric tau species that can constitute both full-length and truncated tau species (62, 63). The majority of insoluble tau in AD brain is intact (13). However, cleaved tau species are prominent in insoluble tau preparations from PSP, CBD, and FTLD-tau brain (13). The increased propensity of caspase-cleaved tau to aggregation (47), and the close association of tau fragments with cell death (64), suggests that although present as a relatively small pool of total tau, cleaved tau may also play an important role in disease. The presence of phosphorylated oligomeric tau species in cortical synapses extracted from AD brain (65) supports a role for highly phosphorylated tau multimers in tau-associated neuronal dysfunction.

THE FORMATION OF INSOLUBLE TAU AGGREGATES

In cell-free systems, soluble tau is a hydrophilic, unstructured, and dynamic protein (66). However, highly ordered aggregated tau filaments constitute the characteristic neurofibrillary lesions observed in tauopathy brain, including NFTs in AD and FTLD-tau, astrocytic plaques in CBD and tufted astrocytes in PSP (67).

There is substantial evidence that tau phosphorylation precedes its aggregation. Highly phosphorylated mouse and human tau undergoes self-assembly *in vitro* (68, 69), and dephosphorylation of soluble tau from AD brain inhibits its polymerization and restores the ability of tau to stabilize microtubules (70). Transgenic mice in which tau kinase activity is increased display increased tau phosphorylation prior to the presence of tau aggregates (24, 25, 58, 71). Furthermore, treating tau transgenic mice with kinase inhibitors results in reduced tau phosphorylation and also a reduced tau aggregate load (22, 72, 73). It should be noted, however, that reduction of tau aggregate load in tau transgenic mice following lithium treatment could result from enhanced autophagy in addition to reduced GSK-3-mediated tau phosphorylation (74). The relationship between tau phosphorylation and aggregation is clearly complex since phosphorylation of tau at specific sites, that are known to result in tau detachment from microtubules, can prevent tau aggregation (75). In addition, disruption to tau phosphatase activity in transgenic mice leads to the development of early disease-like tau abnormalities (76, 77). In particular, tau phosphorylation at the AT100 epitope is apparent in mice with reduced PP2A activity (77), which show cdk5-mediated enhanced activation of GSK3. Phosphorylation at the AT100 site has previously been shown to precede NFT formation (78), thus these findings may also suggest that changes in tau phosphorylation precede its aggregation. However, NFT formation was lacking in mice with reduced PP2A activity, an event attributed to increased clearance of abnormal tau conformers (77).

It is possible that the formation of a small pool of cleaved tau may be critically important in mediating the formation of pathological tau aggregates. Caspase-cleaved tau is prone to phosphorylation at specific epitopes (47, 54) and forms aggregation seeds that sequester full-length tau (51). Indeed, *in vivo* imaging of tau transgenic mice has demonstrated that truncated tau induces the misfolding of soluble tau and leads to the accumulation of hyperphosphorylated tau in tangles (79). Whether or not filamentous tau aggregates are toxic, protective, or inert remains an issue of intense debate (for review, see 80). However, small aggregated tau species have attracted interest recently because of their reported involvement in the propagation/transmission of tau pathology, and this topic is discussed in more detail below.

THE INFLUENCE OF PHOSPHORYLATION ON TAU LOCALIZATION AND FUNCTION

Tau is ubiquitously expressed during early embryonic development, but becomes localized predominantly in axons of mature neurons. The mechanisms underlying the axonal sorting of tau are not fully understood, but might involve selective trafficking of tau mRNA or protein into axons (81, 82), a retrograde transport barrier in the axon initial segment in mice (83), upregulation of tau mRNA translation in axons (84) or selective degradation of tau in dendrites (85). Tau is also found in association with neuronal membranes, in the nucleus, dendrites and synapses, and extracellularly. The localization of tau is altered in disease states. In particular, the redistribution of hyperphosphorylated tau to the somatodendritic compartment is considered a hallmark pathological marker during early tauopathy development (86, 87). The functional consequences of tau phosphorylation-mediated changes in the cellular localization of tau are discussed below.

CYTOPLASMIC TAU: CYTOSKELETAL INTEGRITY AND AXONAL TRANSPORT

A large proportion of tau is found in the cytosolic compartment, where it interacts with microtubules through its C-terminal microtubule-binding domain (**Figure 1**, residues 244–368). The binding of tau with microtubules is regulated by tau phosphorylation status, with *in vitro* phosphorylation of recombinant tau at S262 and S356, orthologous residues in

adjacent microtubule-binding repeats, reducing tau interactions with microtubules and rendering tau less susceptible to aggregation (75). Phosphorylation of tau at residues outside of the microtubule-binding domain of tau, including S214 and T231, have also been shown to reduce its interaction with microtubules (75, 88). These findings suggest that phosphorylation at different tau sites may have opposing effects on the ability of tau to aggregate Furthermore, interaction of the peptidyl-prolyl isomerase Pin1 with phosphorylated T231 mediates the interaction of PP2A with the *trans* configuration of phosphorylated tau, and results in a conformational change that restores the ability of tau to bind to microtubules (89–91). Regardless of the particular sites involved, increased tau phosphorylation that causes tau to detach from microtubules leads to the disassembly of microtubules and disruption to the structure of the neuronal cytoskeleton. In addition, the accumulation of unbound hyperphosphorylated tau in the cytoplasm could cause further microtubule disassembly by sequestering normal tau and other microtubule-associated proteins (92). When tau is in a filamentous state, its interaction with normal (soluble) tau and its inhibition of microtubule stabilization is disrupted (93). Preventing microtubule instability in tauopathies has become an important target for drug development (94, 95).

Alterations in tau phosphorylation also affect its anterograde axonal transport. In general, reducing tau phosphorylation at S/T residues decreases, whereas mimicking tau phosphorylation increases, the rate of axonal tau transport in fly, rodent, and human neurons (21, 96–98). The influence of tau phosphorylation on its transport appears to be associated with differential binding of S/T phosphorylated tau to the molecular motor protein kinesin-1 (97, 98) and differential degradation rates of phospho-tau species through the lysosomal autophagy system (98).

The interaction of tau with microtubules is critically involved in the regulation of microtubule-dependent axonal transport (99), therefore tau phosphorylation also plays a key role in regulating the transport of other important cargoes. Increasing tau phosphorylation at N-terminal Y residues relieves the inhibition of anterograde axonal transport observed in the presence of highly phosphorylated tau aggregates in squid axons (100). However, tau is not usually highly phosphorylated in squid axons and therefore it is unclear whether this provides a good model to examine mammalian tau functions. In mice over-expressing FTLD-tau mutations, there is impaired anterograde axonal transport of vesicles containing the dopamine-synthesizing enzyme tyrosine hydroxylase, which precede the loss of dopaminergic neurons in the substantia nigra (101). The transport deficits reported in this mouse model were shown to be mediated by interactions between phosphorylated tau and JNK-interacting protein 1 (JIP-1) (102). Since JIP-1 regulates the binding of cargo to kinesin, these results further support the idea that increasing tau phosphorylation disrupts axonal transport. Alternatively, reduced degradation or clearance of aggregated or mutant forms of tau might contribute to a "clogging" of microtubules and consequent disruption in axonal transport (103).

Disruption to axonal transport is predicted to be an early event in several neurodegenerative diseases (104) and recent evidence suggests that dysregulated axonal transport may contribute to tau-induced degeneration. Genetic suppression of Miro, an adapter protein essential for mitochondrial axonal transport, exacerbates the neurodegenerative phenotype in *Drosophila* expressing human tau, through a mechanism dependent upon phosphorylation of tau at S262 by PAR-1, the *Drosophila* homolog of MARK kinase (105). Similarly, deletion of kinesin light chain-1 results in accumulation of hyperphosphorylated tau and the appearance of axonal spheroids in mice (106), in line with numerous reports that have characterized the binding of tau to kinesin (21, 96–98).

Finally, alterations in mitochondrial transport and function are intrinsically linked with several neurodegenerative diseases (107). Over-expression of tau *in vivo* results in alterations to mitochondrial distribution that are associated with soluble, rather than fibrillar, tau species (108). In addition, tau phosphorylation alters the axonal transport and distribution of mitochondria in cultured neuronal cells (109, 110), an effect recently attributed to tau phosphorylation-dependent changes in inter-microtubule spacing (110). Furthermore, highly phosphorylated tau has been shown to interact with the mitochondrial fission protein, Drp1 (111), and DuBoff et al. (112) demonstrated that this relationship is important for neurodegeneration. They show that actin is over-stablised in *Drosophila* that express human tau, and that this impairs the actin-based translocation of Drp1 and mitochondria, which reduces their interaction and leads to accumulation of Drp1 on F-actin, mitochondrial elongation, and downstream neurotoxicity (112). Thus tau phosphorylation is closely linked to alterations in the localization and/or function of mitochondria. It is therefore likely that phosphorylated tau influences synaptic dysfunction in tauopathies by contributing to the depletion of functional mitochondria from synapses (113).

MEMBRANE-ASSOCIATED TAU: A CELL SIGNALING ROLE FOR TAU?

Tau interacts with several neuronal membranes, including the endoplasmic reticulum (114), the Golgi network (114), and the plasma membrane (115, 116). An increasing body of evidence shows that the association of tau with plasma membranes is regulated by phosphorylation (116–118). Plasma membrane-associated tau is dephosphorylated at several sites known to be aberrantly phosphorylated in AD brain (116, 117, 119, 120). Indeed, phosphorylation of tau at N-terminal, but not C-terminal, residues prevents its membrane localization in tau-transfected cells, demonstrating that the phosphorylation state of tau directly impacts its positioning at membranes (116).

Tau has also been detected within cell-surface lipid-rich microdomains of the plasma membrane (35, 41, 121), and the amount of tau associated with these lipid rafts is regulated by tau phosphorylation at N-terminal tyrosine residues (121). Tau interactions with the non-receptor tyrosine kinase Fyn are critical for the interaction of tau with lipid rafts (35, 41, 121) and neuronal plasma membranes (116). Tau can interact with Fyn via its SH2 and SH3 domains (121, 122). Phosphorylation of tau at Y18 is important for tau interactions with Fyn-SH2 (121), whereas phosphorylation of S/T residues on tau negatively influences its interaction with Fyn-SH3 (122). Accumulating evidence therefore suggests that targeting of tau to the plasma membrane may be regulated by the interaction of the tau N-terminal projection domain with the SH3 or SH2 domains of tyrosine kinases such as Fyn (118). Furthermore, these data suggest that by binding to

several important signaling molecules in a manner that is regulated by phosphorylation, tau has the potential for a broad role in cell signaling (122).

DENDRITIC TAU AND SYNAPTIC TOXICITY

A number of recent cell and animal studies have shown an important role for tau in dendrites leading to the suggestion that tau-mediated synaptic dysfunction may be one of the earliest events in the pathogenesis of tauopathies. Several studies have indicated that the presence of tau aggregates is detrimental to synaptic health (123, 124), however, soluble tau species are associated with synapse loss in mouse models of tauopathy (125) and phosphorylated tau oligomers have also been detected in synapses in postmortem AD brain (126).

A small amount of tau exisits in dendrites under normal conditions, where it acts to target Fyn post-synaptically, regulating *N*-methyl-D-aspartate (NMDA) receptor subunit 2 phosphorylation and interactions between NMDA receptors and the post-synaptic density protein PSD-95 (3). Disease insults, such as increased concentrations of Aβ in AD, lead to the detachment of highly phosphorylated tau from microtubules and its accumulation in intact dendritic spines (3). This in turn causes local elevations in Ca^{2+} and disruption of synaptic function through impaired trafficking and/or synaptic anchoring of glutamate receptors (3, 127, 128). In a related study, the redistribution of hyperphosphorylated tau into dendritic spines led to reductions in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subtypes that caused impairments in basal synaptic transmission and long term potentiation (129). Thus, there is increasing evidence that tau-mediated synaptic dysfunction might be one of the earliest events in the pathogenesis of tauopathies (reviewed by 130). Therefore, correction of aberrant tau phosphorylation may be therapeutically beneficial during very early stages of disease progression when synaptic deficits first develop. In this respect, it is worth noting that inhibition of GSK3 has previously been shown to attenuate deficits in LTP (131).

NUCLEAR TAU – A ROLE IN DNA PROTECTION

It was first suggested that tau might have novel functions mediated by interactions with DNA or RNA following observations that tau is present in the nuclei of human neuroblastoma cells (132). Full-length tau was identified in neuronal nuclei, where it colocalizes with the chromosome scaffold, nuclear and nucleolar organization centers and can exist as SDS-insoluble species (132, 133). Further studies revealed that the microtubule-binding domain of tau can bind RNA (134), and single and double stranded DNA (135, 136). The interaction of tau with RNA enhances tau polymerization; the RNA acting as a nucleation center for tau aggregation (134), whereas interaction of tau with DNA results in conformational changes in DNA (133) and suppression of DNA amplification *in vitro* (136). Insights into the nuclear function of tau were recently revealed with the observation that tau protects DNA from heat damage and oxidative stress (137). Nuclear tau appears to be largely dephosphorylated (137), suggesting that increased tau phosphorylation in diseased states could interfere with protective functions of non-phosphorylated tau in neuronal nuclei.

EXTRACELLULAR TAU AND THE PROPAGATION OF TAU PATHOLOGY

Tau is present in brain insterstitial fluid in the absence of any neurodegeneration (138). Recent evidence suggests that this extracellular tau is likely to have important functional consequences for neuronal health and for the spread of tau pathology across the brain during disease progression.

To allow investigation of tau pathology spread *in vivo*, transgenic mice have been created with neuropsin-promoter targeted expression of tau in layer II neurons of the entorhinal cortex. These mice demonstrate an age-dependent spread of phosphorylated and aggregated abnormal tau confomers from the site of transgene expression to neighboring neurons and anatomically connected brain regions (139, 140). There are several mechanisms that could account for this observed spread of tau pathology. Firstly, degenerating neurons with high levels of transgene expression might release pathological forms of tau that subsequently propagate in a "prion-like" fashion through their uptake by neighboring neurons. In support of this process, Frost et al. (141) demonstrated that extracellular tau aggregates, but not tau monomers, are taken up by cultured human embryonic kidney (HEK293) cells and neuronal stem cells, leading to fibrillization of full-length intracellular tau. Similarly, small oligomers of tau, similar to those found in human tauopathy brain, can be taken up by cultured neuronal cells via bulk endocytosis (142). It is possible that this process also underlies the postulated prion-like transmission of tau pathology to distal brain regions observed when pathological forms of human tau are injected into mice expressing wild-type human tau (143, 144). Secondly, tau pathology in the neuropsin-promoter regulated tau transgenic mice appears to spread to anatomically connected pathways in the absence of any notable cell loss (139, 140), suggesting that tau is released from intact neurons and then taken up by connected cells. This process is supported in part by recent findings showing endogenous tau release from cultured neurons in the absence of cell death (145, 146). Interestingly, the release of endogenous full-length tau from rat primary neurons was shown to be a dynamic and physiological process that is calcium-dependent and stimulated by AMPA receptor activation and neuronal activity (146), suggesting that tau release may play a role in signaling between neurons. Indeed, exogenously applied tau can interact with muscarinic receptors on the surface of cultured neuronal cells, promoting increases in intracellular calcium that alter cell signaling pathways (147). It is also possible that tau propagation may be mediated via glial cells, since cytosolic tau accumulations are observed in neurons surrounded by activated microglia (148) and astrocytes promote tau phosphorylation in neighboring neurons (54).

The relationship between tau secretion and tau phosphorylation state is not yet established. However, extracellular tau released from primary neurons, neuroblastoma cells and non-neuronal cells is dephosphorylated at several epitopes known to be highly phosphorylated in AD brain (145, 146, 149) and this has been proposed to result from the action of extracellular tissue non-specific alkaline phosphatase (149). How this relates to the phosphorylation state of intracellular tau is not clear, although the secretion of C-terminally cleaved tau from non-neuronal cells can be enhanced by the increased phosphorylation or cleavage of intracellular tau (150). These studies indicate that changes in tau phosphorylation can modulate its release from neurons, and therefore is also likely

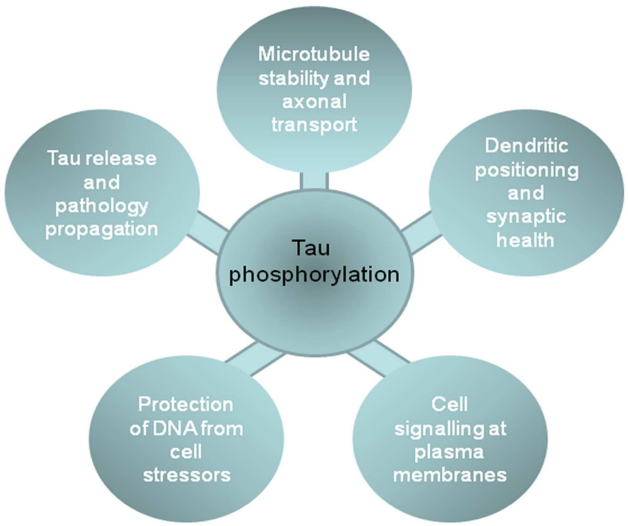

FIGURE 2 | The impact of phosphorylation on tau functions in different cell locations. The figure shows the functions of tau in different cellular compartments that are influenced by tau phosphorylation, and that likely contribute to the development or progression of neurodegenerative tauopathies.

to influence the effects of extracellular tau on neuronal health and the spread of tau pathology in diseased brain.

TAU PHOSPHORYLATION AS A THERAPEUTIC TARGET

As summarized above, tau phosphorylation plays a key role in regulating tau function at different neuronal locations, including the involvement of cytosolic tau in stabilizing the neuronal cytoskeleton and influencing axonal transport; the role of membrane tau and extracellular tau in cell signaling and neurofibrillary pathology spread through diseased brains; the relationship between nuclear tau and protection from DNA damage; and dendritic functions of tau that are involved in synaptotoxicity (**Figure 2**). These data suggest that inhibition of tau phosphorylation could have widespread disease-modifying effects in tauopathies. Therapeutic strategies aimed at targeting tau phosphorylation have been widely reviewed elsewhere (e.g., 8, 9, 67, 151), therefore we will comment only briefly here.

Although several kinases and phosphatases regulate tau phosphorylation, only GSK-3 inhibitors have entered clinical trials for the treatment of AD or rarer tauopathies such as PSP. Based on promising data from animal models (21, 22, 152), the relatively non-specific GSK-3 inhibitor, lithium, was tested in small-scale clinical trials for mild to moderate AD. Whilst lithium did not cause significant adverse effects in an open label study of a year (153), neither did it have any beneficial effects in a short-term trial (154). However, a small trial of lithium in patients with mild cognitive impairment reduced phosphorylated tau in CSF and reported better performance of treated patients in cognitive and attention tasks (155), suggesting that administration of lithium during the early stages of disease could have some therapeutic benefit in defined patient populations.

Tideglusib (NP-12) is a non-ATP competitive inhibitor of GSK3 that has entered clinical trials. Tideglusib has disease-modifying effects when administered to transgenic mice that develop both tau and amyloid pathology (156). Pilot trials for tideglusib in AD and PSP showed good tolerance of tideglusib (157) and phase II studies are underway.

Kinase inhibitors have entered clinical use for conditions unrelated to neurodegeneration (158). However, kinases make for complex therapeutic targets, and probably because of incomplete drug specificity, off-target effects are problematical. An alternative strategy may be to modulate the activity of proteins that directly affect the activity of tau kinases. One interesting target in this respect is lemur tyrosine kinase-2 (LMTK2). LMTK2 phosphorylates PP1C on T320, thereby inhibiting PP1C activity (159–161). PP1 regulates phosphorylation of GSK3β at the inhibitory phosphorylation site S9 (162, 163), and therefore, via its effect on PP1C, LMTK2 regulates GSK-3β phosphorylation at S9, and ultimately GSK-3 activity (160, 161). Therefore, an alternative strategy for inhibiting GSK-3 activity may be to increase LMTK2 expression or activity. Small molecule allosteric agonists for a variety of kinases have now been described, and the development of kinase agonists has been identified as key area for the development of new therapies (164).

Finally, biomarkers are increasingly used to follow the progression of AD, and in some cases to support early diagnosis of the disease (165). However, to accelerate the clinical translation of therapeutics that modify tau phosphorylation, it is essential that sensitive and specific biomarkers are available to allow the measurement of drug–target interactions, and the impact of treatment on downstream pathophysiology. The development of such target validation biomarkers will allow a faster selection of candidate treatments, and appropriate dose ranges. This should accelerate the clinical development of tau phosphorylation inhibitors that are likely to have wide-ranging benefit for the treatment of AD and related tauopathies.

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Tau in MAPK activation

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The nature of "toxic" tau in Alzheimer's disease (AD) has been unclear. During pathogenesis, the importance of tau oligomerization vs. tau phosphorylation is controversial and the investigation of both remains critical toward defining the "toxicity" of tau. The phosphorylation of tau on serines and/or threonines occurs early in the disease course and altering phosphorylation has been shown to disrupt neuropathogenesis. We have recently reported that in PC12-derived cells, tau had a role in signal transduction processes activated by NGF. By depleting tau, NGF-induced MAPK activation was attenuated and by restoring tau, MAPK activation was restored. Furthermore, the phosphorylation of tau on Thr231 was required for tau to potentiate MAPK activation. Here we report the effects of additional disease-related tau phosphorylation sites and tau isoform on the ability of tau to potentiate MAPK activation. Our findings, which tested three other sites of phosphorylation, showed that phosphorylation at these other sites mainly lessened MAPK activation; none potentiated MAPK activation. In comparing 0N3R tau to the other five brain tau isoforms, most showed a trend toward less MAPK activation, with only 2N4R tau showing significantly less activation. Since MAPK activation has been reported in AD brain and is characteristic of cell proliferation mechanisms, tau phosphorylation that promotes MAPK activation could promote cell cycle activation mechanisms. In neurons, the activation of the cell cycle leads to cell death, suggesting that abnormally phosphorylated tau can be a toxic species. The relationship between tau oligomerization and its ability to potentiate MAPK activation needs to be determined.

Keywords: tau, MAPK activation, phosphorylation, signal transduction, NGF

INTRODUCTION

The existence of tau pathology occurs in many age-related neurodegenerative diseases that are now termed "tauopathies." Among these diseases, Alzheimer's disease (AD) is the most prevalent and it has been suggested that the presence of tau is critical for disease progression (1, 2). Neurodegenerative diseases caused by both missense and intronic mutations in the tau gene have indicated the ability of tau to cause disease [reviewed by Ref. (3–5)]. However, the mechanism by which tau leads to neurodegeneration is unknown. For instance, whether there is a loss of function or a gain of toxic function remains controversial. In considering the role of different tau species during neurodegeneration, hyperphosphorylated tau, and tau filaments have long been investigated. Evidence suggesting that neurofibrillary tangles were not a toxic species came from data indicating that tau-induced behavioral deficits could be improved without changing the tangle burden (6). In fact, neuronal loss did not correlate with neurofibrillary pathology (7). Also, in *Drosophila* and *C. elegans*, tau-induced neurodegeneration occurred in the absence of neurofibrillary tangles (8, 9). Most recently, the investigation of tau oligomers has suggested that they may have an early role in neurodegeneration. Tau oligomers correlate with cellular abnormalities (10–12) and neurodegenerative disease (13–16). However, the molecular mechanism by which tau oligomers cause toxicity has not been clearly demonstrated. In addition, in these studies, the tau oligomers were composed of phosphorylated tau, making it difficult to isolate the effects of oligomerization from those of phosphorylation.

Tau phosphorylation is required for its neurotoxic effects (17, 18) and as tau is hyperphosphorylated early in the disease process, it is not surprising that tau oligomers would be formed from phosphorylated tau. Therefore, in determining if tau oligomers have specific function, one could also first determine the function of abnormally phosphorylated tau, then ask if that tau was in the form of oligomers. Recently, we found that tau has the ability to potentiate NGF-induced MAPK activation and that phosphorylation on Thr231 was critical for the activity (19). Since this activity was seen within 3 h after NGF addition, our data identified a new role for tau in signal transduction processes that take place during neuronal differentiation. At the same time, as Thr231 is phosphorylated early during neurodegeneration (20), it raised the question of whether this new tau activity had a role in neurodegeneration. To further probe the relationship between tau phosphorylation and its ability to potentiate MAPK activation, here we investigate the effects of additional phosphorylated sites, focusing on sites relevant to AD. We also investigate the effects of alternative splicing on the ability of tau to affect MAPK activation.

MATERIALS AND METHODS

CELL CULTURE

PC6-3 cells (21) were cultured on collagen (BD Biosciences) coated dishes using RMPI 1640 medium with 10% horse serum and 5% fetal bovine serum. D5 cells, a stable PC6-3 cell line with stable over-expression of the 0N3R isoform of human tau, and rTau4 cells, a PC6-3 cell line with stable expression of hairpin RNAi

targeting endogenous rat tau, were previously described (19). Media for stable cell lines was supplemented with 200 μg/ml G418.

MAPK REPORTER ASSAYS

MAPK activation was measured by a luciferase reporter assay as described by Leugers and Lee (19). The ability of tau mutants to influence NGF-induced MAPK activation was studied by co-transfecting tau plasmids with the MAPK reporter plasmids. Tau plasmids used were pRc/CMV-0N3R, pRc/CMV-0N3R-S214D, pRc/CMV-0N3R-S404D, pRc/CMV-0N3R-S396D/S404D, pRc/CMV-0N3R-S202D, pRc/CMV-0N3R-S199D/S202D, pRc/CMV-0N4R, pRc/CMV-0N4R-S202D, pRc/CMV-0N4R-S199D/S202D, pRc/CMV-1N3R, pRc/CMV-2N3R, pRc/CMV-1N4R, and pRc/CMV-2N4R. (0N3R, 0N4R, etc., denote tau isoforms where 0N3R contains 352 residues with no amino terminal inserts and three microtubule binding repeats; 2N4R contains 441 residues with two amino terminal inserts and four microtubule binding repeats, etc.). Mutant tau plasmids with phospho-mimicking S to D mutations were constructed using site-directed mutagenesis (Stratagene, Inc.); sequences were confirmed by DNA sequencing.

TAU DETECTION IN CELL LINES

D5 cells were grown with or without NGF for 30 min and then harvested in RIPA buffer with protease and phosphatase inhibitors (19). After rocking at 4° for 20 min, lysates were centrifuged 20 min. Supernatants were added to an equal volume of 2× Laemmli sample buffer and boiled 5 min. Cell lysate samples were subject to SDS-PAGE and transferred to PVDF membranes. Membranes were probed with anti-phospho-Ser214-tau (Invitrogen, Inc.), PHF1 (22), AT8 (23), Tau5 (24), Tau12 (25), or anti-GAPDH (Chemicon, Inc.). Signal was visualized using ECL (Western Lightning Plus-ECL, Perkin Elmer, Inc.).

RESULTS AND DISCUSSION

To examine MAPK activation, PC6-3 cells, a PC12-derived cell line (21), were treated with NGF. To probe tau function, we used the rTau4 cell line, a PC6-3-derived cell line that expressed a hairpin shRNA that selectively down-regulated the expression of endogenous rat tau without affecting the expression of human tau mediated by transfection (19). In tau-depleted rTau4, NGF-induced MAPK activation was attenuated and the addition of wild-type human tau (0N3R) was able to significantly restore MAPK activity after growth factor treatment (19). Moreover, a phospho-mimicking mutation at Thr231 brought further increases to MAPK activation while a Thr to ala mutation at Thr231 showed a dominant negative effect on MAPK activation (19). This led us to conclude that tau phosphorylation at Thr231 was required for the effect of tau on MAPK signaling. Based on these findings, and the fact that tau can undergo phosphorylation at a number of sites during early brain development (26) or during neurodegeneration (27), we further investigated the effects of tau phosphorylation on MAPK activation.

To select tau phosphorylation sites to test, we sought sites that were modified in both AD and in the PC6-3 cells. We chose to examine Ser214, Ser396/Ser404, and Ser199/Ser202, all of which are known to be phosphorylated in AD. While tau phosphorylation in NGF-treated PC12 cells has been examined, NGF treatments greater than 24 h were often used and we were interested in earlier time points as our focus was on signal transduction rather than neurite outgrowth. To assess the phosphorylation of both endogenous rat tau and exogenously expressed human tau (0N3R), we utilized the previously described PC6-3-derived cell line D5, that stably expresses human tau (19). In both undifferentiated cells and cells stimulated with NGF, we observed phosphorylation at Ser199/Ser202, detected by AT8, as well as phosphorylation at Ser396/Ser404, detected by PHF1 (**Figure 1**). NGF treatment was performed for 30 min and no further changes in the level of phosphorylation at these sites were observed for up to 3 h (data not shown). The phosphorylation at Ser396/Ser404 was found to occur in both rat tau and human tau species and the addition of growth factor appeared to slightly increase the level of phosphorylation. Phosphorylation at Ser199/Ser202 was also observed in both rat and human tau and did not appear to change upon NGF addition. In contrast, phosphorylation at Ser214 appeared increased after NGF induction (**Figure 1**). Our previous data had shown that phosphorylation at Thr231 also increased after NGF induction (19). These data indicate that these tau sites are being phosphorylated in PC6-3 cells.

To examine MAPK activation, we tested a panel of phosphomimetic mutations at these tau phosphorylation sites. In advance of measuring MAPK activation, protein produced by each plasmid was visualized by western blotting in order to confirm that equivalent amounts of each protein was being expressed in each experiment (**Figure 2A**). In this way, differences in MAPK activation would be attributed to protein identity rather than protein quantity. In each experiment, WT tau was expressed in the tau-depleted rTau4 cells as control. When expressing 0N3R human tau with a phospho-mimicking mutation at Ser214 (S214D, **Figure 2B**), we observed a significant attenuation of MAPK activation relative to WT tau. Next, we tested phosphorylation at Ser404 using S404D and observed a trend of reduction in MAPK signaling (**Figure 2C**), indicating that phosphorylation at this site might also impair the ability of tau to enhance MAPK signaling. As phosphorylation at Ser404 often occurred in conjunction with phosphorylation at Ser396 (22, 26, 27), we also tested a double

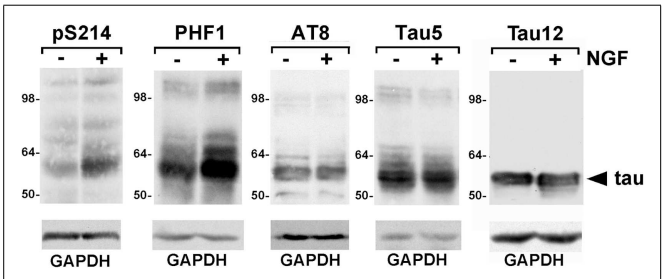

FIGURE 1 | Phosphorylated tau is expressed in PC6-3 cells. Serum starved D5 cells were stimulated with 50 ng/ml NGF for 30 min. Cells were harvested as described in Section "Materials and Methods." Following SDS-PAGE, immunoblotting was performed with antibodies as indicated, total tau being probed by Tau5 and human tau by Tau12. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels are shown as a loading control. Arrowhead indicates human tau; the less abundant rat tau was visualized with Tau5.

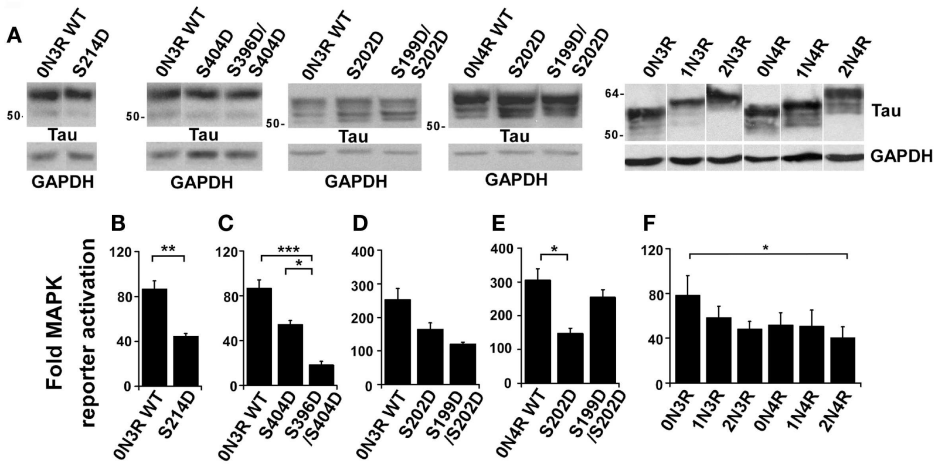

FIGURE 2 | Tau phosphorylation modulates the effect of tau on NGF-induced MAPK signaling. rTau4 was transfected with MAPK reporter system plasmids and tau plasmid indicated. Thirty-six hours after transfection, NGF was added 3 h prior to cell harvest. **(A)** To assure equal tau expression in transfections to be assayed in **(B–F)**, lysates from rTau4 cells transfected under identical conditions were probed with Tau13. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels are shown as a loading control. **(B–F)** Fold-MAPK reporter increase was calculated as previously described (19). (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$). Data shown are mean + SE from three independent experiments; for each experiment, transfections were performed in triplicate for each condition.

mutant, S396D/S404D, and found MAPK signaling significantly impaired relative to both control WT tau and S404D mutant (**Figure 2C**). In addition, the phospho-mimicking substitution at Ser202 was tested and we observed a trend of decreasing MAPK activation (**Figure 2D**). The double mutant S199D/S202D was also tested and we found a similar trend (**Figure 2D**). Together, these findings identified several tau phosphorylation sites where phosphorylation appeared to decrease the ability of 0N3R tau to potentiate MAPK activation. Moreover, these findings indicated that the ability of phospho-Thr231-tau to increase MAPK activation was unique (**Table 1**).

Tau mutations that affect the alternative splicing of tau mRNA can result in increased levels of 4R tau and cause neurodegenerative disease [reviewed in Ref. (28, 29)]. To determine if the isoform identity could alter the ability of tau to affect MAPK signaling, we tested different isoforms of tau for their ability to rescue MAPK activation in rTau4. In comparing the abilities of the wild-type 0N3R and 0N4R tau to restore MAPK signaling, while significant differences were not demonstrated, there was a trend showing that 0N4R tau had reduced activity (**Figure 2F**). In addition, in both 3R and 4R isoforms, we observed a trend of decreased MAPK activation as the N-terminal inserts were added (**Figure 2F**). A significant difference between the largest and smallest isoforms of tau (2N4R vs. 0N3R tau) was observed (**Figure 2F**). These observations demonstrated that the effects of tau on MAPK signaling may be modulated by alternative splicing.

Lastly, we compared the effects of the phospho-mimicking substitutions on 0N3R and 0N4R tau. Comparing the effects of S202D on 0N3R and 0N4R, we found that the mutation inhibited MAPK signaling to a larger extent in 0N4R, where a significant decrease occurred (compare **Figures 2D,E**). However, when the effects of the double mutant S199D/S202D were compared between 0N3R and 0N4R, we found that while 0N3R S199D/S202D resembled 0N3R S202D in its ability to decrease MAPK signaling (**Figure 2D**), 0N4R S199D/S202D appeared to rescue MAPK signaling, yielding levels similar to wild-type 0N4R tau (**Figure 2E**). These findings suggest that in 0N4R, while the phosphorylation of tau at Ser202 decreased the ability of tau to potentiate MAPK activation, additional phosphorylation at Ser199 neutralized the effect, returning MAPK activation levels to WT tau levels. These data indicated that the effect of phosphorylation on NGF-induced signaling depended on the tau isoform used.

Our data shows that phosphorylation differentially affected the function of 3R and 4R tau isoforms. This result resembles previous data reported for the interaction between tau and the SH3 domain of Fyn, where we found that phosphorylation differentially affected the equilibrium binding constant of 0N3R and 0N4R tau for the SH3 domain of Fyn (30). For the Fyn SH3 interaction, phosphorylation at Ser199/Ser202 or at Ser396/Ser404 increased

Table 1 | Phospho-mimicking mutations in 0N3R tau, tested for their ability to potentiate MAPK activation.

	Ability to potentiate MAPK activation
Phospho-Ser214 (S214D)	**↓**
Phospho-Ser202 (S202D)	↓
Phospho-Ser199/Ser202 (S199D/S202D)	↓
Phospho-Ser404 (S404D)	↓
Phospho-Ser396/Ser404 (S396D/S404D)	**↓**
Phospho-Thr231 (T231D)	**↑**

(Bolded larger arrows indicate a statistically significant difference relative to non-phosphorylated tau; non-bolded smaller arrows indicate a trending result.)

the binding of 0N4R to the Fyn SH3 domain whereas phosphorylation at Ser199/Ser202 decreased the binding of 0N3R to the Fyn SH3 (phosphorylation at Ser396/Ser404 did not affect the binding of 0N3R to Fyn SH3). While the details of the effects of phosphorylation are not similar, both the SH3 binding data and the MAPK activation data demonstrate that phosphorylation differentially affected the function of 0N3R and 0N4R tau isoforms. Such findings might help explain why disease could be caused by overexpressing 4R tau relative to 3R tau. In our data, we noted that phosphorylation at Ser199/Ser202 on 4R tau resembled WT tau in its ability to potentiate MAPK signaling whereas the similar modification on 3R tau reduced MAPK activation.

During development, tau phosphorylation changes, with phosphorylation at Ser199, Ser202, Ser214, and Ser404 first increasing, then decreasing while phosphorylation at Thr231 and Ser396 remained unchanged (31). The expression of 4R tau was also up-regulated during development as 0N3R tau was the only tau isoform expressed in fetal brain while the remaining isoforms were expressed in an adult specific manner (32). In our experimental system where NGF was added to initiate neuronal differentiation, phosphorylation could either potentiate or attenuate MAPK activation [**Figure 2**, Ref. (19)]. Phosphorylation at two sites (Ser214 and Ser396/Ser404) significantly down-regulated activation while that at one site (Thr231) up-regulated activation. Therefore, the exact effects of phosphorylation would depend on the quantity of specific phosphorylated tau forms present. This, in turn, would depend on the rate of phosphorylation and dephosphorylation of tau at specific sites. However, the spatial localization of the various phosphorylated species may also be important. Since we measure MAPK activation in a transfected cell, it is possible that the spatial localization of the tau expressed by transfection may not duplicate that of the endogenous tau. If the ability of tau, expressed by transfection, to affect MAPK activation was dependent on a spatial localization not duplicated by endogenous tau, caution needs to be exercised in the interpretation of our results. Nevertheless, a critical role for tau in MAPK activation was confirmed by our experiments where ERK1/2 activation was examined without the transfection of MAPK reporter plasmids (19).

Our tests have investigated the ability of disease-related tau phosphorylation to affect the ability of tau to upregulate MAPK signaling. Among the sites we have investigated, Thr231 was the site whose phosphorylation occurred earliest during neurodegeneration (33, 34). Phosphorylation at Ser262/Ser356 occurred next, with Ser214 close behind; phosphorylation at Ser199/Ser202 and Ser396/Ser404 accumulated latest during neurodegeneration (33). Therefore, as tau phosphorylation changed during disease progression, tau function would similarly change. Our data suggested that phospho-Thr231-tau would potentiate MAPK activation and since phospho-Thr231 occurred early during the neurodegenerative process (20), one could speculate that MAPK activation would also occur. Data reporting the presence of activated ERK1/2 in pretangle neurons and in Braak stage I–III brains (35) supports the hypothesis that phospho-Thr231-tau may potentiate MAPK activation early in the neurodegenerative process. Then, as tau phosphorylation changed during the neurodegenerative process, the capacity for tau to potentiate MAPK activation would also change. Phosphorylation at Ser262/Ser356 would not affect MAPK activation (19) whereas phosphorylation at Ser214 and Ser396/Ser404 would lead to a decrease in the ability of tau to upregulate MAPK activation (**Figure 2**). In tauopathies where the level of 4R tau was increased, the isoform change alone might decrease MAPK activation. However, one could also speculate that the phosphorylation of 4R tau, for instance at Ser199/Ser202, could lead to an increase in MAPK activation, relative to that conducted by similarly phosphorylated 3R tau forms. To further investigate the relationship between tau phosphorylation and MAPK activation during neurodegeneration, tauopathy mouse models and/or human postmortem brain tissue would be probed for phospho-Thr231-tau and activated MAPK. Using immunocytochemistry, if activated MAPK only appeared in the same neurons that were positive for phospho-Thr231 tau, this would suggest that phosphorylation of tau at Thr231 was related to MAPK activation. One would also look for a correlation between activated MAPK and phospho-Ser199/Ser202 in 4R tau isoforms, in addition to a correlation between phospho-Ser214-tau (or phospho-Ser396/Ser404) and a reduction in activated MAPK.

The activation of MAPK can occur in signal transduction pathways where cell proliferation is upregulated [reviewed by Ref. (36, 37)]. Therefore, increasing the activation of MAPK could cause an increase in cell cycle activation. In Drosophila, the ability of tau to cause neurodegeneration was shown to involve cell cycle components (17). Our data supports the hypothesis that tau can potentiate cell cycle mechanisms. In a post-mitotic neuron, the activation of cell division would lead to neurodegeneration [reviewed by Ref. (38)].

It is not known whether tau participates in signal transduction in adult neurons. Gene expression in the tau-depleted mouse was compared to that of WT mouse, using microarray analysis of 8-week-old mice (39). In the tau-depleted mouse, the genes with the highest increase in expression were FosB and c-fos [see Supplemental Data in Ref. (39)]. Since MAPK activation drives fos activation, our data leads us to speculate that the tau-depleted mouse had increased fos expression as a compensatory measure. Since the comparison had used an 8-week-old mouse, it is possible that tau was also necessary for signal transduction in the adult.

While it is clear that phosphorylated tau can form oligomers, we have not determined if the tau that upregulates MAPK activation is a monomer, dimer, trimer, or oligomer. Moreover, the mechanism by which tau affects NGF signaling is under investigation. The ability of proteins to form dimers during signal transduction processes is not unusual and in some cases, dimer formation is linked to phosphorylation. It would be important to determine if tau dimerization or oligomerization occurred in the same manner as disease-related phosphorylation, where dimerization or oligomerization would occur normally during development, then become down-regulated in the adult. If tau oligomers are found during normal development, the toxicity of tau oligomers during neurodegeneration may be related to specific

tau oligomer functions that were inappropriate or abnormal for adult neurons.

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Hyperphosphorylation-induced tau oligomers

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In normal adult brain the microtubule associated protein (MAP) tau contains 2–3 phosphates per mol of the protein and at this level of phosphorylation it is a soluble cytosolic protein. The normal brain tau interacts with tubulin and promotes its assembly into microtubules and stabilizes these fibrils. In Alzheimer disease (AD) brain tau is three to fourfold hyperphosphorylated. The abnormally hyperphosphorylated tau binds to normal tau instead of the tubulin and this binding leads to the formation of tau oligomers. The tau oligomers can be sedimented at 200,000 × *g* whereas the normal tau under these conditions remains in the supernatant. The abnormally hyperphosphorylated tau is capable of sequestering not only normal tau but also MAP MAP1 and MAP2 and causing disruption of the microtubule network promoted by these proteins. Unlike Aβ and prion protein (PrP) oligomers, tau oligomerization in AD and related tauopathies is hyperphosphorylation-dependent; *in vitro* dephosphorylation of AD P-tau with protein phosphatase 2A (PP2A) inhibits and rehyperphosphorylation of the PP2A-AD P-tau with more than one combination of tau protein kinases promotes its oligomerization. In physiological assembly conditions the AD P-tau readily self-assembles into paired helical filaments. Missense tau mutations found in frontotemporal dementia apparently lead to tau oligomerization and neurofibrillary pathology by promoting its abnormal hyperphosphorylation. Dysregulation of the alternative splicing of tau that alters the 1:1 ratio of the 3-repeat: 4-repeat taus such as in Down syndrome, Pick disease, and progressive supranuclear palsy leads to the abnormal hyperphosphorylation of tau.

Keywords: microtubule associated protein tau, abnormal hyperphosphorylation of tau, O-GlcNAcylation of tau, protein phosphatase 2A, alternate splicing of tau, Alzheimer neurofibrillary degeneration, Alzheimer disease, tauopathies

In Alzheimer disease (AD) the oligomer states of Aβ and tau pathologies are believed to cause the neurodegeneration. Oligomer is an intermediate stage between monomer and a large polymer. It consists of a relatively small and identifiable number of monomers, which is usually 3–10 in the case of most proteins. Unlike a polymer, if one of the monomers is removed from an oligomer, its chemical properties are altered. Protein oligomers may be formed by the polymerization of a number of monomers or the depolymerization of a large protein polymer. Protein polymerization is employed by the cell to perform several useful functions, such as neurofilaments and actin filaments serve as cytoskeleton of a neuron and maintain the cell shape. Microtubules that are polymers of tubulin facilitate axoplasmic flow, a vital function of a neuron. Some protein polymerization reactions are very efficient and almost all the protein in the cell is seen as polymers, as is the case with neurofilaments. In contrast, microtubule assembly and disassembly are extremely dynamic to meet the axoplasmic transport needs of a neuron. The oligomers of neurofilaments and microtubules are apparently very short-lived and are, to date, of no known deleterious consequence.

In AD, Aβ and, in the case of tau also in tauopathies, the protein polymerization is apparently employed as a detoxifying process to get rid of the toxic protein oligomers, which seem to stay in the diseased brain and have been isolated and studied. Tau oligomerization is increasingly being suspected as a prion-like phenomenon. This article, which is an update of our previous article on this subject (1), discusses the tau oligomers seen in AD brain and how they differ from Aβ and PrP oligomers.

In human brain tau is alternatively spliced into six isoforms and the ratio of the 3-repeat: 4-repeat protein is altered in different tauopathies. The alternative splicing of human tau pre-mRNA results in six molecular isoforms of the protein (2). These six tau isoforms differ in containing three (3R) or four (4R) microtubule binding repeats (R) of 31–32 amino acids in the carboxy-terminal half and one (1N) or two (2N) amino-terminal inserts (N) of 29 amino acids each; the extra repeat in 4R tau is the second repeat (R2) of 4R taus. This alternative splicing of tau pre-mRNA results in the expression of three 3R taus (0N3R, 1N3R, 2N3R) and three 4R taus (0N4R, 1N4R, 2N4R). The 2N4R tau is the largest size human brain tau with a total of 441 amino acids (tau_{441}) in length. The smallest size tau isoform, which lacks both the two amino-terminal inserts and the extra microtubule binding repeat (0N3R; tau_{352}) is the only isoform that is expressed in fetal human brain. Tau has little secondary structure; it is mostly random coil with β structure in the second and third microtubule binding repeats.

In a normal mature neuron almost all tau is bound to microtubules; tubulin is present in over 10-fold excess of tau. The concentration of tau in a neuron is ∼2 μM (3, 4) and it binds

to microtubules at a kd (dissociation constant) of ∼100 nM (5). Overexpression of tau causes microtubule bundling in cultured cells. However, neither in AD nor in any tauopathy has microtubule bundling been reported.

Neurofibrillary degeneration not only is seen in AD and Down syndrome (DS) but also in a family of related neurodegenerative diseases called tauopathies. These include frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) caused by tau mutations, Pick disease, corticobasal degeneration, dementia pugilistica, and progressive supranuclear palsy. In every one of these tauopathies the neurofibrillary pathology is made up of abnormally hyperphosphorylated tau and these pathological changes in the neocortex are associated with dementia; in a large number of supranuclear palsy cases the tau pathology in the brain stem is associated with motor dysfunction.

OLIGOMERIZATION OF TAU AND HOW IT DIFFERS FROM THAT OF Aβ AND PrP

In 1986 we discovered that not only Alzheimer neurofibrillary tangles were made up from abnormally hyperphosphorylated tau protein (6) but also the altered tau was present in AD brain cytosol and was responsible for the inhibition of microtubule assembly (7). In subsequent studies we showed that the cytosolic AD abnormally hyperphosphorylated tau (AD P-tau) sequestered some of the normal tau and sedimented at 200,000 × *g*, whereas most of the non-hyperphosphorylated tau from the same AD brains remained in the 200,000 × *g* supernatant (8, 9). The AD P-tau showed up as globular particles by negative stained electron microscopy (**Figure 1**). The sedimentable AD P-tau is increasingly being referred to as the oligomeric tau or granular tau (10). *In situ* demonstration of oligomeric tau seen immunohistochemically as amorphous aggregates in the neuronal cytoplasm was described at "stage 0" tangles for the first time by Bancher et al. (11). Biochemical analysis of AD P-tau sedimented from AD brain showed that it co-sedimented some of the non-hyperphosphorylated tau, suggesting that the AD P-tau oligomers are hetero-oligomers of hyperphosphorylated and non-hyperphosphorylated tau (8). Furthermore, normal tau was found to co-aggregate with and promote the aggregation of AD P-tau into filaments (12). As much as 40% of abnormally hyperphosphorylated tau in AD brain is seen as AD P-tau (8).

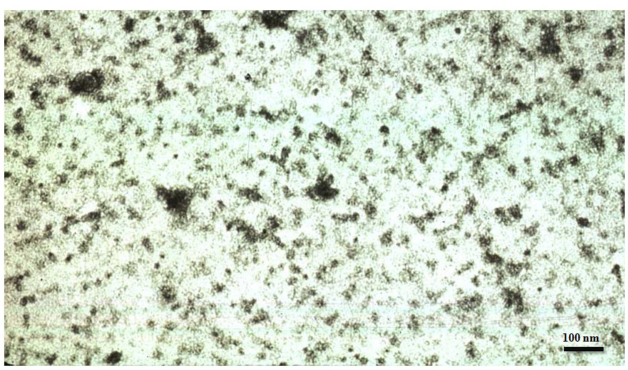

FIGURE 1 | Electron micrograph showing tau oligomers from an Alzheimer disease brain negatively stained with phosphotungstic acid.

Unlike normal tau which binds to tubulin and promotes its assembly into microtubules, the AD P-tau, instead of interacting with tubulin, binds to normal tau as well as MAP1 and MAP2, and causes depolymerization of microtubules (9, 12, 13). *In vitro* hyperphosphorylation of tau revealed that the oligomeric tau was an intermediate stage between monomeric and filamentous state because at 4–6 mol phosphate/mole protein it became oligomeric and microtubule-assembly inhibitory whereas further hyperphosphorylation made it polymerize into filaments. Neither the *in vitro* formed hyperphosphorylated tau filaments nor PHF isolated from AD brains had any detectable effect on tau-promoted assembly of microtubules (14–16). While normal tau promoted GTP binding to tubulin and its assembly into microtubules, the AD P-tau inhibited this activity. AD-PHF had no effect on GTP binding but on *in vitro* dephosphorylation it promoted GTP binding to tubulin (17). On dephosphorylation with protein phosphatase 2A (PP2A) the AD P-tau oligomers are converted into normal-like non-sedimentable protein that, like normal tau, promotes microtubule assembly (9, 12, 18). PP2A was also found to dissociate Alzheimer neurofibrillary tangles, releasing protein which behaved like normal tau in promoting microtubule assembly (19). Thus, the AD P-tau oligomerization is unique because it is solely induced by abnormal hyperphosphorylation and is reversible on dephosphorylation of the protein (20).

In the AD field the interest in oligomers started with the initial report of Lambert et al. (21) who showed that diffusible, non-fibrillar ligands from $A\beta_{1-42}$ were potent central nervous system toxins. Though Aβ oligomers are toxic, in contrast to tau oligomerization, they are formed by the strong hydrophobic nature of this peptide and this process is not initiated or promoted by phosphorylation. Similarly, the PrP oligomers are formed at acidic pH and on removal of denaturants such as sodium dodecyl sulfate or salt from the protein solution (22, 23). Unlike AD P-tau and $A\beta_{1-42}$ oligomers, the PrP filaments are the infective state and their depolymerization into oligomers results in the loss of the infectivity (24). Most recently PrP cellular has been reported to promote the Aβ oligomerization (25).

ROLE OF O-GLcNAcylation IN TAU OLIGOMERIZATION AND NEURODEGENERATION

In addition to phosphorylation, tau is also modified by O-GlcNAcylation, a dynamic protein posttranslational modification, by which O-linked β-*N*-acetylglucosamine (O-GlcNAc) is transferred enzymatically from a UDP-GlcNAc donor to the hydroxyl group of serine or threonine residues of proteins. In contrast to glycosylation of secreted and membrane proteins, which occurs in the endoplasmic reticulum and Golgi apparatus, O-GlcNAcylation modifies nucleocytoplasmic proteins and is more like protein phosphorylation (26). O-GlcNAcylation and phosphorylation sometimes occur at identical or proximal sites of a protein and thus are reciprocal to each other. The crosstalk between O-GlcNAcylation and phosphorylation has been implicated to be essential for the control of vital cellular processes and for understanding the mechanisms of certain diseases (27, 28). O-GlcNAcylation also serves as a sensor of intracellular

glucose metabolism (29), because the UDP-GlcNAc donor for O-GlcNAcylation is formed from glucose metabolism via the hexosamine biosynthetic pathway.

Tau is highly modified by O-GlcNAc, on average, with four O-GlcNAc groups per tau molecule at more than 12 serine/threonine residues (30, 31). Five O-GlcNAcylation sites (Thr123, Ser208, Ser238, Ser400, and one site at Ser409, Ser412, or Ser413) have been mapped to date (32–34). We previously demonstrated that inhibition of O-GlcNAcylation leads to hyperphosphorylation of tau in cultured cells and in rat brain slices (31). Experimental reduction of brain glucose metabolism leads to decreased O-GlcNAcylation and increased phosphorylation of tau *in vivo* (27, 35), and inhibition of protein O-GlcNAcylation induces hyperphosphorylation of tau in rat brain (27). Furthermore, we discovered that the global O-GlcNAcylation of proteins, especially of tau, is decreased, which likely results from impaired brain glucose metabolism, and that the decrease in O-GlcNAcylation correlates to hyperphosphorylation of tau in AD brain (27). Furthermore, hyperphosphorylated tau purified from AD brains contains approximately five times less O-GlcNAc than normal tau (27). Therefore, we postulate that tau pathology and neurodegeneration can be caused by impaired brain glucose metabolism via the down-regulation of tau O-GlcNAcylation in AD (27).

O-GlcNAcylation may also inhibit tau oligomerization directly. The fourth microtubule binding repeat of tau self-aggregates at a slower rate *in vitro* when it is modified by O-GlcNAc at Ser356 than the unmodified counterpart, as determined by turbidity, precipitation assay, and electron microscopy (36). A recent study showed that O-GlcNAcylation inhibits tau aggregation in rodents (37). O-GlcNAcylation also modulates proteotoxicity in *C. elegans* models of human neurodegenerative diseases (38, 39). Therefore, decreased O-GlcNAcylation may promote tau-mediated neurodegeneration through promoting tau oligomerization directly and also indirectly by inducing its abnormal hyperphosphorylation.

ABNORMAL HYPERPHOSPHORYLATION OF TAU CAUSES NEURODEGENERATION AND COGNITIVE IMPAIRMENT

Protein phosphatase 2A accounts for ∼70% of the total tau phosphatase activity in the brain (40). A cause of the abnormal hyperphosphorylation of tau in AD and adults with DS is a decrease in the brain PP2A activity (41–43). PP2A activity is negatively regulated by two inhibitor proteins, I_1^{PP2A} and I_2^{PP2A} in a substrate-specific manner (44, 45). Both I_1^{PP2A} and I_2^{PP2A} inhibit PP2A activity toward AD hyperphosphorylated tau (46) and these inhibitors are predominantly localized in the hippocampus and the cerebellum (47). I_1^{PP2A}, which is also known as PHAP-1, is a 239 amino acid long cytoplasmic protein (48). I_2^{PP2A}, also known as SETα, PHAP-II, and TAF1β, is primarily a nuclear protein of 277 amino acids in length with an apparent molecular weight of 39 kDa on SDS-PAGE (45, 49, 50). mRNA and protein expression levels of both I_1^{PP2A} and I_2^{PP2A} are selectively increased in the affected areas of AD brain. I_2^{PP2A}, which is a 39 kDa and a primarily nuclear protein, is selectively cleaved at N175 into an amino-terminal (I_{2NTF}) and a carboxy-terminal (I_{2CTF}) fragment and translocated from the neuronal nucleus to the cytoplasm in AD brain (51). Both I_{2NTF} and I_{2CTF} interact with the PP2A catalytic subunit PP2Ac and inhibit its activity toward hyperphosphorylated tau (52). Transduction of the brains of newborn rats with adeno associated virus serotype 1 vector carrying human I_{2CTF} (53) or I_{2NTF} and I_{2CTF} transgenes was found to induce AD-like abnormal hyperphosphorylation and aggregation of tau, a loss of neuronal plasticity, and cognitive impairment in these animals at 5–12 months post-infection (54); however, no neurofibrillary tangles or Aβ plaques were detected in the brains of AAV1-$I_{2NTF\text{-}CTF}$ rats up until 13 months. These findings suggest a deleterious role of the abnormally hyperphosphorylated oligomeric tau.

The inhibitory activity of the non-fibrillized abnormally hyperphosphorylated tau has been confirmed in yeast, drosophila, and in mouse models that express human brain tau. The expression of the longest human brain tau (2N4R tau) in yeast produces pathological phosphoepitopes, assumes a pathological conformation, and forms aggregates. These processes are modulated by yeast kinases Mds1 and Pho85, orthologs of GSK-3β and cdk5 (55, 56). In yeast the aggregation of tau increases with increasing hyperphosphorylation and the mobility in SDS-PAGE retards. The hyperphosphorylated tau isolated from the stably transfected yeast is able to assemble into filaments, and nucleate the assembly of the normal non-phosphorylated tau. These yeast studies, like those carried out previously using AD P-tau, suggest that the hyperphosphorylated tau works as a nucleation factor that initiates and promotes the aggregation of tau (12, 15).

In wild-type human tau- and mutated human tau-transgenic *Drosophila*, the accumulation of the abnormally phosphorylated tau in the absence of its fibrillization into neurofibrillary tangles leads to neurodegeneration (57). In a P301L tau inducible transgenic mouse model, cognitive improvement was observed when expression of human tau, which became abnormally hyperphosphorylated, was suppressed although neurofibrillary tangles continued to form, suggesting that the accumulation of the cytosolic abnormally hyperphosphorylated tau, and not its aggregation, was apparently involved in behavioral impairment in these animals (58). Reduction of soluble Aβ and soluble abnormally hyperphosphorylated tau, but not soluble Aβ alone, was found to ameliorate cognitive decline in 3xTg mice that express both plaque and tangle pathologies (59). Furthermore, *in vitro* dephosphorylation of neurofibrillary tangles disaggregates filaments and, as a result, the tau released behaves like normal protein in promoting microtubule assembly (19).

Hyperphosphorylation of tau, though not to the same level as in AD, is not only associated with the disease as in tauopathies, but is also employed by the neuron to down regulate its activity transiently and reversibly where required. For instance, during development the level of tubulin in the brain is at its highest, i.e., almost 33% of total cytosolic protein, which is almost 1.5-fold the critical concentration of 4 mg/ml tubulin required for its polymerization into microtubules (60). Probably to avoid microtubule bundling, the fetal tau is transiently hyperphosphorylated during development. However, the level of hyperphosphorylation of tau in fetal brain is far less than that seen in AD brain. Similarly, anesthesia and hypothermia induced by hibernation in animals induces transient hyperphosphorylation of tau (61–64). The molecular mechanism of the transient hyperphosphorylation of tau observed during development is, at present, not understood. However, during hypothermia the activity of PP2A is transiently and reversibly reduced and is believed to cause the hyperphosphorylation of

tau (62, 63). In AD and DS the decrease in brain PP2A activity apparently involves different molecular mechanisms, and occurs in a non-transient and irreversible manner (41–43). It is the non-reversible nature of the abnormal hyperphosphorylation of tau in AD, DS, and related tauopathies which results in an involuntary slowing down of neuronal activity and a consequent chronic progressive neurodegeneration and its clinical phenotype, the dementia.

There is approximately as much tau in the somatodendritic compartment as in the axon (65). In the somatodendritic compartment tau is associated with rough endoplasmic reticulum and Golgi apparatus (7, 8, 66, 67). The abnormal hyperphosphorylation of tau and its accumulation in the somatodendritic compartment in AD might have been responsible for the morphological alterations of the RER and the Golgi apparatus and the abnormal N-glycosylation of tau in AD (68–71). In AD brain abnormally hyperphosphorylated tau, in addition to forming neurofibrillary tangles, is associated with granulovacuolar changes (6, 72–74). Overexpression of tau, which results in its hyperphosphorylation, has been found to induce fragmentation of Golgi both in neuronal cultures and in neurons in JNPL3 P301L tau-transgenic mice (66). In P301S tau-transgenic mice, which show abnormal hyperphosphorylation of tau, a selective decrease in mitochondria and RER has been observed (75). The chronic accumulation of the hyperphosphorylated tau as a misfolded protein in the ER could cause neurodegeneration due to protracted ER stress (76). Hyperphosphorylation of tau might also be involved in neurodegeneration through alterations of RER and Golgi and a consequent reduction in RER and mitochondria.

In addition to abnormal hyperphosphorylation, truncation of tau has been found in neurofibrillary tangles in AD and in mutated tau overexpression transgenic mouse models [e.g., (77–82)]. Of all the proteases that can cleave tau, the role of caspases has been studied the most (83, 84). Caspase 3 and caspase 6 cleave tau at D421 and D13, respectively, and treatment with Aβ can induce the D421 cleavage in cultured neurons (78, 80, 81). Truncation of tau, along with its hyperphosphorylation, promotes its aggregation into fibrils (85, 86). Although only a small fraction of tau is truncated in AD, the truncated protein can apparently recruit the full-length protein to co-aggregate with it in both tau-transgenic rat and mouse models (87, 88). To date, the bulk of the evidence suggests the soluble hyperphosphorylated tau is neurotoxic and upstream of truncation and aggregation of this protein into neurofibrillary tangles [e.g., (89, 90)].

ROLE OF MUTATIONS AND ALTERNATIVE SPLICING OF TAU IN NEURODEGENERATION

In FTDP-17 several mutations in tau co-segregate with the disease (91–93). Four of these missense mutations, G272V, P301L, V337M, and R406W, which have been most studied to date, all make tau a preferable substrate for abnormal hyperphosphorylation *in vitro* (94). Some of the tauopathies are associated with altered alternate splicing of tau. In normal human brain the 3-repeat and 4-repeat taus are expressed in 1:1 ratio.

In some of the FTDP-17 mutations, i.e., tau_{K257T} (95), tau_{G272V} (96), $tau_{\Delta K280}$ (97), tau_{E10+19}, and tau_{E10+29} (98), and in Pick disease most of the tau is 3R isoforms due to the exclusion of axon 10 which codes for the second microtubule binding repeat (R_2). In contrast, in other FTDP-17 mutations, cortical basal degeneration and progressive supranuclear palsy, most of the tau is 4R (99, 100).

How the imbalance of 3R tau/4R tau leads to neurofibrillary degeneration and dementia is currently not understood. The 4R taus bind microtubules more readily than 3R taus. Thus, a change in 3R:4R ratio of 1:1 in tauopathies results in free tau that is unbound to microtubules and free tau becomes a favorable substrate for abnormal hyperphosphorylation (101). In DS brain an increase in 3R:4R ratio combined with an extra copy of Dyrk1A, which can hyperphosphorylate tau, results in tau pathology during the fourth decade of life which is almost two decades earlier than the average age of onset of AD (102, 103).

Hyperphosphorylation by brain protein kinases induces the self-assembly of all six human brain tau isoforms into tangles of PHF/SF under physiological conditions of protein concentration, ionic strength, pH, temperature, reducing conditions, and the absence of any cofactor (9). The hyperphosphorylation of tau is catalyzed by one or more combinations of the proline-directed protein kinases (PDPKs) and non-PDPKs. Phosphorylation of tau by non-PDPKs generally primes taus for hyperphosphorylation by PDPKs (20, 104–106). Tau isoforms *in vitro* might be phosphorylated differentially. 2N4R tau is a more favorable substrate for phosphorylation by rat brain protein kinases and is phosphorylated faster and to a higher extent than 2N3R tau at Thr181, Ser199, Ser202, Thr205, Thr212, Ser214, Thr217, Thr231, Ser235, Ser262, Ser396, Ser404, and Ser422 (94). The differential phosphorylation of 3R and 4R taus involves a combination of non-PDPKs and PDPKs because, GSK-3β alone phosphorylates tau isoforms similarly (107). Pseudophosphorylation of seven GSK-3β phosphorylation sites S199, S202, T205, T231, S235, S396, and S404, affects the aggregation of tau isoforms differently; the pseudophosphorylation at these seven sites was found to enhance arachidonic acid-induced polymerization of 0N4R tau while greatly inhibiting the aggregation of the 3R isoforms (108). Thus, phosphorylation generated by the same set of kinases could be sufficient to increase the propensity of some isoforms to aggregate while reducing the aggregation of others, resulting in the differential isoform inclusion in pathological tau aggregates (108).

Aggregation of tau isoforms is affected by the type of inducer for aggregation used. Arachidonic acid induces 4R tau to polymerize to a greater extent than 3R tau (107). 0N tau requires higher concentration of arachidonic acid to get maximal polymerization. The concentration of arachidonic acid for reaching a maximal polymerization of 1N tau and 2N tau were reported to be similar, suggesting addition of exon 3 containing isoforms does not further reduce inducer concentrations needed for maximal polymerization (107). Similar results were obtained for the heparin induction of tau isoform polymerization (107). The 2N4R tau required less heparin inducer for maximal polymerization than 1N4R and 0N4R taus. Aggregation of six tau isoforms by thiazine red inducer was also reported in a tau isoform-dependent manner. Tau exons 2 and 10 were found to promote aggregation, whereas exon 3 depressed it with its efficacy dependent on the presence or absence of a fourth microtubule binding repeat (109).

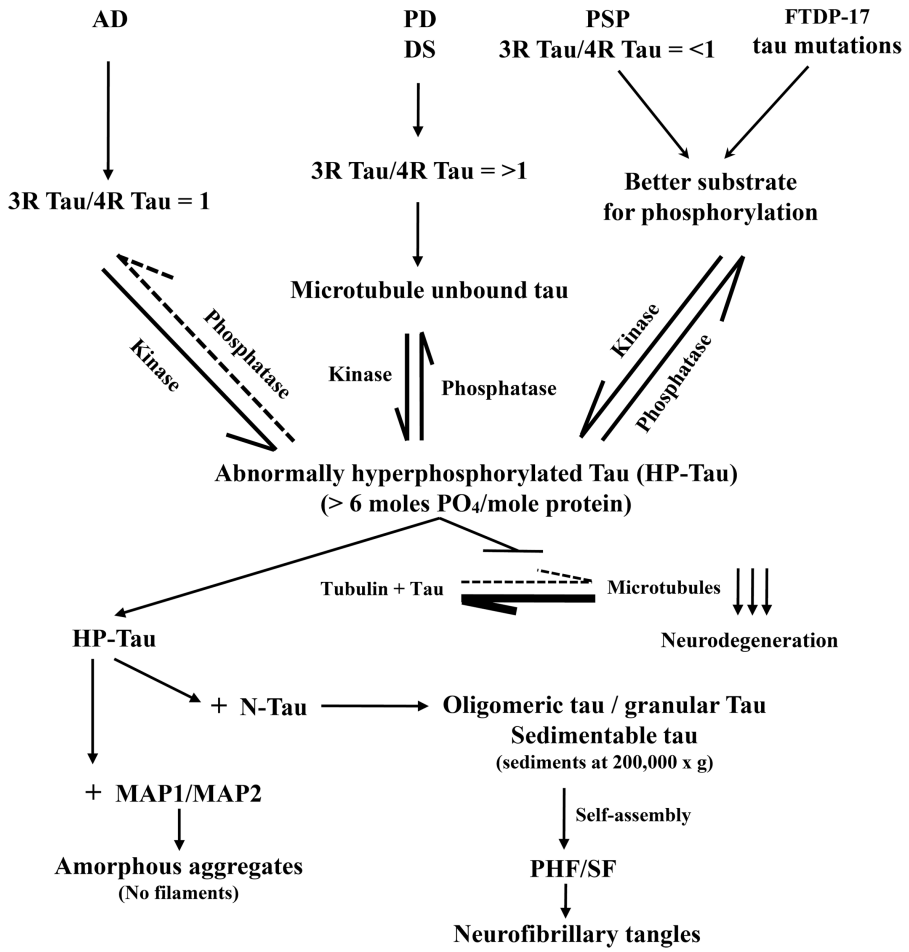

FIGURE 2 | Abnormal hyperphosphorylation of tau promotes its oligomerization and self-assembly into paired helical filaments, forming neurofibrillary tangles. A protein phosphorylation/dephosphorylation imbalance apparently caused by a decrease in protein phosphatase 2A (PP2A) activity leads to abnormal hyperphosphorylation of tau in AD brain. The abnormally hyperphosphorylated tau binds to normal tau (and not to tubulin) and this sequestration leads to the disruption of microtubules and the formation of oligomers which can be sedimented at 200,000 × *g*; the tau oligomers show up as granular structures by negative stain electron microscopy. The abnormally hyperphosphorylated tau isolated from AD brain cytosol readily self-assembles into paired helical filaments *in vitro* under polymerizing conditions.

Alzheimer disease P-tau sequesters normal tau, MAP1, and MAP2 and disassembles microtubules and that the dephosphorylation of AD P-tau eliminates this toxic property (9, 13). Tau isoforms bind to AD P-tau deferentially. The binding of AD P-tau to 4R tau tends to be greater than to the corresponding 3R tau and its binding to normal human recombinant tau was found to be 2N4R > 1N4R > 0N4R and 1N4R > 1N3R > 0N3R (110). AD P-tau interacts preferentially with the tau isoforms that have the amino-terminal inserts and four microtubule binding domain repeats and that hyperphosphorylation of tau appears to be sufficient to acquire AD P-tau characteristics. Thus, lack of amino-terminal inserts and extra microtubule binding domain repeat in fetal human brain might be protective from Alzheimer's neurofibrillary degeneration.

CONCLUSION

In conclusion, in AD and related tauopathies the abnormal hyperphosphorylation of tau promotes its oligomerization (**Figure 2**). The tau oligomers sequester normal tau as well as MAP1 and MAP2 and can be separated from normal tau by sedimentation at 200,000 × *g*. The abnormal hyperphosphorylation of tau seen in AD is different from the normal and from the transient hyperphosphorylation of this protein that occurs during development, anesthesia, or hypothermia. The oligomeric cytosolic AD

P-tau probably causes neurodegeneration by sequestering normal MAPs and disrupting the microtubule network. Tau mutations found in frontotemporal dementia may cause neurodegeneration through promoting abnormal hyperphosphorylation of tau. AD P-tau self-assembles into PHF/SF, forming neurofibrillary tangles. Tau truncation found in AD brain promotes its self-assembly into PHF/SF. Unlike AD P-tau, the tangles neither show any detectable activity to sequester normal MAPs nor inhibit microtubule assembly. Inhibition of abnormal hyperphosphorylation of tau offers a promising therapeutic target for AD and related tauopathies. Animal models that recapitulate various disease mechanisms seen in AD and related tauopathies are no less valuable for preclinical studies for drug development than transgenic mouse and rat models in which one or more mutated human proteins are overexpressed to produce Aβ plaques and/or tau neurofibrillary tangles.

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