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Commentary

Commentary on Ubiquitin is Associated with Early Truncation of Tau Protein at Aspartic Acid421 during the Maturation of Neurofibrillary Tangles in Alzheimer Disease

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Commentary

In Alzheimer Disease (AD), the sequence of changes that tau protein undergoes during the formation and maturation of neurofibrillary tangles (NFT) has long been investigated. Certainly, several modifications in tau structure lead this molecule to alter its normal microtubule-binding properties and the consequent selfaggregation into abnormal Paired Helical Filaments (PHFs) in the cytoplasmic space of specific neurons in the hippocampus and cerebral cortex. The major post-translational modification in the tau molecule that affects its capabilities to stabilize microtubules is abnormal phosphorylation [1-4], which may also increase its accumulation and aggregation into NFT [5,6]. By studying the dissected brain of AD cases, several groups have reported that early non-fibrillary aggregates of tau protein and considerable numbers of NFTs were recognized by antibodies at distinct phosphorylation sites. Multiple kinases appear to be involved in the phosphorylation of tau, including GSK3ß, cdk5, MAPK, and PKA [7-10]. Hyperphosphorylation of tau may direct this protein to adopt distinct conformational changes that, in turn, may also contribute to its abnormal aggregation. Despite that tau protein displays no complexity in secondary structure, some local and structural changes have been proposed as being adopted by this molecule when it is phosphorylated and early accumulated in the form of both fibrillary and non-fibrillary aggregates in the brain of patients with AD [11]. The most well-known conformational change adopted by tau protein during its aggregation in AD is that recognized by the conformational and specific N-terminus folding marker Alz-50 antibody [12]. This antibody recognized a discontinuous epitope spanned along the N-terminus and the second repeated domain of the tau molecule [12]. In the previous work of our group, we characterized this conformational change by monitoring the state of the tau molecule in double- and triple-labeling immunofluorescence experiments, and found that the majority of tau aggregates displaying this conformation contained an N- and C- terminus-intact molecule proliferating at early AD stages [13]. Proteolytic cleavage has been proposed as an alternative tau processing mechanism that may increase its loss of function and concomitantly the gain of abnormal aggregation properties in affected neurons in AD. Because abnormal proteolytic processing of proteins occurs as part of the aging process and in several neurodegenerative diseases [14-17], some participants belonging to the family of cysteine-aspartyl proteases, referred to as caspases, have been reported as active and increased in AD [18-21].

A relevant cleavage site has been found in the tau molecule, mainly occurring at the C-terminus residue aspartic acid-421 (Asp421), for which caspase-3 activity was found to be mainly responsible [22,23].

Abnormal properties for this truncated variant of tau indicated that this protein product polymerizes at a higher rate than that reported for the wild-type, full-length tau, and that it is an apoptotic generator when overexpressed in cultured neuronal and non-neuronal cells [24-27]. More recently, our group has further investigated the pathologic effects when full- length tau and its Asp421-truncated variant were overexpressed in neuroblastoma cells, and found that these proteins induced severe lobulations of the nuclear membrane, associated with alterations in the microtubule lattice [28]. Moreover, when expressed in C6 glial cells, these proteins also produced pathologic lobulations in the plasma membrane leading the cells to reduce their migration *in vitro*. These effects, which were independent of fibrillary aggregation of tau, if they truly occur in affected neurons in the brains of those undergoing AD, may alter neuronal functioning and contribute to the early symptoms of the disease.

In the brains of patients with AD, Asp421-truncated tau was visualized as a component of the neurofibrillary pathology by employing the Tau-C3 antibody, an immunological tool that specifically recognizes this cleavage site [14,23]. Asp421-truncated tau was mostly found in NFT, but also in neuritic plaques, neuropil threads, and amorphous non-fibrillary aggregates [29-31]. The load and accumulation of these NFTs significantly correlated with the clinical manifestation of dementia in patients with AD. Cleavage of tau at Asp421 depends completely on caspase activity; however, permanent activation of these proteases is unlikely to occur during the development of a long-term disease. We have probed that capase-3 may not only process tau in a monomeric state; for instance, this enzyme was able to cleave tau and generate the Asp421 residue, even in full-length tau oligomers and polymers already formed [31]. This result indicated that induction of this pathologic truncation in long-lasting structures, such as NFTs, may increase permanent toxicity for neurons in the brains of patients with AD.

By carrying out an early biochemical analysis of pronase-treated PHFs purified from the brains of patients with AD, a minimal structural-core predominantly comprised of tau protein cleaved at Glutamic acid-391 (Glu391) was found [32]. Although this truncation has been proven to have a clinicopathological meaning during the progression of the disease [29,30,33], to date there are no candidate proteases that produce this specific cleavage at the Glu391 site.

By analyzing the time course for the occurrence of tau truncations during the formation and maturation of NFTs, we proposed that this proteolytic process is orderly and that it developed from the extreme C-terminus toward the region of the repeated domains, which also correlated with the progression of the disease [30,34]. These

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truncations are associated with conformational changes in tau, and early and intermediate NFTs are characterized not only by displaying the N-terminus folding recognized by the Alz-50 antibody, but also by coexisting with the early truncation of the molecule at Asp421. A considerable number of chimeric NFTs, composed of full-length tau and the Asp421-truncated variant, predominated along early and intermediate disease stages [30].

From intermediate-to-advanced stages of NFTs maturation, the tau protein adopts a new conformation in which a new folding takes the proline-rich region to make contact with the third repeated domain [35]. In NFTs with advanced stage of maturation, this conformation is conserved; however, the C-terminus of tau is further cleaved at the Glu391 position, which is conserved onward to the latest stages of NFTs maturation [30,34]. As a terminal marker of neurofibrillary-pathology neurodegeneration, the occurrence of Glu391-truncation of tau highly correlated with the advanced manifestation of dementia in cases of AD [30,33].

Proteolytic processing of tau protein has been studied for some time *in vitro*, and the reported susceptibility for the action of different proteases mostly includes calpain [36], cathepsins [37], and caspases [22,23]. Among these, the sole tau-truncated product accurately observed in association with AD neurofibrillary pathology is that obtained by caspase activity (Asp421-truncated tau).

In the neurons of AD brains, all of these modifications in tau leading to misfolding may be perceived as attractive candidates for a multiple degradative process converging in the same substrate. However, few reports have analyzed and integrated the timing of distinct patterns of tau processing that contribute to its aggregation in AD, and this issue remains open to investigation.

The role of the Ubiquitin-Proteasome System (UPS) in AD and other neurodegenerative disorders has been long studied and reviewed [38-40]. Several components of this degradative pathway have been detected in association with pathological markers of the disease [38]. In particular, the first connection between UPS and tau composing the neurofibrillary pathology arose when ubiquitin was found in association with PHFs [41,42] and NFTs [43,44] in the brain of subjects with AD.

However, prior to our investigation in 2012 [45], the precise stage of tau ubiquitination during the NTF formation and maturation, according to the evolution of phosphorylation and proteolytic processing of tau in AD, had not been entirely demonstrated.

In this work, we aimed to determine the possible contribution of ubiquitin during the formation of NFTs and the evolution of AD. We focused on the relationship between ubiquitination and C-terminus truncation of tau.

Our interpretations were based on the combination of fluorescent markers with affinity to β -sheet conformation fibrillary structures, such as Thiazine red and Thioflavin-S [16] and the use of immunological markers that recognize different modifications of tau protein, including ubiquitination. Thus, we were able to predict the state of maturation of the NFTS.

As previously reported, we found that ubiquitin was a marker of NFTs, occurring in 30% of these structures. This fraction was calculated in relation to the total number of Thioflavin-S-positive NFTs, a more accurate evaluation compared with previous studies using individual antibodies to tau to determine the total load of NFTs. Due to proteolytic processing, it is currently understood that labeling

with a single antibody to tau protein may underestimate the total amount of NFTs.

From the total number of NFTs positive for ubiquitin antibodies, we found that distinct populations of these structures were labeled or not by several antibodies to tau protein that mapped the entire molecule. In this regard, NFTs conformed by tau protein undergoing abnormal phosphorylation at Ser396, 404 residues were highly associated with ubiquitin targeting. This result was not a novelty; however, the finding that nearly at the same degree, ubiquitin-targeting of tau protein was also associated with tau truncated at Asp 421, represents a relevant indication that this protein can be a dual substrate for degradation through apoptotic and proteosomal pathways. Yet to be understood is the order by which apoptotic-to-proteosomal pathways, or vice versa, guides the processing of tau protein to promote its aggregation. Controversy remains regarding the thought that ubiquitinated tau in NFTs instead represents a way by which this protein eludes its ubiquitin-guided traffic to the proteasome. We also discarded that the proteolytic processing of tau generating truncation at Glu391 in NFTs was associated with proteosomal degradation; thus, these structures exhibited few signs of ubiquitin-targeting. We concluded that the ubiquitination of tau could be associated to a greater extent with early and intermediate maturation of NFTs, which continue to display conformational changes and phosphorylation. This modification may either precede or follow the proteolytic processing of tau by caspases, which generates Asp421 truncation. These modifications may be crucial for inducing changes in the structure of tau that, in an attempt for these to be eliminated, these modifications rather increase tau insolubility and toxicity. Because proteolysis of tau appears to contribute to its aggregation and toxicity in AD, more studies are needed to better elucidate the role of the UPS in this disease.

Currently, we continue to analyze the role of ubiquitination of tau in AD, but we now focus on its accumulation in the neuritic compartment. In this regard, we have found that the burden of ubiquitin-positive structures increases according to the load of neuritic pathology. The occurrence of neuritic structures followed the emergence of NFTs, and ubiquitin-targeting of these structures appears to follow the same pattern (Figure 1). We found that not all ubiquitin-targeted neuritic pathology is accumulated in the form of positive β -sheet conformations, which may indicate that ubiquitin labeling in these structures may be an early marker of tau processing. Whether Asp421 or Glu391 truncations are associated with different affinity to ubiquitinated neurites, is a non-addressed issue that at present remains elusive.

More evidences of ubiquitin-targeting of tau aggregates, proteosomal degradation of tau, and altered proteosomal activity by tau accumulation in different cells models and transgenic mice have arisen since our study by 2012 [45]. Exogenous proteasome complexes were introduced into stable tau-expressing Hela and other nonneuronal cells, and these structures were capable of degrading tau and of reducing the levels of tau aggregates [46]. In mouse neuroblastoma N2a cells, inhibition of Ubiquitin C-terminal Hydrolase L1 (UCH-L1), which is critical for protein degradation, reduced the microtubulebinding ability and increased phosphorylation and the accumulation of ubiquitinated tau aggregates [47]. However, to date neither evidences of alterations in UCH-L1 levels nor an association with tau pathologies in the brain of AD cases has been demonstrated. On the other hand, by investigating the effects of tau accumulation on proteasome function in a mouse model of tauopathy, reduced peptidase activity of brain 26s

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proteasome and higher levels of ubiquitinated proteins were observed in these animals [48].



Figure 1: Neuritic pathology composed of ubiquitin follows accumulation of neurofibrillary tangles (NFTs) in Alzheimer disease (AD). A-B, C-D, and E-F, represent pairwise combination of double labeling immunofluorescence with anti-phosphorylated-tau (PHF1) and anti-ubiquitin antibodies in the CA1 sector of the hippocampus of three AD cases. In a representative mild case (A-B), neuritic pathology is scarce (arrows) and most of the immunolabeling with both antibodies corresponds to NFTs (asterisks). In a moderate case, while short neuritic processes appear positive for ubiquitin (arrows in D), phosphorylated tau structures (NFTs) seem to reduce their number (asterisks in C). In a representative severe case (E-F), the number of NFTs positive for ubiquitin and tau antibodies is reduced (asterisks); however, high amount of large neuritic processes composed of ubiquitin proliferate in the same region (arrows in F). At this stage, note that NFTs display an extracellular profile (double asterisks in E). Scale bar. 40 µM for all panels. Images were collected by epifluorescence by using a digital camera AxioCam MRc5 coupled to a Zeiss Axio Imager.z1 upright microscope (Carl Zeiss Imaging Solution, GmgH, Germany).

When chemical activation of the proteosome in these animals was induced, lower levels of aggregated tau and improvements in cognitive performance were observed. Despite this body of evidence, more studies in the human brain of AD and cases of other tauopathies need to be conducted to corroborate these findings. It would be interesting to evaluate other modifications of tau involving ubiquitin-like modifiers that may also link altered tau protein with proteosomal degradation. One example of these modifiers is the cytokine-inducible modifier HLA-F Adjacent Transcript 10 (FAT10), which consists of two ubiquitin-like domains. By contrasting, the repeated ubiquitin association that warrants the proteosomal processing of many proteins, a single, conjugated FAT molecule is sufficient to bind to the proteasome in order to mediate the degradation of the bounded substrate [49]. In this regard, it was recently demonstrated that Small-Ubiquitin-related MOdifier (SUMO)ylation of tau at K340 residue increases tau hyperphosphorylation and reduces its ubiquitin-targeting and proteosomal degradation in HEK293 cells expressing tau protein. In this study, the coexistence of SUMOylation and phosphorylated tau was corroborated in the neurofibrillary pathology of AD cases [50].

In summary, ubiquitin-targeting of tau is a relevant modification that may contribute to the degradation of this molecule when it is accumulated in the brain of AD cases; however, by mechanisms yet to be clarified, aggregation of modified tau may impair proteosomal activity, thus leading to an accumulation of multiple, overubiquitinated substrates. Truncation of tau conducted by caspases activity appears to be coincidental with ubiquitin-targeting; therefore, a dual processing of this protein may lead to increasing its aggregation and toxic effects in the brain of patients with AD. Further investigation may provide additional insights concerning the mechanisms underlying ubiquitin-targeting and proteosomal processing of tau to develop alternative therapeutic strategies for treating or preventing AD.

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