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Phosphorylation of Tau Protein Associated as a Protective Mechanism in the Presence of Toxic, C-Terminally Truncated Tau in Alzheimer's Disease

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1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly and is characterized by progressive memory loss leading to a gradual and irreversible deterioration of cognitive function. The neuropathology of AD is characterized by the accumulation of fibrillary lesions in the form of neuritic plaques (NPs, Fig. 1A), neurofibrillary tangles (NFTs, Fig. 1C,D; small arrow) and dystrophic neurites (DNs, Fig. 1; arrows) in neocortex, amygdala and hippocampus [1]. The density of the NPs and NFTs correlate with the degree of dementia in AD [2]. The accumulation of these lesions does not occur at random; the presence of NFTs is associated with vulnerability of the perforant pathway [3]. NPs are comprised of extracellular deposits of amyloid- β peptide fibrils that are associated with DN of dendritic and axonal origin (Fig. 1A; arrows). Intracellular NFTs selectively kill neurons in specific brain areas. In AD, the distribution of NFTs follows a stereotypical profile arising first in layer II of the entorhinal cortex, hippocampal region and CA1/subicular layer IV of the entorhinal cortex and then neocortex (mainly in fronto-temporal and parietal areas). This pattern of distribution was first described by Braak and Braak in 1991 [4], and provides the most important neuropathological criteria for a definite diagnosis of AD (Fig. 2) [5]. Ultrastructurally, NFTs are composed of dense accumulations of structures known as paired helical

filaments (PHFs) [6, 7], which are mainly distributed in the perinuclear area of the neuron and in proximal processes (Fig. 1C). Tau protein is the major structural constituent of the PHF subunits [7-9]. Normally, tau protein exists as a family of microtubule-associated protein (MAPs) that are found predominantly in axons. Through repeated domains located toward the carboxy-terminus of the protein, tau provides stability to the microtubule and this process can be regulated through a balance in the phosphorylation/dephosphorylation process of tau protein [10]. In AD, tau protein accumulates as PHFs in the somatodendritic compartment, with consequent destabilization of axonal microtubules. Tau is further post-translationally modified in AD, with modifications of ubiquitination [11, 12], glycation [13, 14], glycosylation [15], nitration [16], polyamination [17], hyperphosphorylation [18, 19] and proteolysis [7, 20-24]. The latter two changes occur throughout the tau molecule [25-27].

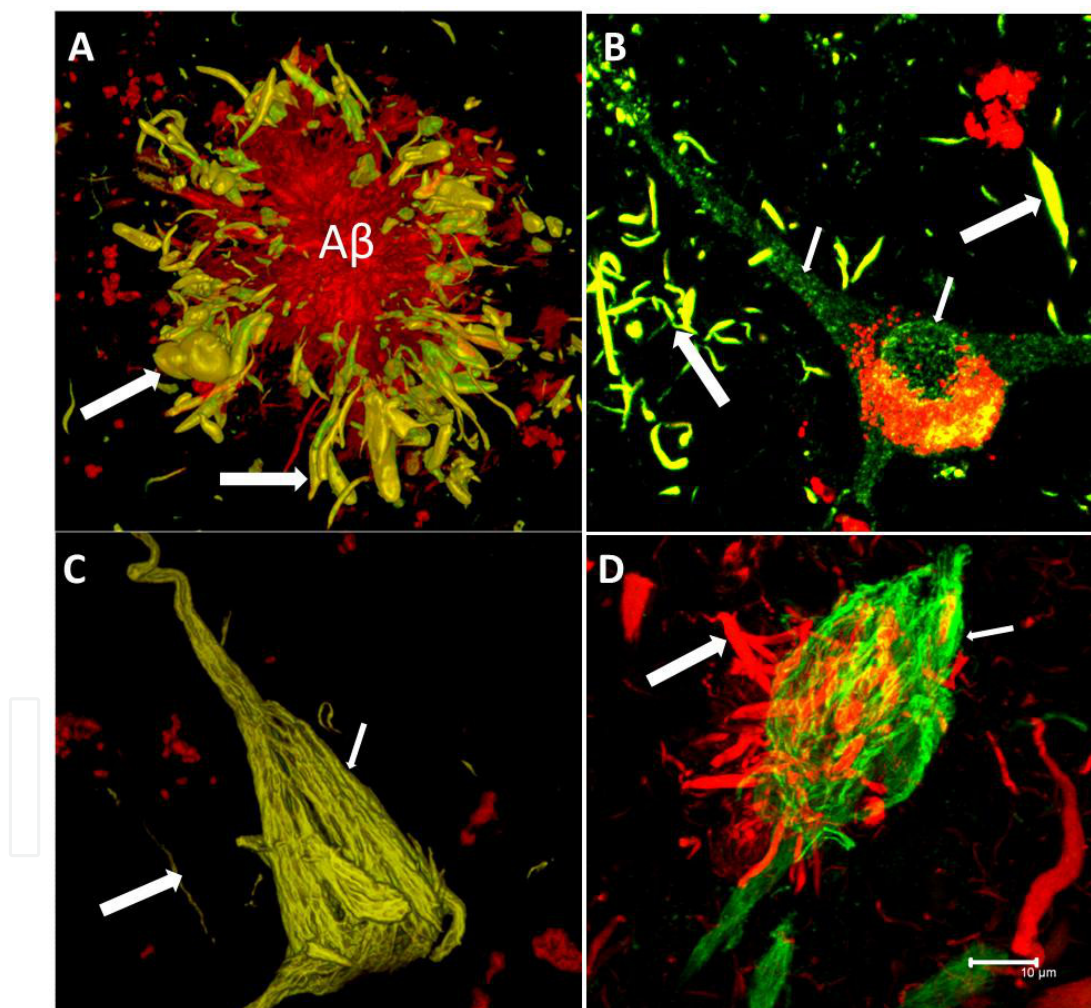


Figure 1. Neuropathological hallmarks of Alzheimer's disease. Double labelling with tau antibody (green channel) counterstained with thiazine red (red channel). A) A classical neuritic plaque in which an amyloid fibrillar plaque ($A\beta$), recognized by thiazine red, is associated with dystrophic neurites (arrows). B) Pre-tangle cells are characterized by diffuse granular deposits throughout the perinuclear area (small arrow) and proximal processes. C) A neurofibrillary tangle that is strongly labeled by tau antibody and colocalized with thiazine red. (A,B) projection of 20 and 9 confocal microscopy sections, respectively, each of 1.0 μm thickness.

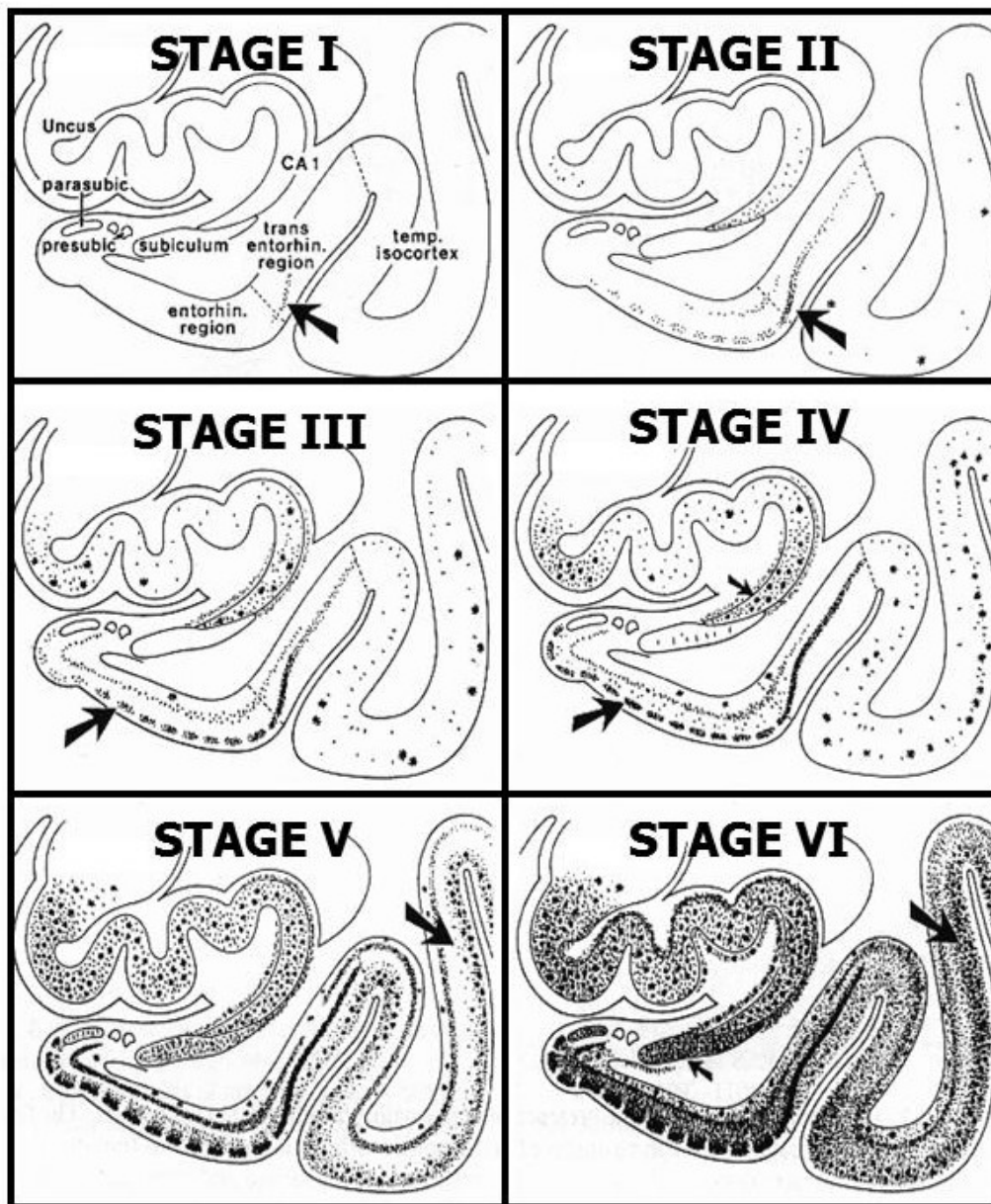


Figure 2. Braak stages of AD neuropathology base on the pattern of neurofibrillary change (NFT, Neuropil threads and plaques dystrophic neurites) [4], Although clinic-pathological correlations were not made, Braak and Braak did speculate that the entorhinal stage (I-II) represents clinically silent periods of the disease with NFT involvement confined to trans-entorhinal layer pre-alpha. Limbic stages (III/IV) correspond with clinically incipient AD, and NFT involvement of CA1, and neocortical stages (V/VI) represent fully developed AD, with NFT involvement of all areas of association cortex.

Tau protein can be phosphorylated at multiple sites. While there is evidence that phosphorylation of tau protein promotes its assembly into PHFs [18, 19, 28, 29], the role of phosphorylation in the genesis of PHFs has been limited to the analysis of "mature", intracellular NFTs (NFT-I). By this stage in the disease, tau protein will have been affected by many different pathological processes, several of which may be associated with the hyperphosphorylation process [25, 26, 30].

Another post-translational modification found in AD is the proteolytic truncation of the C-terminal portion of tau protein [7, 20, 21, 23, 31, 32]. It has been proposed that such truncation unlike hyperphosphorylation, favours polymerisation of tau [33, 34][35].

In recent years, evidence from both *in vitro* and *in vivo* studies[36, 37], suggests that hyperphosphorylation of tau protein has a protective role. In this review, we analyze the protective effects of hyperphosphorylated species of tau protein and their relationship to toxicity, and the participation of truncated species of tau in the formation of PHFs.

2. Tau protein

The cytoskeleton is formed by three types of filaments: microfilaments, intermediate filaments and microtubules [38]. The cytoskeleton provides a dynamic scaffold to proteins, vesicles and organelles, essential for proper cell function and changes in the state of its polymerization, play an important role in neuronal process such as polarization, axonal transport, maintenance of neuronal extensions, synaptic plasticity and protein sorting [39]. Tau protein functions as a regulator of microtubule assembly [40]. Tau protein participates in microtubule polymerization [41], regulation of axonal diameter [42], regulation of axonal transport [43], neurogenesis and the establishment of neuronal polarity in development [44]. Furthermore, tau participates in the regulation of signaling pathways by acting as a protein scaffold.

The gene that encodes for tau consists of 16 exons and is located at the chromosomal locus 17q21 [45]. Through alternative splicing, six tau isoforms are generated in the CNS, varying from 352-441 amino acids in length (Fig. 3). Tau protein can be divided into three domains: an acidic region in the N-terminal projection, a proline-rich domain and a microtubule-binding domain (Fig. 4) [46]. The alternative transcription of exons 2, 3 and 10 modifies the presence of repeats in the N-terminus of tau (0-2N) and the number of microtubule-binding repeat domains (3R or 4R), respectively.

3. Tau protein metabolism

The *MAPT* (tau) gene is transcribed mainly in neurons and a promoter that confers neuronal specificity has been described [47]. It has been reported that the presence of a tau promoter lacking neuronal specificity might account for the expression of tau in peripheral tissue [48]. In both cases, sequences containing binding sites for transcription factors AP2 and Sp1 were described. Whereas tau protein synthesis is unaffected by microtubule polymerization or depolymerization, degradation of tau is stimulated by microtubule depolymerization [49].

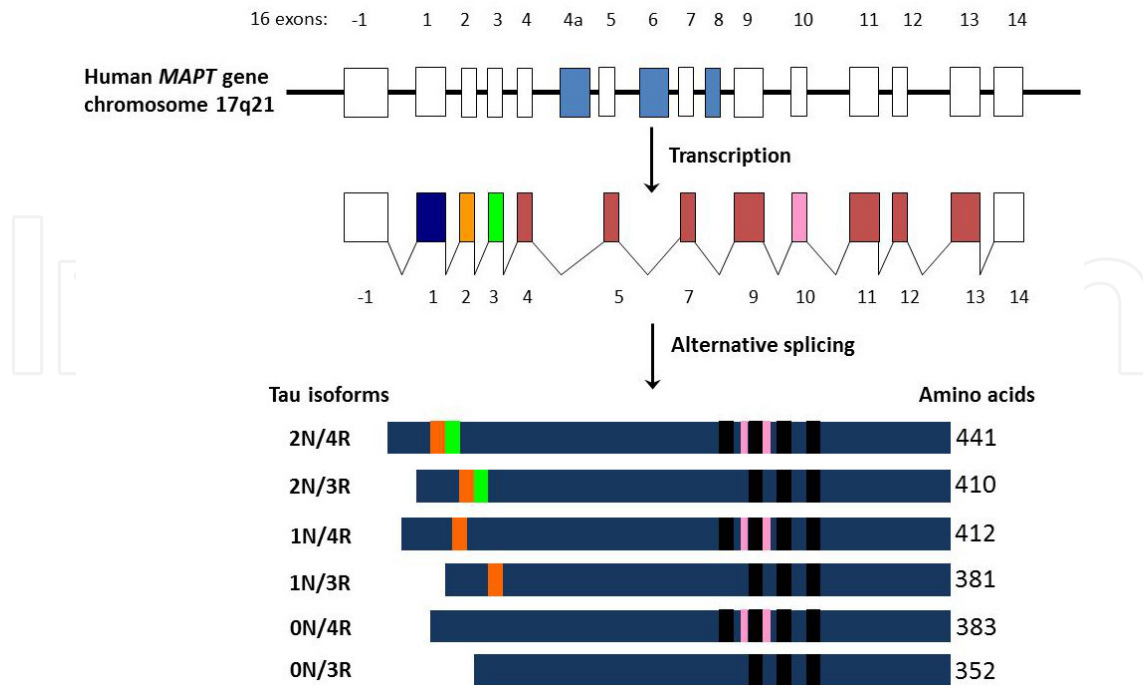


Figure 3. Schematic representation of the human *MAPT* (tau) gene, the primary tau transcript and the six CNS tau protein isoforms. The *MAPT* gene is located over 100kb of the long arm of chromosome 17 at position 17q21. It contains 16 exons, with exon -1 is a part of the promoter (upper panel). The tau primary transcript contains 13 exons. Exons -1 and 14 are transcribed but not translated. Exons 1, 4, 5, 7, 9, 11, 12, 13 are constitutive, and exons 2, 3, and 10 are alternatively spliced, giving rise to six different mRNAs, translated in six different CNS tau isoforms (lower panel). These isoforms differ by the absence or presence of one or two N-terminal inserts of 29 amino acids encoded by exon 2 (yellow box) and 3 (green box), in combination with either three (R1, R3 and R4) or four (R1–R4) C-terminal repeat-regions (black boxes). The additional microtubule-binding domain is encoded by exon 10 (pink box) (lower panel). Adult tau includes all six tau isoforms, including the largest isoform of 441-amino acids containing all inserts and other isoforms as indicated. The shortest 352-amino acids isoform is the only one found only in fetal brain.

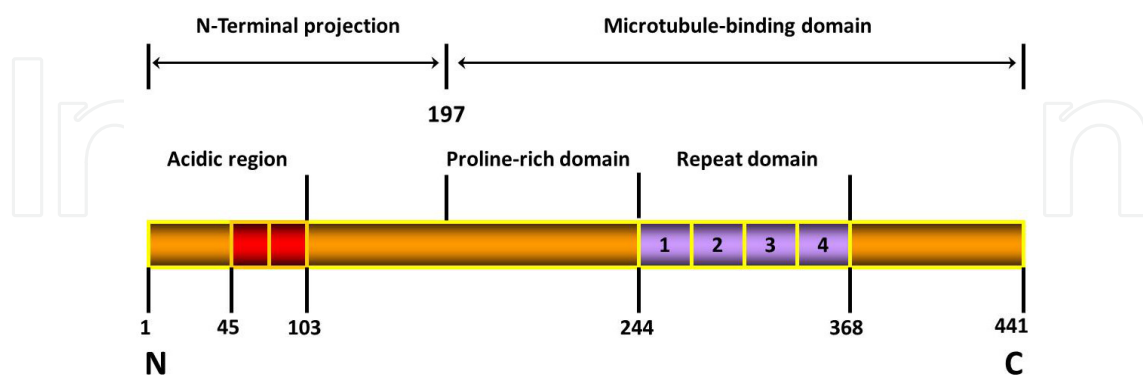


Figure 4. Schematic representation of the functional domains of the largest tau isoform (441 amino acids). The projection domain, including an acidic and a proline-rich region, interacts with cytoskeletal elements to determine the spacing between microtubules in axons. The N-terminal part is also involved in signal transduction pathways by interacting with proteins such as PLC- γ and Src-kinases. The C-terminal part, referred to as the microtubule-binding domain, regulates the rate of microtubules polymerization and is involved in binding with functional proteins such as protein phosphatase 2A (PP2A) or presenilin 1 (PS1).

It is technically difficult to determine the half life of the different tau isoforms and several factors may regulate tau degradation such as, for example, the extent of phosphorylation and acetylation of tau (see below). The half life of tau decreases in rats by neonatal period P20 and there is less demand for tau in non-dividing, mature neurons [50].

Two main mechanisms for tau protein degradation have been documented: 1) the proteasomal ubiquitin pathway and 2) the lysosomal autophagic pathway. Proteasomal degradation of tau protein has been described by 20S proteasomal processing [51], although there have also been reports suggesting that tau is not normally degraded by the proteasome [52]. Tau, modified by phosphorylation, can be ubiquitinated by the CHIP-hsc70 complex and degraded by the proteasome [53]. Furthermore, acetylation of tau can regulate its proteasomal degradation by modifying those lysine residues needed for ubiquitination. In this way, acetylation of tau inhibits its degradation through a competition between ubiquitination and acetylation [54].

On the other hand, tau may get processed through a lysosomal autophagic mechanism. It has been reported that tau can be degraded by lysosomal proteases [55] and, more recently, it was shown that tau fragmentation and clearance can occur by lysosomal processing [56].

Tau protein is a microtubule-associated protein. It's mostly abundant in neurons in the Central Nervous System (CNS). The main function of Tau protein is to interact with tubulin to stabilize microtubules and promote tubulin assembly into microtubules. Tau protein controls microtubule stability in two different ways : isoforms and phosphorylation.

Normally, the tau protein is very important, as it manages the transport of materials within soma and other cellular regions through the myelin sheaths. Once it spotted something suspicious or irrelevant, it stops the information sending process automatically. However, in Alzheimer's disease, the tau proteins started to perform uncommon reaction, where it transmitting the information to the brain simultaneously, regardless of its validity.

Once the above problem happening, it causes the brain overloading with information and might lead to inflammation, clumps or tangles, which kill most of the brain cells (Fig. 5).

4. Phosphorylation of tau protein

Protein phosphorylation is the addition of a phosphate group, by esterification, to one of three different amino acids: serine, threonine and tyrosine. Phosphorylation is the most common post-translational modification of tau described and increased tau phosphorylation reduces its affinity for microtubules leading to cytoskeletal destabilization. Eighty-five putative phosphorylation sites on tau protein have been described in AD brain tissue (Fig. 6). The formation of fibrillar aggregates of post-translationally modified tau protein in the brain are characteristic of AD and other tauopathies. The phosphorylation of tau protein affects its solubility, localization, function, interaction with partners and susceptibility to other post-translational modifications. However, the role of specific sites of tau phosphorylation in early neurodegenerative mechanisms is unknown. The molecular mechanisms of aggregation

of tau into insoluble forms may help to account for the different dementias in which both clinical symptoms and age of onset differ.

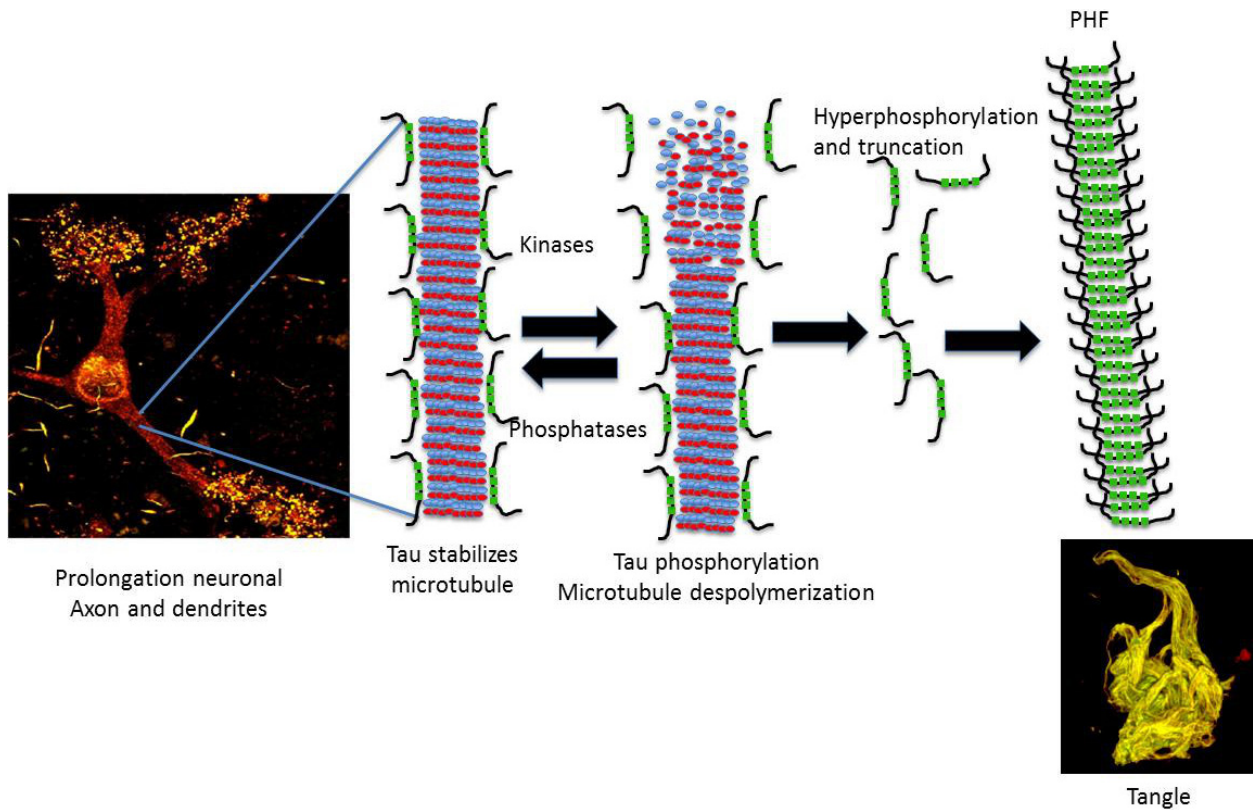


Figure 5. tau protein, which forms part of a microtubule. The microtubule helps transport nutrients and other important substances from one part of the nerve cells to another. Axons are long threadlike extensions that conduct nerve impulses away from the nerve cells; dendrites are short branched threadlike extensions that conduct nerve impulses toward the nerve cell body. In Alzheimer's disease the tau protein is abnormal and the microtubule structures collapse.

5. C-terminal truncation of tau protein in AD

5.1. PHF-core concept

In 1988, Wischik et al, [7, 22] identified tau protein as the major constituent of Pronase-resistant PHFs and tau was characterized by a specific C-terminal truncation of the protein at Glu-391. This truncation is identified by the monoclonal antibody (mAb) 423 [23, 31], and the acid-reversible occlusion of the intact core tau domain. PHFs are labeled by the fluorescent dye, thiazin-red, a dye which can be used to differentiate between amorphous and fibrillar states of tau and amyloid proteins in AD. The minimum tau fragment in the PHF [20, 24] corresponds to the tandem repeat region in the C-terminal domain of tau protein, a species having a molecular weight of 12.5 kDa. Characteristically, this fragment is highly stable to proteolysis, insoluble and toxic and is referred to as PHF-core tau [22, 57, 58]. PHF-core

tau and mAb 423 immunoreactivity of NFTs, have a close clinical-pathological relationship; the density of NFTs immunolabelled with mAb 423 is correlated with the progression of neurofibrillary pathology, as determined by Braak staging criteria (Fig. 2). Most significantly, there is a correlation between mAb 423 immunoreactivity and both clinical severity and progression to dementia [3]. On the other hand, over-expression of PHF-core tau, in cell culture, favors a programmed cell death or apoptosis, which shows that it is highly toxic[59]. Recombinant tau protein truncated at Glu-391 also shows increased rates of polymerization compared with recombinant full-length tau. From confocal microscopy studies, it has been shown that this fragment of tau is hidden within the PHF-core and exposed by formic acid treatment [57]. In the cytoplasm of susceptible neurons, this truncated tau triggers an auto-catalytic process in which the fragment has a high affinity for full length tau and, once bound, leads to cycles of proteolysis and further tau binding to form a proto-filament [60]. In this scenario, the initiating tau species that gave rise to the filament is hidden within a large number of covering tau molecules, some of which become hyperphosphorylated. This would correspond to the early aggregation of tau protein associated with PHF in small NFT. Tau molecules of the NFT would become exposed on death of the neuron to reveal the extracellular NFT, or "ghost tangle" (Fig. 1D, small arrow) which shares the properties of being stable, insoluble and immunoreactive with mAb 423 [57, 61]. The proteases responsible for truncation at Glu-391 are not known.

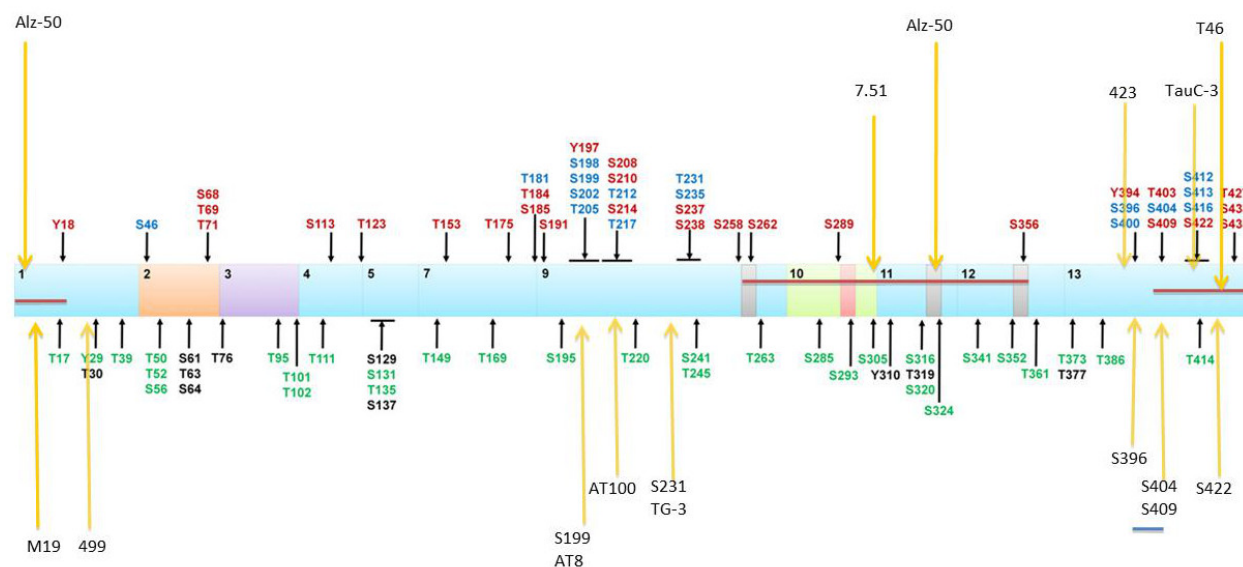


Figure 6. Location of tau phosphorylation sites and epitopes for tau antibodies. Multiple amino acids are phosphorylated with some those observed in AD brain [5], normal brain (green) and both normal and AD brains (blue). Putative phosphorylation sites that have not yet been demonstrated *in vitro* or *in vivo* (black). Localization of antibody epitopes are indicated arrows. Residues are numbered according to the longest tau isoform.

6. Truncation of tau protein at Asp-421

In 2003, a second truncation of tau protein was found to be associated with PHFs [62-65]. This truncation is found at position Asp-421 in the C-terminus of the tau molecule and its presence can be detected specifically by using mAb TauC-3 [25, 63]. Unlike truncation at Glu-391, for which the protease responsible is unknown, caspase-3 (an enzyme involved in the apoptotic pathway) is responsible for the truncation at Asp-421 *in vitro* [59, 62, 63]. This suggests that cleavage of the carboxyl terminus of tau protein, could result as a neuronal response to prevent or control the polymerization of tau in PHF [58]. In 2005, Binder and colleagues discovered a truncation at the amino terminus of the tau protein associated with PHFs. This cut corresponds to Asp-13, which is produced by caspase-6, another enzyme involved in the apoptotic pathway [66]. An antibody to detect this cleavage site of the amino terminus of tau protein has not yet been generated.

Tau-C3 has an affinity for NFTs, NDs and neuropil threads in AD brains. Immunohistochemical studies indicated that truncation at Asp-421 occurs after conformational change; the antibody binds with greatest affinity when the amino terminus of tau molecule contacts the third microtubule binding repeat (MTBR), as recognized by mAb Alz-50 [26]. However, other studies have shown Tau-C3 immunoreactivity in pre-tangle cells before they become Alz-50 immunoreactive and in the absence of PHFs [64, 67].

7. Impact of phosphorylation and truncation on the abnormal processing of tau protein in AD

7.1. A neuroprotective mechanism for the phosphorylation of tau protein in the AD brain

During neurodegeneration in AD, tau protein is abnormally phosphorylated in the proline-rich region at Ser and Thr residues [68], and such phosphorylation sites can be identified using highly specific antibodies such as: AT8 (Ser-202/Thr-205) AT100 (Ser-212 and Ser-214), TG3 (Thr-231/Ser-235) and PHF-1 (Ser-396/Ser-404), among others (Fig. 6). However, NFTs are found in viable neurons at late stages of the disease, and they persist in neuronal cells for decades with a significant number of NFTs being found in the cognitively intact elderly [69, 70]. Such NFT-bearing neurons contain normal content and structure of microtubules [68]. The findings from studies in transgenic mice and human data, suggest that tau accumulation in the somatodendritic compartment may represent the manifestation of a protective mechanism or a cellular adaptation that arises with advancing age. An increase in tau phosphorylation in AD brain has been associated with a protective mechanism against oxidative stress [71]. In another study, intact microtubules were found in NFT-bearing neurons [8], calling into question whether accumulation of phosphorylated tau and destabilization of microtubules are necessarily linked. Although microtubules are depolymerized in neurons with fibrillary degeneration, one study found evidence that the reduction of microtubules in AD is marked and specifically limited to vulnerable pyramidal neurons, and that even these alterations were observed in the absence of PHF [72]. This finding is also consistent with

previous work by one of the authors noting a microtubule decrease of nearly 50% in dendrites that did not correlate with either PHFs or age [73], suggesting that a proportion of phosphorylated tau protein is associated with microtubules [71]. In animal models, it has been confirmed that axonal transport is not affected by either over-expression or reduction of tau protein *in vivo* [42, 74]. Another study found evidence that axonopathy precedes the formation of NFTs in a transgenic mouse [75]. A transgenic mouse expressing a human tau isoform developed NFTs, neuronal loss and behavioral impairments [58]. After suppression of tau expression, the behavioral deficits stabilized yet NFTs continued to accumulate, suggesting that NFTs are not sufficient to cause cognitive decline or neuronal death.

Within NFTs, different species of tau protein associated with phosphorylation are observed, but the neurodegenerating neurons still appear to be functional [75]. These observations suggest that the cytoplasmic accumulation of hyperphosphorylated tau protein is non-toxic, similar to the accumulation of lipofuscin that does not alter cellular metabolism [68]. It is generally assumed that disintegration of microtubules is associated with an imbalance between kinase and phosphatase activities, which lead to an alteration in the stability of microtubules, disruption of cell function and culminate in neuronal death. The data, however, suggest that, at least, a subpopulation of hyperphosphorylated NFTs may be not toxic. This is controversial, given the fact that the hyperphosphorylation of tau and NFTs are considered to be toxic. However, the ability of tau protein fractions, purified from AD brains, to alter microtubule assembly, *in vitro*, has been attributed to sequestration of normal tau molecules [18]. Alonso and colleagues [28] demonstrated that recombinant hyperphosphorylated tau, *in vitro*, decreases the breakdown of the recombinant microtubule when assembled into small aggregates [19, 28]. On this basis, the authors suggested that hyperphosphorylated tau protein plays a protective role against the disintegration of the microtubule.

Tau that has been hyperphosphorylated with GSK3- β kinase becomes immunoreactive with mAbs AT8, PHF1 and TG-3, antibodies whose epitopes are very closely related to AD [29, 76]. The fact that GSK3- β is capable of creating epitopes considered pathological in AD suggests that there are other participants that require to be considered. These data suggest the possible existence of a toxic species of non-phosphorylated tau protein, which would be responsible for capturing further molecules of tau in PHFs, yet would not be exposed on the filament [7, 57, 61]. It is possible that, by hiding the toxic form in PHFs could protect the neuron [77]. It is important to note that the presumed "intermediaries" are present in the cytoplasm of the neuron when it is still viable. Another study showed that NFTs (and presumably tau oligomers) could remain in the cytoplasm of the neuron for decades [78], an observation that would further argue against a primary toxicity of phosphorylated tau protein in AD.

7.2. Hyperphosphorylation of tau protein protecting neurons from apoptosis

It is also proposed that apoptosis plays an important role in neuronal damage in AD. This proposal is based on the detection of fragmented DNA and expression of apoptosis signaling proteins such as caspases 3, 6, 8 and 9, Bax, Fas and Fas-L, in the cortex and hippocampus, in postmortem brain tissue [79, 80] and observations that amyloid- β can induce

neuronal apoptosis [81]. Apoptosis is a process that usually occurs over a period of hours, whereas the accumulation of tangles found in AD brains occurs over a period of years or decades [78]. It has been suggested that hyperphosphorylation of tau protein is a mechanism used to evade cell death by apoptosis. Cells over-expressing hyperphosphorylated tau appear to avoid the apoptotic process [82].

8. Participation of hyperphosphorylated and truncated tau species in the early formation of PHFs

8.1. Model for the mechanism of assembly

Despite suggestions of a neuroprotective role for tau protein in AD, links between phosphorylated species (that are presumed to be protective) and the complex assembly of toxic, truncated tau into insoluble PHFs is not clear. In recent years, we have characterized the early stages of tau protein processing in neurons (pre-tangle state) (Fig. 1B, small arrows) and have described accumulations of tau that possess pathological species present in NFT, yet which do not show the presence of assembled structures in PHFs [67, 83]. The pre-tangle (Fig. 1B) is the first step in non-fibrillar aggregation of tau protein in AD and one in which at least 5 different changes take place (Fig 1 C,D). These events include: a) the presence of a C-terminally truncated and toxic tau species (Glu-391); b) a cascade of specific phosphorylation of tau protein in the N-terminus; c) C-terminal truncation via the action of caspase-3; d) oligomerization and aggregation of tau species and e) assembly of tau into PHFs.

A model to accommodate the observations are represented schematically in Fig. 7. In this model, the first event to occur would be the emergence of tau oligomers or a PHF subunit (Fig. 7 A,B). The mechanism whereby this is initiated is unknown, but its toxicity and high affinity for binding to intact tau molecules would trigger an immediate need for the cell to protect itself. That would be reflected by hyperphosphorylation of the molecule in a failed attempt to hide the PHF and prevent the capture of further intact tau molecules (Fig. 7 B,C). In AD, this protective function of the phosphorylated species favors more molecules becoming available for sequestration and formation into PHFs (Fig. 7D). Gradually, phosphorylated tau will be affected by exogenous proteolysis to re-expose the PHF-core (Fig. 7E). These steps follow as a molecular consequence of the catastrophic fragmentation of the microtubule, synaptic dysfunction, oxidative stress and post-translational modifications of tau. This model emphasizes that polymerization and neuroprotective mechanisms are both involved in the development of PHFs. The phosphorylated species of tau protein play a role in the initial protective response of the neuron to prevent the assembly of these filaments [35]. Thus NFTs, in which externally available tau is hyperphosphorylated, represents a mechanism whereby the neuron may try to protect itself from neurofibrillary degeneration and further studies to confirm this hypothesis are warranted.

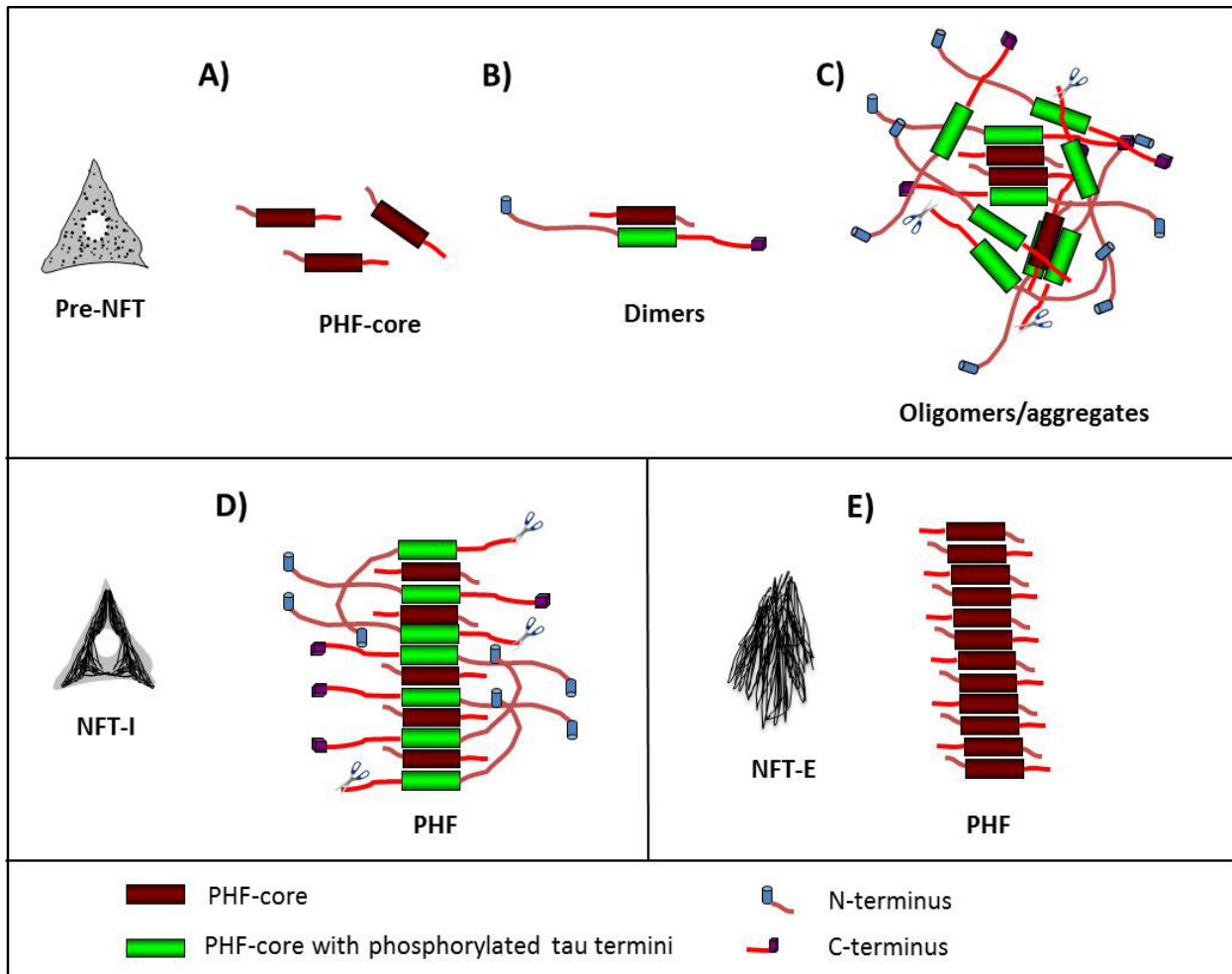


Figure 7. Scheme illustrating the early steps of aggregation and polymerization of tau protein in Alzheimer's disease. (A) The model starts with the appearance of PHF-core tau in cytoplasm of susceptible neurons. (B) The high binding capacity of PHF-tau results in the assembly of dimers of PHF-core and intact tau molecules in the cytoplasm. (C) The phosphorylation of intact tau would be an early event to hide the toxic soluble aggregates of molecules. (D) The high affinity and stability of the proto-filaments that make up the mature intracellular NFT allows tau molecules to form PHFs. (E) With the death of the neuron, the PHF-core subunit becomes exposed again in the extracellular space following proteolysis. Further details are described in the text.

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