Pathological Stages of Abnormally Processed Tau Protein During Its Aggregation into Fibrillary Structures in Alzheimer’s Disease

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1. Introduction

Abnormal aggregation of tau protein within the cytoplasm of susceptible neurons has been considered one of the major hallmarks that define the neuropathology of Alzheimer’s disease (AD) (Iqbal et al., 2010; Pritchard et al., 2011). At early stages of neuronal degeneration tau protein is accumulated in the form of early non-assembled aggregates that may alter the normal functioning of affected neurons (Hoozemans et al., 2009; Luna-Munoz et al., 2007). Nonfibrillar aggregation of tau protein as a pre-tangle state has been reported to occur early in the disease but also observed in nondemented very old individuals (Garcia-Sierra et al., 2000). Some studies have reported that oligomeric species of tau protein represent the toxic structures rather than fibrillar structures (Berger et al., 2007; Maeda et al., 2006), however few studies have analyzed and determined a positive correlation between the load of pre-tangle carrying neurons and the clinical symptoms of AD. Further alterations in neurons may occur when the soluble aggregates of tau become assembled into insoluble polymers referred to as paired helical filaments (PHFs) that may also obstruct the transit and distribution of intracellular components, modify the neuronal morphology and alter the cytoskeleton (Ballatore, Lee & Trojanowski, 2007; Kidd, 2006). These filaments progressively coalesce into neurofibrillary tangles (NFTs) which eventually lead to the neuronal death (Guo & Lee, 2011). It is generally accepted that in AD cases, the density of NFTs is the best correlate with the dementia score (Arriagada, Marzloff & Hyman, 1992; Gomez-Isla et al., 1997).
The description about the progression of the neurofibrillary pathology in AD has been proposed by Braak and Braak (Braak et al., 1993, 1996), which states that there is a stereotype in the appearance and distribution of NFTs along entorhinal, limbic and isocortical areas. It is well known that in AD, NFTs and dystrophic neurites (DNs) are mostly composed of tau protein which has undergone several posttranslational modifications such as abnormal phosphorylation, conformational changes and truncation (Du et al., 2007; Novak et al., 1991; Wischik et al., 1988a). In this regard, we have previously proposed that a continuous and specific pathway of conformational changes and truncation of tau protein is occurring during the maturation of NFTs (Binder et al., 2005; Guillozet-Bongaarts et al., 2005, Mondragon-Rodriguez et al., 2009).

The sum of our data support that not only the number of NFTs defines the progression of AD, but also the state of proteolysis of the C-terminus which is associated with conformational changes, i.e. structural modification along the tau molecule (Garcia-Sierra, Mondragon-Rodriguez & Basurto-Islas, 2008). In distinct populations of NFTs, the occurrence of mutually exclusive truncations at either acid aspartic-421 (Asp\(^{421}\)) or glutamic acid-391 (Glu\(^{391}\)) correlated well with the evolution of the disease (Basurto-Islas et al., 2008). This cascade of pathological molecular events may give a better correlation with the neuropathological progression of the disease. In our model of pathologic processing of tau we pointed out the existence of chimeric NFTs which are composed of diverse molecules of tau characteristically in different stages of proteolysis.

2. The neurofibrillary pathology of AD

The observed neuropathological changes in patients with AD are at least partly the result of the accumulation of NFTs (Fig. 1, arrows) and amyloid-\(\beta\) (A\(\beta\)) deposits (A\(\beta\)-plaques) (Fig. 1, asterisk) around the hippocampal area (Braak & Braak, 1994; Tseng, Kitazawa & LaFerla, 2004). However, the numeric correlation between A\(\beta\)-plaques and clinical symptoms in AD barely represents what is happening in the neurodegenerative process. Also the simple A\(\beta\)-accumulation per se is not the only etiological factor to trigger AD abnormalities (Lee et al., 2005). Cited studies explain why many aged individuals, despite the presence of high numbers of senile plaques, show little or no cognitive decline (Lee et al., 2004). With these data in mind, the belief that the A\(\beta\)-accumulation is a consequence rather that a cause is gaining more support. However, the pathology of the NFTs, mainly composed of tau protein, remains as a relevant criterion for the diagnosis of AD after death, because of the spatial correlation, albeit not perfect, between the number of NFTs and the clinical symptoms of this disease (Braak & Braak, 1991; Murayama & Saito, 2004). The support for this asseveration is mainly based on Braak's study, in which the occurrence and progression of NFTs along allocortical and isocortical areas was described (Braak & Braak, 1991). This study evaluates the density and distribution of NFTs along the brain and classifies them into I, II, entorhinal; III, IV, limbic; and V, VI, neocortical stages. These three groups of stages correspond to normal cognition, cognitive impairment, and dementia (Braak & Braak, 1997). Note that this correlation is not sufficient to demonstrate a cause and effect relationship between NFTs and cognitive deficits. In this regard, the obvious question raised is why do brains of AD patients show the pathology of NFTs? Despite all the information available the answer is not even close to being addressed. However, a huge number of strategies focused on the mechanisms governing the aggregation of the tau protein into PHFs and NFTs (Kosik, Joachim & Selkoe, 1986) have attempted to address this. Those
efforts have been directed to unmask the mechanisms involved in tau dysregulation and its abnormal processing during AD (Kidd, 2006).

Fig. 1. Neurofibrillary pathology in the brain of patients with Alzheimer’s disease. NFTs (arrows) are triple labeled with antibodies to tau protein (blue and green channels) and thiazin red (red channel). An amyloid-β-core plaque is visualized in the red channel (asterisk). Neuropil threads are mostly observed in the green channel. Images taken from the hippocampus.

3. Abnormally processed tau protein is the major component of the neurofibrillary pathology in AD

There is a considerable amount of data consistent with the hypothesis that the aggregated state of tau could be functioning as a potential mediator to neurodegeneration, by either creating novel toxic species (gain of function) or by interfering with the normal function of the tau protein itself (loss of function) (Honson & Kuret, 2008). The aggregated state of tau interferes with the axonal transport, a result that further supports the crucial role of aggregated tau in the pathogenesis of AD (Stokin & Goldstein, 2006).

The growing facts showed that the aggregation certainly contributes to neurodegeneration, however the data also showed that the final aggregated state (NFTs) during AD, by bringing some compensatory properties, could exert a beneficial role (Congdon & Duff, 2008; Gotz et al., 2008). Despite this controversial fact, the aggregation debate has pointed out three posttranslational modifications as the key events; hyperphosphorylation, conformational changes, and cleavage, all of them taking place in the tau molecule and that are believed to promote the pathological and aggregated state of this protein (Chun & Johnson, 2007; Du et al., 2007; Yin & Kuret, 2006).

Aberrant phosphorylation of some sites as Ser262 has been proposed to play a crucial role in reducing the binding properties of the tau protein to the microtubules, driving the tubulin-unassociated tau protein to a soluble state that in turn could become an aggregated entity (Biernat et al., 1993; Gustke et al., 1992). Some reports had set the path for aberrant phosphorylation as a strong candidate promoting the formation of tau oligomers, and sequentially to the assembly into PHFs. Thus phosphorylation at sites such as Ser396, 404 has been proposed to cause PHF structures in a model in vitro (Schaffer et al., 2008). Following the approaches in vitro, dysregulation of protein kinases and protein phosphatases that control tau phosphorylation has been reported (Mandelkow et al., 1992; Schaffer et al., 2008). However, some data came from the approach in vitro and do not necessarily reproduce the cytoplasmic conditions seen for tau protein in the disease. Nevertheless, it has long been reported that in the brains of AD patients the tau protein residing in the PHFs is abnormally
phosphorylated at several residues, therefore, it has been concluded that this modification plays an important role during the formation of NFTs (Iqbal, del & Grundke-Iqbal, 2008; Wang, Grundke-Iqbal & Iqbal, 1996).

It has also been postulated that structural changes in the molecule of the tau protein can be caused by phosphorylation of specific residues, which then cause local modifications, which in turn transiently lead to extensive folding of the N-terminus (Luna-Munoz et al., 2005, 2007). It was also suggested that phosphorylation at site Ser\(^{356}\) conditioned by the fourth microtubule-binding domain may be responsible for conformational changes of tau and self-aggregation (Du et al., 2007). Moreover, the conformational changes in this molecule have been proposed to mediate dimerization of tau and the subsequent formation of NFTs. The support for this hypothesis comes from the theory that the extreme ends of the tau protein may reduce the aggregating properties of the protein if they had an unfolded conformation (Gamblin, Berry & Binder, 2003a). Conformational changes in tau protein leading to its abnormal aggregation in the AD brain has been monitored by using conformational-dependent antibodies such as Alz-50, MCI, and Tau-66 (Carmel et al., 1996; Ghoshal et al., 2001; Jicha et al., 1997a). These antibodies, with discontinuous epitopes along the tau molecule, only recognize tau protein when these residues approach one another, which only occurs through the folding of the N-terminus over the repeated domains. This conformational alteration is mostly found when tau is abnormally aggregated into affected neurons in the brain of AD patients (Garcia-Sierra et al., 2003).

The cleavage of the tau protein has also been implicated in the abnormal processing of tau protein that contributes to its self-assembly into PHFs and increased toxicity (Gamblin et al., 2003b; Wischik et al., 1988b). It has been proposed that tau protein is a substrate of several intracellular proteases associated with the turnover of proteins such as cathepsins, calpains, and caspases (Gamblin et al., 2003b; Rissman et al., 2004; Wang et al., 2009; Yang & Ksiezak-Reding, 1995). Interestingly, cleavage of the tau protein is also related to apoptosis, because this action is specifically caused by caspase-3 generating a large truncated product preserving its N-terminus, but proteolytically truncated at the position Asp\(^{421}\) at the C-terminus (Fasulo et al., 2000; Gamblin et al., 2003b). This truncation was also involved with both nucleation and extension of tau fibrilization in vitro (Gamblin et al., 2003a; Rissman et al., 2004) and is closely related to toxicity in cell and animal models (Garcia-Sierra et al., 2008). The relevance of truncation of tau protein has also been demonstrated to have a positive correlation with the clinical symptoms of AD (Basurto-Islas et al., 2008).

Furthermore, it has been shown that cleavage and phosphorylation together can alter the tau microtubule interaction (Ding, Matthews & Johnson, 2006), however in a nonpathological role it was recently shown that phosphorylation at site Ser\(^{422}\) prevents cleavage of the tau protein at site Asp\(^{421}\) (Guillozet-Bongaarts et al., 2006).

To date both conformational changes and cleavage have been involved in the pathological processing of tau protein, which made them relevant candidates as potential therapeutic targets. The current scenario that attempts to elucidate how these postranslational modifications interact with one another to drive the fibrillar aggregation of tau and the timing of occurrence along the evolution of AD disease still remains elusive. However, we believe that some answer could be gotten from the chronological characterization at the molecular level of the major lesions, the NFTs that correlate better with the cognitive impairment observed in AD patients. This approach may turn the present scenario into a more promising pathway to the understanding of the genesis and maturation of the NFTs.
4. Abnormal hyperphosphorylation of tau protein and its relationship to the pathology of the neurofibrillary tangle

In 1986, abnormally phosphorylated tau protein in the brains of AD patients was reported (Grundke-Iqbal et al., 1986b). Back then it was found that tau was a phosphodependent protein regulated via coordinated actions of kinases and phosphatases, which mostly preserve this protein in a low phosphorylated state in association with microtubules (Liu, Liang & Gong, 2006; Mandelkow et al., 1995). In AD, either dysregulation of phosphatases or upregulation of kinases such as calcium and calmodulin-dependent kinase II (CaM Kinase II), protein phosphatase 2A (PP2A), mitogen-activated protein kinase (ERK II), glycogen synthase Kinase-3β (GSK-3β), protein phosphatase-1 and -2C, p70 kinase, cyclic-AMP-dependent protein kinase, protein kinase C, and casein kinase II may lead to the formation of hyperphosphorylated tau protein (Avila, 2008; Iqbal & Grundke-Iqbal, 2008).

Moreover, in AD the tau protein is abnormally phosphorylated not only in those sites traditionally controlling its microtubule binding properties but more unconventional residues increase its phosphorylated state, such as Ser396, Ser214, and Ser262 (Biernat et al., 1993; Bramblett et al., 1993; Schneider et al., 1999). Additionally, it has been proposed that cytosolic-phosphorylated tau is able to recruit normal tau and MAP protein causing microtubule disruption (Alonso et al., 1997, 2001; Iqbal et al., 2008). Besides these modifications that lead to the loss of function of tau, hyperphosphorylation also causes the tau protein to self-aggregate into nonfibrillary or fibrillary aggregates that are not easily eliminated from the cytoplasm of altered neurons in AD. An increased phosphorylation of tyrosine residues was found to correlate to the formation of tau aggregates (Vega et al., 2005). Concomitantly from experiments in vitro, it is long been reported that pseudophosphorylation of tau protein increases its ability to polymerize (Alonso et al., 2001). When the process of tau aggregation was evaluated in vitro in the presence of methylglyoxal and acrolein (drugs that cause lipid peroxidation) the fibrilization was slightly enhanced in phosphorylated tau species (Kuhla et al., 2007).

Soluble aggregates composed of abnormally phosphorylated tau were isolated from the brains of AD patients and were referred to as the A68 fraction, because of its electrophoretic mobility compared to normal tau protein (Brion et al., 1991; Lee et al., 1991; Vincent & Davies, 1990). In accordance with these data, it was shown that tau protein in the PHFs, which are the main component of the NFTs, was abnormally hyperphosphorylated at several residues (Grundke-Iqbal et al., 1986b). At the neuropathological level, large amounts of NFTs seem to progress according to the evolution of AD with early invasion into those areas that start the neurofibrillary degeneration along the perforant pathway, correlating with the cognitive decline of patients with dementia (Garcia-Sierra et al., 2000). In agreement with these findings, CaM Kinase II that phosphorylates tau at site Ser416 was found to be closely related to the accumulation of tau in the soma of affected neurons in the brain of AD patients (Yamamoto et al., 2005). By using transgenic models, phosphorylation of tau was found to represent an essential modification that causes self-aggregation and abnormalities in the normal functioning of this protein (Delobel et al., 2008).

In contrast to pathological properties, some authors using polymerization models have suggested that abnormal phosphorylation of tau protein enhances, but does not trigger, fibrilization (Necula & Kuret, 2004). Other investigators have shown that abnormally phosphorylated tau protein residing in the PHFs can be released and rescued from this...
inactive state to recover normal functions, such as microtubule binding after dephosphorylation mainly caused by active phosphatases (Wang et al., 1998). Despite this controversy and noting its pathological role as a major issue, the phosphorylated state of tau protein has been associated with neurodegeneration in AD, likely by causing the impairment of the axonal transport, as was demonstrated in both the aggregated and nonaggregated state (Lapointe et al., 2008; Mandelkow et al., 2003). Accumulation of tau aggregates inside neurons may represent either a physical impediment for the cytoskeletal organization that affects the organelle transport or a recruiting structure that binds diverse molecular components in a nonspecific fashion. Despite the controversy about the toxicity of the nonaggregated state of the phosphorylated tau versus fibrillary structures as the major pathological players, the well-described NFTs remain as the stronger candidate.

5. Abnormal phosphorylation and conformational changes of tau protein during the formation and maturation of neurofibrillary tangles

In some neurodegenerative disorders it is well-known that the misfolding of linear proteins causes them to adopt conformations that may affect their normal functioning and cause them to self-aggregate. This is common for AD and prion diseases, in which proteins prone to folding attain an altered conformational state with properties that inevitably lead to cell death (Carrell & Gooptu, 1998; Soto, 1999). It is believed those conformational changes could cause the proteins to adopt an amyloid-conformation and finally to disease, although the mechanisms remain unknown (Kelly, 1996). It has been postulated that the conformational changes in tau protein could actually be driving a nucleation phenomenon similar to that occurring in the brain of AD patients (Fox, Harvey & Rossor, 1996).

The tau protein under physiological conditions has a random coil structure (Barghorn, Davies & Mandelkow, 2004; Sadqi et al., 2002), but in AD tau develops conformational changes that alter its normal function and increases its aggregation properties into a pathological ß-sheet conformation. Large amounts of this conformationally altered protein have been reported to aggregate during the progression of AD (Ghoshal et al., 2002). The mechanisms driving conformational changes are still elusive, however data referring to local alterations in the tau structure showed abnormal phosphorylation of specific residues as a major factor (Jicha et al., 1997b; Luna-Munoz et al., 2007). The description of the first conformational change discovered in the tau molecule was based on the characterization of the Tau-2 monoclonal antibody, which was developed by immunizing mice with tau protein purified from bovine brain (Papasozomenos & Binder, 1987). When this antibody was tested against tau residing in PHFs purified from the brains of AD patients, immunoreactivity was stronger than that shown by human monomeric tau. From this result it was concluded that Tau-2 recognized a conformational shift occurring in polymeric tau that mimicked the original residue contained in bovine tau. The normal monomer of tau contains a proline residue in the site of the recognition of Tau-2, however during tau fibrilization this residue adopts a serine-like conformation that is now detected by this antibody. Following the same trend, immunization of mice was also done with brain extracts from AD patients, producing a new conformational antibody named Alz-50 (Wolozin et al., 1986). Immunoreactivity of this antibody was based on the extensive folding of the N-terminus of the tau molecule over the third microtubule-binding domain, which was revealed when the epitope mapping of this antibody reported a discontinuous sequence of recognition (residues 7-9 and 312-342) (Carmel et al., 1996). Thereafter the Alz-50
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conformational antibody was described as a reliable marker of tau neuropathology in AD (Brady & Mufson, 1991; Hyman et al., 1988; Tourtellotte et al., 1990). Since then, conformational changes have gained more attention from the AD-study community, which has proposed this modification to be related to transcriptional abnormalities during the genesis of tau protein in human brain neurons (Hyman, Augustinack & Ingelsson, 2005). In contrast, a variable role has been attributed to the Alz-50 conformation of tau protein because the normal activity of neurons was reported to take place in the presence of tau protein attaining the Alz-50 conformation (Salehi et al., 1995; van de Nes et al., 1998). These data are intriguing and crucial because the Alz-50 conformational change is present as an early event during the pathology of AD. Later there were claims that the Tau-66 antibody could recognize a discontinuous sequence (residues 155-244 and 305-314) in the tau molecule, which was developed by immunizing mice with three repeated domains of tau polymers assembled in vitro (Ghoshal et al., 2001). Supporting the conformational change as a pathological event, different structures, called granulovacuolar, and fibrillar lesions that correlated with several determinations of episodic memory, were detected (Ghoshal et al., 1999, 2002). The epitopes of both conformational changes recognized by Alz-50 and Tau-66 partially overlap in a common region located at the third repeated domain, suggesting this part of the molecule as a relevant site for the protein folding. Local conformational changes in the repeat-domain region have also been reported (Du et al., 2007). The local and structural conformational changes adopted by the tau protein can be explained in part by phosphorylation of specific amino acids that change the charge and thermodynamic stability of an unfolded conformation. It has also been suggested that phosphorylation of the tau protein at site Thr$^{231}$ precedes the Alz-50 conformational change (Luna-Munoz et al., 2005, 2007).

The main data about the relationship between phosphorylation and conformational changes come from AD tissue, which somehow yielded the question about how phosphorylation was able to generate those foldings in the proteins. By asking this question and by using two test proteins, cystatin and NtrC, it was reported that phosphorylation is able to change the free energy landscape, which in turn modifies the original structure by changing it into a folded conformation (Latzer, Shen & Wolynees, 2008). More related to AD, phosphorylation of Ser and Thr residues in the Pro-rich region of the tau protein causes this protein to have a polyproline II helix conformation (Bielska & Zondlo, 2006). The ability of tau to aggregate in vitro is increased once phosphorylation at the site Ser$^{356}$ occurs. Additionally this phosphorylation favored a local conformation (Du et al., 2007). More recently, pseudophosphorylation in vitro of proline residues along the epitopes of AT8 and PHF1, two tau phosphodependent antibodies, caused the pathological conformation in the tau molecule that is recognized by the MC1 antibody (Jeganathan et al., 2008). So far the data set out clearly those phosphorylations are highly related to the conformational change seen during AD, though the time-course of the appearance of both events is not clear.

By attempting to determine the time-course by which phosphorylation of the tau protein and conformational changes are associated to one another in the authentic disease, we have analyzed the profile of the immunoreactivity of NFTs to tau antibodies that map the entire molecule and recognize both conformational changes and diverse phosphorylation residues during the progression of AD. Our data have shown a strong relationship between the Alz-50 conformational change and the phosphorylation of multiple residues in the tau molecule such as Ser$^{396}$, $^{404}$ (labeled by the AD2 antibody) at the C-terminus. Both events may occur in the same molecule of tau protein, which is shown by the close colocalization observed in a large number of NFTs. Other phosphorylation sites such as Ser$^{199}$, $^{202}$ Thr$^{205}$, Ser$^{262}$, and Ser$^{422}$ were also linked to the Alz-50 conformation.
These observations demonstrated a close relationship between conformational changes and phosphorylation, with the latter as a possible cause of the folded state of the tau protein. These NFTs are the common neuropathological features during early stages of AD, in which most of these structures are composed of C-terminus intact tau protein (immunoreactive to Tau-46.1 antibody) and also having the Alz-50 conformational change (arrows in Fig. 2). The fibrillar state of these structures is clearly seen by the positive signal to TR, a fluorescent marker for a β-pleated sheet conformation as shown by already assembled PHFs (Mena et al., 1995). The light-blue to white pseudocolor of these structures observed in the merge channel of Fig. 2, indicates the existence of C-terminus intact and conformationally altered tau protein in an assembled state.

It can be also seen that some NFTs are only detected by TR (asterisks), which may imply that these structures have been further processed and both the Alz-50 conformation and the C-terminus of tau molecule are likely lost by proteolysis. Further, it can be seen that the neuritic component along the neuropil is also composed of C-terminus intact and conformationally altered tau protein, though the state of assembly does not yet show a fibrillar aggregation (thiazin-red negative). This result indicates a more delayed accumulation and processing of tau protein along this component. According to our data, we have found that most of these abnormal phosphorylation and conformational changes in the full-length tau protein occur in the early stages of AD.

Fig. 2. The Alz-50 conformation of tau in NFTs is associated with preservation of the C-terminus. At early stages of AD, in most of the Alz-50 positive NFTs (green channel), tau preserves its extreme C-terminus intact (Tau-46.1 labeling). Arrows indicate the double labeling of these NFTs (note also the triple labeling with TR as a white pseudocolor in the merge channel). In more advanced NFTs these epitopes are missed and they are labeled only by TR (asterisk). Images taken from the hippocampus.

Maturation of NFTs has been associated with the transformation of morphology and composition of these structures (Garcia-Sierra et al., 2001). After the maturation of the early NFTs described above, we have observed that the tau protein in a fibrillar state is further processed to yield a new conformation as observed by using the conformationally dependent antibody Tau-66 (Garcia-Sierra et al., 2003; Ghoshal et al., 2001, 2002). At the intermediate stages of AD, early NFTs now progress from entorhinal areas to the hippocampal formation. The more advanced NFTs that follow the maturation process are recognized by the Tau-66 antibody, that in this case the N-folding conformation adopted by tau has also lost its extreme C-terminus. Although the adoption of the Tau-66 conformational change may imply the loss of some phosphorylated residues in the tau protein, the AD2 epitope (pSER396, 404) is preserved at least in some NFTs.

Thus, it is clear that the Tau-66 conformational change is not associated with the full-length tau protein. As initially reported (Garcia-Sierra et al., 2003), we also found that both
conformations of tau in the NFTs, the Alz-50 and the Tau-66, were mutually exclusive, because they were not synchronized along the time-course and area of distribution. Moreover, Alz-50 and Tau-66 never were colocalized in the same NFT. These data also support our proposal for the Alz-50 conformational changes as an early event, but more importantly also show the nonsynchronous stage of the neuropil pathology compared to the NFTs formation.

These particular changes in the tau molecule also occur in the temporal cortex. It has been reported that NFTs in this area display a laminar distribution occurring mostly in cortical layers II, III, V, and VI (Thangavel et al., 2008). These structures are also conformed of tau protein phosphorylated at several domains (Thangavel et al., 2008; Guillozet-Bongaarts et al., 2006) and some showing immunoreactivity to Alz-50 antibody (de la Monte et al., 1992). Summarizing, during the progression of early and intermediate stages of AD, the discriminatory presence of each conformational event sets the dynamic behavior of tau during the maturation of NFTs, started by the N-terminus folding that is caused by the phosphorylation of specific domains. These time-dependent conformational changes also occur during the aggregation of the tau protein into the neuritic component, but not synchronized in time with those changes shown in the NFTs.

6. Cleavage of tau protein and the maturation of the NFTs

The pathologic processing of the tau protein, in which several residues are lost, has been attributed to intracellular proteolysis (Gamblin et al., 2003b; Novak, Kabat & Wischik, 1993; Rissman et al., 2004). The early biochemical analysis of the insoluble fraction of homogenates of the brain of AD patients revealed an enriched fraction of PHFs (Greenberg & Davies, 1990; Grundke-Iqbal et al., 1986a; Wischik et al., 1988b). When this fraction was completely digested using pronase, a minimum PHF-core remained as an insoluble protease-resistant fraction (Novak et al., 1993; Wischik et al., 1988b). The remaining PHF-core comprised a 12 kDa portion of the tau protein beginning in the vicinity of histidine-268 and containing the tubulin-binding domains.

This fragment ended at the site glutamic acid-391 (Glu\textsuperscript{391}) towards the extreme C-terminus (Novak, 1993,1994). No morphological differences were found between native PHFs and the remaining PHF-core when analyzed by electron microscopy, but the latter was antigenically deprived of the N- and C-termini commonly found in the tau protein during its early aggregation in AD (Wischik et al., 1995). Mice were immunized with this PHF-core fraction to develop the monoclonal antibody MN423 that specifically labeled the Glu\textsuperscript{391}-truncated residue in the tau protein (Novak et al., 1989, 1991, 1993). Validating the importance of the truncated tau protein at site Glu\textsuperscript{391}, correlation of this event with the NFT progression, and the clinical symptoms were demonstrated (Garcia-Sierra et al., 2001). Despite the promising alternative for the role of the Glu\textsuperscript{391} cleavage of tau during the formation of NFTs in AD, a limited relevance has been attributed to the cleavage because so far no intracellular proteases have been described to be responsible for this event. Even though the polymeric state of the Glu\textsuperscript{391}-cleaved tau inside the cytoplasm of affected neurons in AD has been associated with toxicity in vulnerable areas of the brain, such as those corresponding to the perforant pathway (Garcia-Sierra et al., 2001), it is well-documented that an abnormal proteolytic processing of proteins occurs as part of the aging process and cell death in several neurodegenerative diseases (Cotman et al., 2005; Newman et al., 2005; Rubinsztein, 2006). One of the most cited participants is a family of cysteine-aspartyl proteases, referred
to as caspases, that are reported to be active during apoptosis and have an increased expression in AD (Dickson, 2004; Rubinsztein, 2006).

Some other cleavage sites have been found in the tau protein, mainly occurring at the C-terminus residue aspartic acid-421 (Asp\textsuperscript{421}), for which caspase-3 was found mainly responsible for this action (Gamblin et al., 2003b; Horowitz et al., 2004). From the previous data we can see that the cleavage of the tau protein has been strongly related to the apoptosis process, which is also thought to contribute to the neurodegeneration during AD.

Fig. 3. Apoptotic nuclei are disseminated in the hippocampus of AD cases. Arrows in A indicate TUNEL-positive nuclei in cells lacking NFTs (neurofibrillary tangles). In panels B and C, two NFTs composed of Asp\textsuperscript{421}-truncated tau (positive to Tau-C3 antibody) are in close association with TUNEL-positive nuclei (arrows). Images obtained by confocal microscopy represent the merge channel of double labeling samples with TUNEL (green) and Tau-C3 antibody (red).

In the brain of AD patients, increased amount of cell undergoing apoptosis was reported in comparison to age-matched nondemented individuals (de la Monte, Sohn & Wands, 1997; Lucassen et al., 1997; Nagy & Esiri, 1997). Because apoptosis is characterized by several biochemical changes involving activation of proteolytic caspases, it was thought this mechanism could generate Asp\textsuperscript{421}-truncated tau \textit{in situ}. By using the \textit{in situ} cell death detection kit TUNEL (deoxynucleotydyl transferase dUTP end labeling), which detects apoptotic nuclei (Gold et al., 1994), we found mostly in the hippocampus of AD patients that a large population of neurons contained apoptotic nuclei in the absence of intracellular NFTs (arrows in panel A, Fig. 3). However in some specific populations of apoptotic neurons, NFTs composed of Asp\textsuperscript{421}-truncated tau were detected in the cytoplasmic space (Arrows in panels B and C, Fig.3).

However, some investigators have postulated that apoptosis during AD could also be a consequence instead of being the causal event (Dickson, 2004). A simple nonmathematic and chronological relationship between AD and apoptosis has been proposed suggesting that this process does not change or affect the course of AD (Zhu et al., 2006). In sum, the contradictory data about the apoptosis during AD and the discovery of proteolytic cleavage of the tau protein by multiple caspases raised the question whether or not these families of enzymes were also responsible for the truncation at Glu\textsuperscript{391}. However, neither caspases nor calpain (Newman et al., 2005) were directly associated with the formation of this truncated epitope. Despite the controversy about the genesis of the truncated species of tau protein, experiments \textit{in vitro} corroborated that both truncated tau variants, the Asp\textsuperscript{421} and Glu\textsuperscript{391}, were able to polymerize at a faster rate than the normal C-terminus-intact tau protein (Abraha et al., 2000; Rissman et al., 2004). This result corroborated the
abnormal properties of the truncated tau protein compared to those of the wild-type molecule. For the Asp^{421} cleavage, it was found in the brain of AD patients by using a monoclonal antibody called Tau-C3, which was developed by immunization of mice with this truncated variant and proved to be specific for the Asp^{421} ending residue. By using this antibody further support for a relevant role of Asp^{421}-cleaved tau in AD was corroborated by the positive correlation found between the density of NFTs recognized by Tau-C3 and the neuropathological progression of the disease described by Braak and Braak (1991), and to the clinical severity of dementia (Basurto-Islas et al., 2008). Additionally, a close relationship to the presence of the Apolipoprotein-E (ε4) allelic variant was found in cases with an increased density of NFTs immunoreactive to the Tau-C3 antibody (Basurto-Islas et al., 2008).

7. Unified model of tau processing during the evolution of AD

All the previous evidence highlighted the important role for the cleavage of tau protein as a relevant mechanism leading to the well-described AD pathology. However, how these truncations could interact with another to determine the formation of the fibrillary pathology, and how they may affect conformational changes of the tau protein, are important questions that require further investigation. Trying to address these questions we turned back to the postmortem analysis of the brain of AD patients with varying degrees of the clinical manifestation of dementia, in which the authentic disease can be analyzed as a progressive process. We were able to describe that NFTs composed of either Asp^{421} or Glu^{391}-truncated tau were mutually exclusive in the brain of AD patients at any stage of AD progression, and that the advanced Glu^{391}-truncated variant of tau is a common feature of AD but not for other tauopathies (Basurto-Islas et al., 2008; Mondragon-Rodriguez et al., 2008). Interestingly we found that the initial Alz-50 conformational stage of tau protein residing in early NFTs is transiently transformed to the Tau-66 conformation, possibly caused by subtle truncation of the extreme C-terminus at the position Asp^{421} (Fig. 4 and Fig. 5). It is clearly seen that Alz-50 colocalizes with Tau-C3 in some of these structures (arrows in Fig. 4), but in others the Alz-50 conformation is lost coincidentally with the presence of truncation at the Asp^{421} residue (arrowheads in Fig. 4).

This result is intriguing because the major contributor for the Alz-50 folding is the N-terminus, completely opposite to the truncation site at the C-terminus. From these data, we have only inferred that the loss of these 20 amino acids may cause a rearrangement in the N-folding of the tau protein because it coincides with the increase of NFTs developing a new conformation now detected by the Tau-66 antibody (arrows in Fig. 5).

In relating cleavage and phosphorylation, abnormal phosphorylated sites in the tau protein, such as Ser^{199}, 202 Thr^{205}, Ser^{396}, 404, and Ser^{262}, were found coexisting in the Tau-C3 structures. Moreover and supporting those previous findings that come from AD brain tissue, chimeric NFTs composed of full-length tau and C-terminal-truncated tau at Asp^{421} were also found in this study (Guillozet-Bongaarts et al., 2005). Abnormal phosphorylation of tau protein has also been found in other tauopathies, such as Pick disease in which Asp^{421} cleavage of tau was colocalized into the characteristic Pick bodies (Mondragon-Rodriguez et al., 2008). These data suggest a close relationship of phosphorylation and cleavage for the tau processing (Guillozet-Bongaarts et al., 2007; Mondragon-Rodriguez et al., 2008).
Fig. 4. Subtle truncation of the extreme C-terminus is occurring in tau protein adopting the Alz-50 conformation. In some intermediate-stage NFTs (arrows), the tau molecule adopting the Alz-50 conformation is subjected to proteolysis that cleaves the extreme C-terminus at the position Asp$^{421}$ (Tau-C3 immunolabeling). In contrast, other NFTs have either the Alz-50 conformation (asterisk) or the truncation at the Asp$^{421}$ (arrowhead). Images taken from the hippocampus.

Fig. 5. Asp$^{421}$-truncation links a new conformation of tau protein in NFTs. From intermediate to advanced stages in AD, some NFTs are carrying tau molecules that have a new conformation recognized by the Tau-66 antibody and are also undergoing truncation at position Asp$^{421}$ (arrows). In contrast other NFTs have either the Tau-66 conformation (asterisk) or the truncation at the Asp$^{421}$ (arrowhead). Images taken from the hippocampus.

In contrast, phosphorylation of tau is able to prevent apoptosis in growing cells (Li et al., 2007) and the cleavage at the Asp$^{421}$ residue is reduced in vitro by the pseudophosphorylation of the site Ser$^{422}$ (Guillozet-Bongaarts et al., 2006). Similarly, dephosphorylation of the molecule is crucial for tau protein to undergo cleavage (Rametti et al., 2004). However, other experiments in vitro recapture the pathologic relationship between phosphorylation and the cleavage of tau protein. Alterations in the microtubule-binding
properties were found in tau protein when the molecule was phosphorylated at specific sites, such as Ser\textsuperscript{396, 404}, and cleaved at the site Asp\textsuperscript{421} (Ding et al., 2006). It was also found that the combination of phosphorylated and truncated tau contributes to tau oligomerization (Cho & Johnson, 2004). Supporting the phosphorylation as a pathological event, upregulation of c-jun N-terminal kinase leading sequentially to tau phosphorylation and cleavage has also been reported (Sahara et al., 2008). Overall, and affirming the initial idea, it is clear that phosphorylation is highly related to the cleavage during tau processing, although the precise role still remains to be clarified.

As our data have shown, it is clear that a tau molecule truncated at Asp\textsuperscript{421} also attains a conformation recognized by Tau-66 (merged white tangle in Fig. 5), an event that can be the link between the Alz-50 and Tau-66 conformational stages. Different NFTs are not synchronized along the time-course of maturation and have either the truncation at the Asp\textsuperscript{421} of tau protein (arrowhead) (and probably are Alz-50 positive) but are negative to Tau-66 or have attained the Tau-66 conformation and are truncated at the C-terminus (asterisk in Fig. 5) (negative to Tau-C3). Putting these data together, we have concluded that the switching of conformations in the tau molecule in a polymeric state is a transient process occurring during the maturation of NFTs from early to intermediate stages of the disease. During this maturation, early NFTs will colonize new areas that follow the important perforant pathway of communication from the entorhinal cortex to the hippocampus, and later from this area to the isocortex via the subiculum and entorhinal cortex layer IV (Braak & Braak, 1991; Garcia-Sierra et al., 2001; Van Hoesen, Hyman & Damasio, 1991). The most mature structures and consequently the more proteolytically processed remain at the earlier invaded areas. From intermediate to advanced stages of AD, the Tau-66 conformation attained by tau protein after Asp\textsuperscript{421} truncation is altered by further truncation of the remaining C-terminus. This means the conformational folding and partial truncation of tau protein by caspases may cause rearrangements in the polymeric state of tau protein to expose occluded epitopes that in turn may be the target for other intracellular proteases, such as cathepsins, calpains, or more nonspecific carboxy-peptidases. Whatever the responsible enzyme is, the result is that tau protein is later truncated at the Glu\textsuperscript{391} site, which is still observed in NFTs carrying tau molecules having the Tau-66 conformation (arrows in Fig. 6).

Frequently from the intermediate to the late stages of AD, Tau-66 and MN423 are observed to colocalize in both intracellular and extracellular NFTs, the latter commonly recognized by MN423 and having less intensity for TR staining (arrows in Fig. 6). The Tau-66 conformation, even when present at late stages of AD, is finally lost when the NFTs are severely proteolyzed, leaving only a remainder PHF-core that is mostly composed of truncated tau at the Glu\textsuperscript{391} site (Mena et al., 1996; Novak et al., 1993). In the hippocampus of AD patients the maturation of NFTs is reported to be unsynchronized, therefore these structures have different stages of tau processing (Basurto-Islas et al., 2008). We found different populations of NFTs in the same hippocampal area that were mutually exclusive when they were composed of either Glu\textsuperscript{391}-truncation or Alz-50 conformation, but not colocalized at any single point during the maturation of the NFTs (Fig. 7).

Although phosphorylation of tau residues is believed to be reduced during the progression of the disease, NFTs recognized by the conformation-dependent antibody Tau-66 and tau truncated at Glu\textsuperscript{391} still preserve the phosphorylated residues pSer\textsuperscript{199, 202} and pSer\textsuperscript{396, 404} (arrows in Fig. 8), which is explained by the existence of chimeric NFTs composed of independent molecules of tau having different degrees of truncation. A clear example of how tau pathology is not synchronized in the hippocampal area is shown in Fig. 8, where...
somatic aggregation of advanced truncation of the tau protein (Glu$^{391}$) is observed in one NFT (asterisks), which is innervated by dystrophic neurites composed of phosphorylated tau (pSer$^{396, 404}$) (green channel), with the conformationally altered tau protein distributed only in the apical neurite (arrowheads in the blue channel).

Fig. 6. Tau-66 conformation is strongly associated with truncation of tau protein at Glu$^{391}$. At intermediate and advanced stages of AD, significant numbers of NFTs acquire the conformational change detected by the Tau-66 antibody (channel green). In this conformation the tau molecule has lost both the N- and C- termini, and truncation of tau protein in some of these NFTs has reached the Glu$^{391}$ position recognized by the MN423 antibody (arrows). In other NFTs, conformationally altered tau protein is expressed, but the Glu$^{391}$ truncation is not yet reached (asterisks). Images taken from the hippocampus.
Fig. 7. Alz-50 conformation is not associated with truncation of tau at Glu\(^{391}\). As truncation of tau at Glu\(^{391}\) was progressively occurring during the maturation of the NFTs, the conformational change recognized by Alz-50 was becoming lost. NFTs recognized by MN423 (to Glu\(^{391}\)-truncated tau) (blue channel) were never detected with the Alz-50 antibody (green channel), which mostly detected dystrophic neurites. The picture corresponds to NFTs (asterisks) and neuritic components surrounding a \(\beta\)-amyloid plaque (see the red core of the \(\beta\)-amyloid in the merge channel) taken from the hippocampus.
Fig. 8. Chimeric NFTs are composed of truncated and phosphorylated tau molecules. Some NFTs at advanced stages of AD show a chimeric profile (arrows) composed of truncated tau protein at Glu391 and more intact molecules phosphorylated at Ser396,404. In another example, a well-defined extracellular NFT is composed of Glu391 truncated (asterisk) that is innervated by dystrophic neurites conformed by phosphorylated tau (Ser396,404) (green channel) and the apical neurite filled with tau molecules displaying the Tau-66 conformational change (arrowhead in merge channel). Images taken from the hippocampus.

When we look at the entorhinal cortex at advanced stages of AD, the common scenario is the presence of extracellular NFTs formed inside multipolar neurons that are morphologically distinct from NFTs appearing in pyramidal neurons of the hippocampus. NFTs in the entorhinal cortex preserve the characteristic columnar arrangements shown by the islands of multipolar cells (Fig. 9). It is clearly seen how these NFTs strongly colocalize with TR, which indicates a well-synchronized processing mostly containing Glu391-truncated tau (Fig. 9d-f). Interestingly, those NFTs mainly composed of Glu391-truncated tau showed a distinct pattern around the hippocampal area (Fig. 9a-c), in which part of the whole population had only the Glu391 truncation of tau and the other is only recognized by TR, which suggests the presence of more intact tau protein in these structures. These data clearly show the unsynchronized, by time, processing of NFTs in the hippocampus and the late time processing of NFTs in the ERC-II area, emphasizing that this is the more vulnerable area for the onset of the AD neurofibrillary pathology. Regarding isocortical areas, which are the later affected regions in AD progression, most of the changes in tau protein that are seen in the NFTs, mostly correspond to increased phosphorylation (Thangavel et al., 2008). However along the progression of the disease, truncation of tau protein at Asp421 also has
been described in some of NFTs proliferating in the temporal isocortex (Guillozet-Bongaarts et al., 2006). Moreover, at very advanced stages of the disease, some of these NFTs in the temporal isocortex also display tau molecules truncated at the Glu\textsuperscript{391} (Garcia-Sierra et al., 2001).

Fig. 9. Columnar arrangements of NFTs along the entorhinal cortex of AD patients. In the hippocampus of AD patients the neurofibrillary pathology is unsynchronized and selected populations of NFTs (a-c) have advanced truncation of tau protein at Glu\textsuperscript{391} (yellow NFTs in the merge channel). In contrast, in the layer II of the entorhinal cortex NFTs distributed along the columnar arrangements of neurons (d-f) are synchronically proteolyzed and have a uniform labeling with the MN423 antibody (note that most of the NFTs have a yellow pseudocolor in the merge channel).

8. Conclusion

We summarize that the chronological formation and maturation of NFTs in AD is made by a sequence of well-ordered events involving conformational changes in the tau molecule that may be driven by specific phosphorylations and sequential truncation of its C-terminus (Fig. 10). Early truncation at Asp\textsuperscript{421} is a pivotal modification that may produce more alterations on the tau structure, facilitating the exposure of different domains that can be lately proteolyzed by other non-apoptotic enzymes. \textit{In situ}, we demonstrated a close association between Asp\textsuperscript{421}-truncated tau and several apoptotic markers. For diagnostic purposes, NFTs now can be classified according to their chimeric character that better predict the neuropathological evolution of AD. We currently are trying to reproduce this scenario by analyzing the polymerization properties of chimeric tau filaments assembled \textit{in vitro}. Moreover we aim to evaluate the toxic effects of double expressed truncated and nontruncated tau variants in cultured neurons to validate the pathologic interaction of these proteins observed \textit{in situ}. 
Fig. 10. Chronological formation and maturation of NFTs in AD. Formation and maturation of NFTs is based on conformational changes of tau protein (Alz-50) developed early by phosphorylation (p-p) of specific domains (A-C). These changes are modified at intermediate stages of the disease by early cleavage of tau protein at the Asp\textsuperscript{421} residue (D), leading to a new conformation (Tau-66) that is associated with further truncation of the molecule at the Glu\textsuperscript{391} (E-F). Advanced stages are characterized by the presence of the minimum PHF-core composed only of Glu\textsuperscript{391}-truncated tau protein (F). Tau protein in neuropil threads also undergoes a similar processing, however it is delayed in time and only reaches early conformational changes (Alz-50) and truncation at the Asp\textsuperscript{421} (A-D).

9. Acknowledgment

The authors thank Dr. Virginia Lee for the use of Tau-46.1 and PHF1 antibodies, Dr. Peter Davis for the use of Alz-50 and PHF1 antibodies, Michael Novak for the use of MN423 antibody. We also want to thank to Dr. Ellis Glazier who edited this English-language text. Financial support by CONACyT-México to FG-S grants 59651, 113772. RM was supported by CONACyT-México, grant 102278. Support from the Ministry of Health of the Czech Republic was for ZK and DR, project MZ0PCP2005.

HCC was supported by ICyT-México

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