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Neurofibrillary degeneration of the Alzheimer-type: an alternate pathway to neuronal apoptosis?

Malika Hamdane[a], Patrice Delobel[a], Anne-Véronique Sambo[a], Caroline Smet[b], Séverine Bégard[a], Anne Violleau[a], Isabelle Landrieu[b], André Delacourte[a], Guy Lippens[b], Stéphane Flament[c], Luc Buée[a,*]

[a]*INSERM U422, Institut de Médecine Prédictive et Recherche Thérapeutique, Place de Verdun, F-59045 Lille Cedex, France*
[b]*UMR CNRS 8525, Institut de Biologie de Lille, Institut Pasteur de Lille, F-59021 Lille, France*
[c]*UPRES EA 3442, Université de Nancy I, F-54505 Vandœuvre les Nancy, France*

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Abstract

Neuronal death is a process which may be either physiological or pathological. Apoptosis and necrosis are two of these processes which are particularly studied. However, in neurodegenerative disorders, some neurons escape to these types of death and ''agonize'' in a process referred to as neurofibrillary degeneration. Neurofibrillary degeneration is characterized by the intraneuronal aggregation of abnormally phosphorylated microtubule-associated Tau proteins. A number of studies have reported a reactivation of the cell cycle in the neurofibrillary degeneration process. This reactivation of the cell cycle is reminiscent of the initiation of apoptosis in post-mitotic cells where G1/S markers including cyclin D1 and cdk4/6, are commonly found. However, in neurons exhibiting neurofibrillary degeneration, both G1/S and G2/M markers are found suggesting that they do not follow the classical apoptosis and an aberrant cell cycle occurs. This aberrant response leading to neurofibrillary degeneration may be triggered by the sequential combination of three partners: the complex Cdk5/p25 induces both apoptosis and the ''abnormal mitotic Tau phosphorylation''. These mitotic epitopes may allow for the nuclear depletion of Pin1. This latter may be responsible for escaping classical apoptosis in a subset of neurons. Since neurofibrillary degeneration is likely to be a third way to die, molecular mechanisms leading to changes in Tau phosphorylation including activation of kinases such as cdk5 or other regulators such as Pin1 could be important drug targets as they are possibly involved in early stages of neurodegeneration.

Keywords: Neuronal death; Cell cycle; Cdk5; Pin1; Tau phosphorylation; Neurofibrillary tangles (NFT)

1. Alzheimer's disease

AD is a progressive neurodegenerative disorder that leads to dementia, and affects approximately 10% of the population older than 65 years of age. Memory loss is the first sign of cognitive impairment, followed by aphasia, agnosia, apraxia and behavioral disturbances. These symptoms are explained by a severe neuronal loss and the presence of two brain lesions: senile plaques and NFT. Senile plaques result from the extracellular accumulation of a peptide referred to as Aβ into amyloid deposits. Aβ derives from a precursor, the APP. In cases of familial AD, mutations have been found on APP gene, suggesting that it plays a central role in the etiopathogenesis. Senile plaques are diffusely and variably distributed throughout the cerebral cortex and in subcortical structures. NFT correspond to the aggregation of abnormally phosphorylated Tau proteins into filaments referred to as PHFs, within certain vulnerable neuronal populations. At the microscopic level, NFT are preferentially observed in the large pyramidal cells of the hippocampus and the entorhinal cortex, and the supragranular (II–III) and infragranular (V–VI) layers of the association cortical areas, while primary sensory and motor cortices are relatively spared. Many cortical and subcortical areas, such as nucleus basalis of Meynert, amygdala, locus coeruleus

* Corresponding author. Tel.: +33-320-622074; fax: +33-320-622079.
E-mail address: buee@lille.inserm.fr (L. Buée).
Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Cdk, cyclin-dependent kinase; NFT, neurofibrillary tangles; PHF, paired helical filaments.

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and dorsal raphe, are also affected by NFT formation. The demonstration of both senile plaques and NFT within specific regions of the cerebral cortex is necessary to establish the diagnosis of definite AD. However, NFT lesions with a lower density are also present in entorhinal cortex and hippocampus of elderly normal brains [1].

There is almost a consensus that the ''amyloid cascade'' hypothesis, with Aβ neurotoxicity, is the unique and central etiological factor of AD. Most of the therapeutic research is devoted to this dogma [2]. However and unexpectedly, recent molecular findings from human brain and models suggest that ''Tauopathy'' is the motor of AD, upstream of the amyloid cascade but fuelled by it [3].

Recently, it has been shown that more than 20 neurological disorders with dementia were due to a direct or indirect Tau defect [4]. Furthermore, the close relationship between the extension of Tau pathology in polymodal association areas and cognitive deficits demonstrate that AD belongs also to Tauopathies [3,5,6]. Moreover, a detailed spatiotemporal quantification of both lesions shows that Tau pathology can be observed without Aβ deposits in the entorhinal and hippocampal area of non-demented patients. Conversely, each case with Aβ deposits had at least mild Tau pathology in these brain areas. However, Tau pathology is progressing in polymodal association cortical areas, a stage associated with severe cognitive impairment, only in the presence of cortical Aβ42 deposits [5]. These findings are likely to reflect the basic mechanisms of all forms of AD, familial and non-familial: Alzheimer's disease is the result of a potentiation of Tau pathology by APP dysfunction. At last, recent papers demonstrate that APP and its relevant secretases are transported via the anterograde transport along microtubules. Tau proteins being the regulator of microtubule stability, all defects on Tau should alter the axonal transport of vital factors, including APP [7]. Here again, Tau stands upstream of APP dysfunctions.

Therefore, the central question to fully understand AD is related to the molecular mechanisms that lead to Tau pathology and cell death.

2. Apoptosis and cell death in Alzheimer's disease

The notion of apoptosis in the neuropathology of AD is still controversial. Aβ neurotoxicity can be explained by apoptotic mechanisms. Furthermore, amyloid-related genes including APP and presenilins can also modulate apoptosis susceptibility in cell models (for reviews [2,8]). Since apoptotic process is completed within 16–24 hr, it would not be surprising to underscore the apoptosis in postmortem specimens. However, a number of groups have detected evidence of apoptosis including DNA fragmentation and apoptotic markers [9–11]. At least, two hypotheses could be proposed. (1) The high number of apoptotic neurons observed in AD is related to postmortem and oxidation artifacts. In fact, DNA fragmentation can also be caused by oxidative damage or postmortem autolysis [12,13]. (2) Apoptosis is not completed and only early apoptotic markers are observed in AD brain.

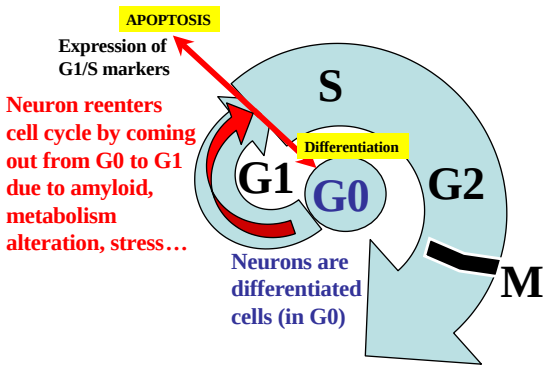

Fig. 1. Reactivation of the cell cycle in neurons leading to apoptosis. Neurons are coming out from G0 to G1. At the G1/S checkpoint, neurons are either coming back in G0 by differentiation or entering apoptotic process. At that time, they express a few markers including cyclin D1 and E2F-responsive gene products. G0: quiescent state; G1: the first gap phase of the cell cycle; S: DNA replication phase; G2: the second gap phase of the cell cycle; M: mitosis.

This second hypothesis may be of particular interest. In fact, several recent findings demonstrate increased expression of cell cycle-related proteins in the degenerating neurons found in AD. This apparent attempt to re-enter cell cycle (coming out from G0 to G1 with cyclin D1 expression) is one of the mechanisms that leads to apoptosis [14–16] (Fig. 1). In AD, this apoptotic process may be aborted and may explain G2/M cell cycle markers in neurofibrillary degeneration.

3. Aberrant cell cycle and neurodegeneration in Alzheimer's disease

First, neurons of the adult brain are in G0: they do not divide and are differentiated. Therefore, the re-expression of cell cycle proteins has to be considered as pathological. It should be noted that re-expression of G1/S markers is well correlated with the appearance of apoptosis in many systems including neurons (Fig. 1). It is characterized by the formation of the cdk4/6–cyclin D1 complex, phosphorylation of Rb, dissociation of the Rb–E2F complex and activation of genes leading to apoptosis [17]. For instance, in ischemia, the surrounding region referred to as the penumbra displays numerous apoptotic features in both neurons and glial cells. In this process, an increase in cyclin D1 expression if often used as an early apoptotic marker [18]. However, in AD, both G1/S and G2/M markers are found in neurons undergoing neurofibrillary degeneration [19–24]. Differentiated cells coming out of G0 into G1 are usually stopped at the G1/S checkpoint and then undergo either re-differentiation or apoptosis [14–18]. Thus, the detection of G2/M markers indicates that anarchical cell

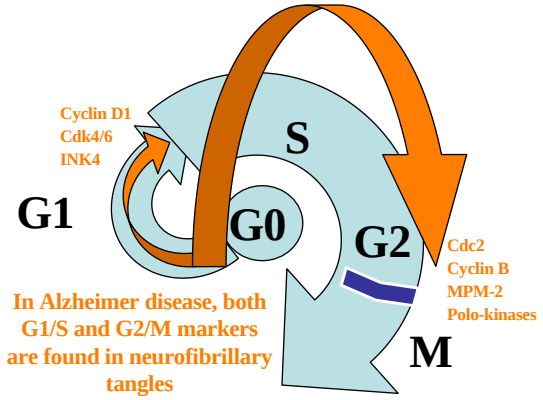

Fig. 2. Reactivation of the cell cycle in neurons in Alzheimer's disease. Neurons are coming out from G0 to G1. Both G1/S and G2/M checkpoint markers are found in neurofibrillary tangles suggesting that neurons bypass the classical neuronal apoptosis.

cycle occurs in Alzheimer-type neurodegeneration [20,21]. In this regard, reexpression or deregulation of the genes involved in G1/S and G2/M transition controls may be some of the mechanisms that facilitate neurofibrillary degeneration in AD and thus, allow escaping apoptosis (Fig. 2).

What are Tau proteins? Which processes lead to Tau pathology? Do Tau proteins lead to cell death and how? Is reentry of the neuron in aberrant cell cycle one of the mechanisms that activates anarchic transduction pathways leading to the abnormal phosphorylation of Tau proteins and their aggregation in Tauopathies?

4. Tau proteins and phosphorylation

In the adult human brain, Tau proteins are found essentially in neurons. Six Tau isoforms are produced by alternative mRNA splicing. Three isoforms contain in their carboxy terminal part 3 repeats of a microtubule-binding domain whereas the three other Tau isoforms contain four of these repeats. Tau proteins bind microtubules through the microtubule-binding domains. However, microtubule assembly depends partially upon the degree of phosphorylation since hyperphosphorylated Tau proteins are less effective than hypophosphorylated Tau on microtubule polymerisation (Fig. 3; for review [4]).

Among the 80 Ser/Thr residues on Tau, at least 30 phosphorylation sites have been described, most of which occur on Ser–Pro and Thr–Pro motives. In fact, phosphorylation of Ser262, located in the first microtubule-binding domain, dramatically reduces the affinity of Tau for microtubules *in vitro*. Nevertheless, this site alone is insufficient to abolish Tau binding to microtubules. Thus, phosphorylation outside the microtubule-binding domains may also strongly influence tubulin assembly by modifying the affinity between Tau and microtubules (Fig. 3).

Tau proteins are found in all cell compartments, but in different phosphorylation states. Within the same compartment, variability in the degree of phosphorylation is

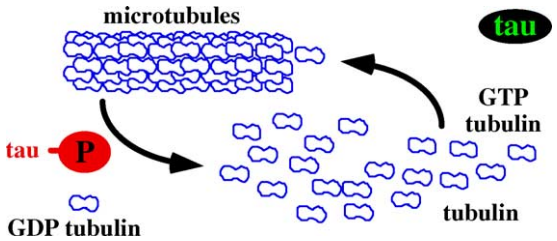

Fig. 3. Tau phosphorylation and microtubule polymerization. Polymerization of tubulin into microtubules is regulated by the state of phosphorylation of Tau proteins. Hypophosphorylated Tau (green) induce tubulin polymerization and microtubule stabilization whereas hyperphosphorylated Tau (red) lead to the depolymerization of microtubules into tubulin.

observed during development. Phosphorylation seems to affect simultaneously several sites. However, this has to be clarified, using a panel of monoclonal antibodies against different phosphorylation sites and following their fates during development. Furthermore, the state of phosphorylation is strongly modified during development, due to the expression of several specific adult isoforms, and because the ratio between kinases and phosphatases is modified. Phosphorylation, in combination with the type of isoform, can modulate the properties of Tau proteins. In turn, Tau proteins provide the microtubule with its own identity and physical characters (rigidity, length, stability, interactive capacity with other organelles). Therefore, by regulating microtubule assembly, Tau proteins have a role in modulating the functional organization of the neuron, and particularly in axonal morphology, growth, and polarity (for review [4]).

Altogether, these observations indicate that Tau phosphorylation is a key posttranslational modification for Tau biological functions and structure (Fig. 4). In AD, Tau is abnormally phosphorylated at several sites thought to be disease-specific.

5. Tau proteins, abnormal phosphorylation and neurofibrillary degeneration

Aggregation of Tau proteins into filaments is a common feature encountered in AD and other neurodegenerative disorders referred to as Tauopathies. Abnormal phosphorylation is the major modification of these aggregated proteins [4]. Their biochemical characterization by immunoblotting reveals the presence of a triplet of proteins (Tau60, 64 and 69) also referred to as A68, or PHF-Tau (Fig. 4). However, a 72–74 kDa component is also present in only very low amounts and corresponds to the longest Tau isoform. Using PHF-Tau preparations, Goedert *et al.* showed that dephosphorylated PHF-Tau proteins have a similar electrophoretic mobility than the six recombinant Tau isoforms [25]. However, it is likely that both the size of Tau isoforms and phosphorylation are responsible for variations in their electrophoretic mobility. For instance, phosphorylation of the longest Tau isoform may lead to the formation of

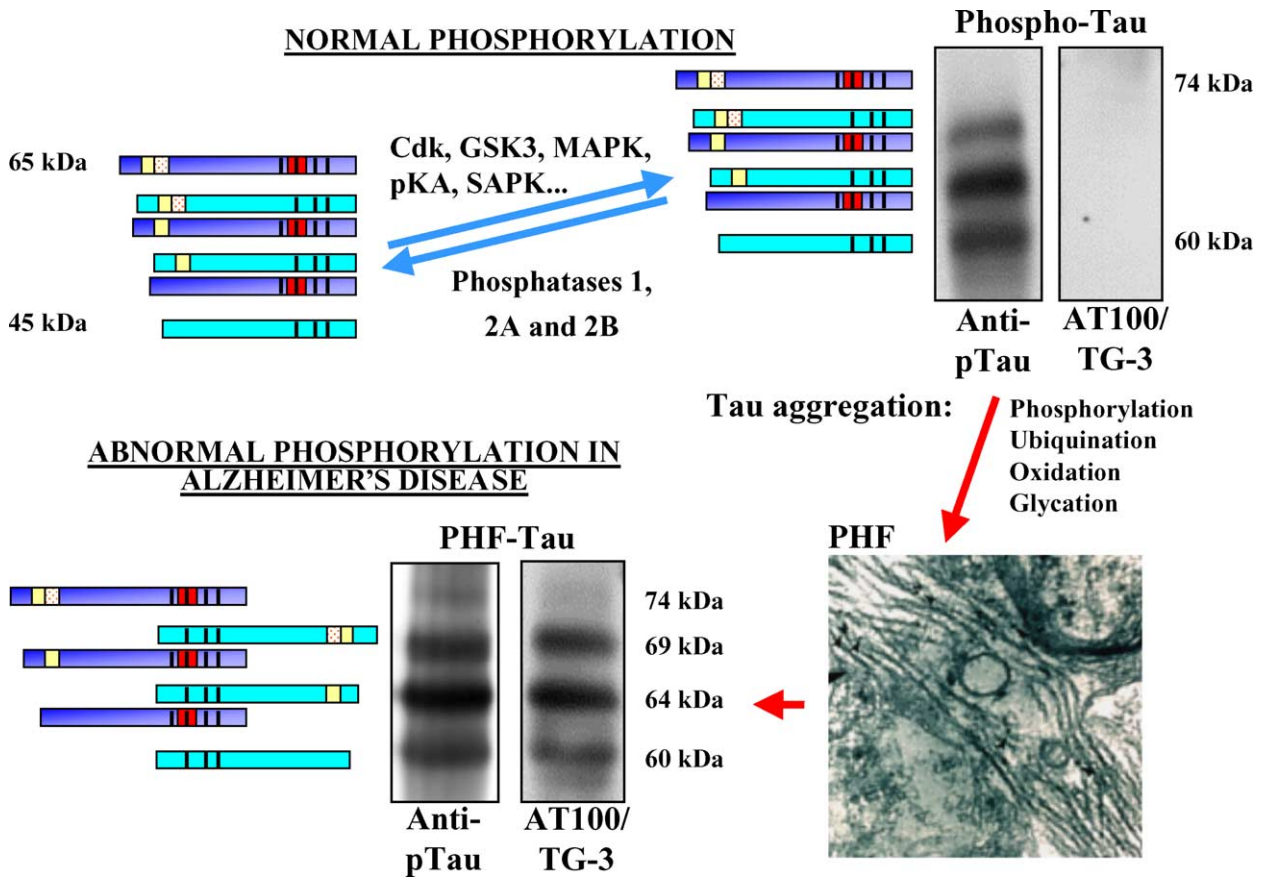

Fig. 4. Schematic representation of the modifications leading to Tau proteins aggregation in Alzheimer's disease. In human brain, there is a balance phosphorylation–dephosphorylation of Tau proteins allowing microtubule dynamics. Tau proteins are detected by anti-phospho-Tau antibodies but do not display Alzheimer-type epitopes including those of AT100 and TG-3. In the brains of patients exhibiting Alzheimer's disease, Tau proteins aggregate into paired helical filaments (PHF). Aggregated Tau proteins are found abnormally phosphorylated within these filaments and exhibit Alzheimer-type epitopes (AT100/TG-3).

Tau variants with molecular weights ranging from 68 to 74 kDa according to their degree of phosphorylation (Fig. 4).

In AD, three phosphorylated Tau epitopes are clearly identified on residues Thr212/Ser214, Thr231 and Ser422 and recognized by the following antibodies AT100, TG-3 and 988/AP422, respectively. This abnormal phosphorylation associated with AD may be related to either an increase in kinase activity or a decrease in phosphatase activity (for reviews, see [4,26]). Among the numerous kinases that have been implicated, glycogen synthase kinase 3β may be involved in AD pathology [26]. However, it is still a controversial candidate [27]. A combination of glycogen synthase kinase 3β and other kinases activity may also allow for the genesis of disease-specific epitopes [28]. Stress-activated protein kinases are also of interest since all of them have been shown to phosphorylate Tau proteins [29]. Conversely, Tau hyperphosphorylation may be related to a decrease in phosphatase activity. Phosphatase inhibition in cell models allows the formation of specific AD-type epitopes such phosphorylation of Thr212/Ser214 and Ser422 [30,31]. Finally, mitotic protein kinases may also play a major role in Tau phosphorylation since many mitosis-specific epitopes such as MPM-2 are found in NFT [24,32]. Furthermore, phosphorylation-dependent anti-Tau antibodies such as TG-3 that recognize conformation-dependent epitopes can visualize phosphorylated Tau aggregated into filaments. TG-3 epitope (phosphorylated Thr231) is expressed in mitotic cells but not in quiescent cells [32] suggesting that mitotic phosphoepitopes may lead to conformational changes [33] and aggregation into filaments. In this respect, one can ask if many of the key proteins involved in mitosis and found within neurofibrillary tangles including Cdc25, cyclin-dependent kinases (Cdks)/cyclins, the peptidyl prolyl *cis/trans* isomerase Pin1 and polo-like kinase [24,34,35] are related to this abnormal Tau phosphorylation.

6. Any link between abnormal Tau phosphorylation and mitosis?

A direct link between M-phase and the appearance of abnormal Tau phosphorylation was recently documented using the *Xenopus laevis* oocyte maturation [36]. Tau protein was microinjected into prophase I oocytes that were then stimulated by progesterone that activates cyclin-dependent kinase pathways. Oocyte maturation is characterized by two steps: meiosis I and II. Surprisingly, abnormal Tau

phosphorylation was observed in meiosis II, indicating that molecular events of the second division of meiosis, which is considered as mitosis, are sufficient to produce the Alzheimer-type phosphorylation.

These abnormally phosphorylated epitopes include Thr212/Ser214, Thr231 and Ser422. Regarding Thr231, it is clear that $p34^{cdc2}$ (Cdc2) is responsible of the immunoreactivity recognized by TG-3 antibody since experiments done in meiosis I stage allowed discrimination between this kinase and classical MAPK pathway. Moreover, Cdc2 is still activated in meiosis II and may be responsible of TG-3 epitope genesis. However, other phenomena may also be implicated. For instance, phospho-Thr231 is a binding site of Pin1, an enzyme involved in G2/M transition [34,37].

The results obtained with oocyte maturation were also confirmed using stable transfected neuroblastoma cells. In these cells, abnormal Tau phosphorylation was detected in mitotic cells. Finally, treatments by nocodazole or taxotere ascertain these results. Indeed, these drugs provide G2/M arrest by polymerisation or depolymerization of microtubules. In both cases, G2/M arrested cells exhibited abnormal Tau phosphorylation. Finally, it should also be noted that no abnormal phosphorylation of Tau proteins was observed when the cells undergo apoptosis and especially after taxotere or nocodazole treatment.

Thus, abnormal Tau phosphorylation is linked to mitotic mechanisms. Abnormally Tau phosphorylation may be the first step in Tau aggregation.

7. Cdk5, a link to explain abnormal Tau phosphorylation without mitotic mechanisms

Among abnormal Tau phospho-epitopes, TG-3 that is exclusively found in mitotic cells but not in quiescent ones [32,36] seems to be of great interest. Indeed, TG-3 immunoreactivity is specifically evidenced in degenerating neurons of AD and seems to be associated with early stages of disease [24,38]. The Cdc2 kinase generates TG-3 epitope in mitotic cells [23,36] but the kinase(s) responsible for this phosphorylation in neuronal cells remain(s) unknown. A potential candidate is Cdk5, the neuronal Cdc2-like kinase. Several data support the idea of Cdk5 involvement in AD [39,40]. Cdk5 mediates extracellular amyloid-induced neuronal death, and phosphorylates the amyloid precursor protein and Tau [41,42]. The kinase is activated by a protein, p35, and even more potently by p25, a proteolytic byproduct of p35 [42]. Apoptotic inducers including Aβ, glutamate excitotoxicity and oxidative stress lead to the calpain activation and the formation of the p25 byproduct [39,43]. The complex cdk5/p25 induces apoptosis in most cells [39,42,43]. Finally, Tau is phosphorylated by Cdk5/p25. One of the *in vitro* phosphorylation site of the complex Cdk5/p25 is Thr231 [44] (Fig. 5). This phosphorylated epitope (TG-3 epitope) is also the only binding

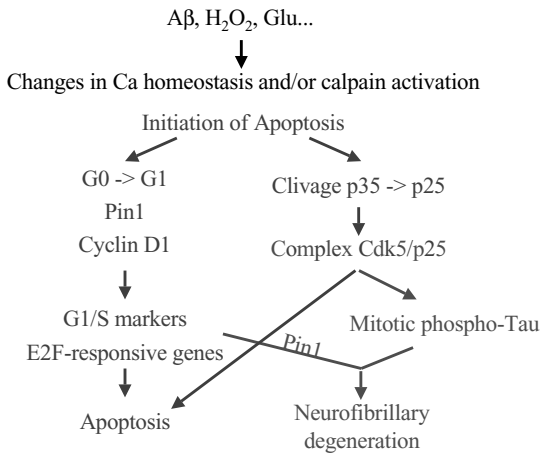

Fig. 5. Apoptotic inducers including Aβ, hydrogen peroxide (H_2O_2) and Glu allow for changes in calcium (Ca) homeostasis and/or calpain activation. In a classical apoptosis (blue pathway), neurons are coming out of G0 to G1 phase with the appearance of specific markers such as cyclin D1 and Pin1. It is followed by the expression of G1/S markers and E2F-responsive genes. Induction of apoptosis could also lead to calpain activation and the formation of the p35 proteolytic product, p25 and a deregulated Cdk5 activity. The complex Cdk5/p25 allows Tau phosphorylation leading to "mitotic phospho-Tau". Pin1 binds "mitotic phospho-Tau epitopes" allowing its nuclear depletion and thus does not bind to its classical nuclear substrates (*jun*, p53, etc.) (red pathway). This latter may be responsible for escaping the classical apoptosis in a subset of neurons and allow neurofibrillary degeneration through an alternate pathway to apoptosis.

site of Pin1 [34,37,45]. Altogether, these data suggest that cdk5/p25 may be a key actor in cell death in Alzheimer's disease. This complex is implicated at the interface apoptosis-neurofibrillary degeneration.

8. Abnormal Tau phosphorylation and changes in Tau conformation: role of Pin1

The phospho-epitope mapping by immunochemistry heavily relies on a number of specific antibodies that recognize the unphosphorylated Tau protein, certain of its phospho-epitopes or Tau aggregated into PHF. The latter are also categorized as conformation-dependent antibodies including TG-3 and AT100, although the conformational changes that they detect are still not elucidated. Because many of the phosphorylation sites are proline directed, one possible hypothesis is that the proline conformation would change upon phosphorylation and/or aggregation. The proline residue is indeed unique in the sense that the energetic barrier between its *trans* and *cis* conformation is significantly lower than for all other amino acids, leading to a higher population of the *cis* form. In small peptides, this population is typically of the order of a few percents, but in the context of a folded protein, proline residues can be found that are totally in the *cis* conformation. Indirect evidence for a role of the proline conformation comes from the recent finding that Pin1, a prolyl *cis*/*trans* isomerase essential for the cell cycle, interacts with Tau

[34,37,45]. Pin1 is a recently characterized human peptidyl-prolyl *cis*/*trans* isomerase that modulates the assembly, folding, activity and transport of cellular proteins. It is a cell cycle regulator interacting with a range of proteins that are phosphorylated prior to cell division [20]. Pin1 recognizes a specific motif of a phosphorylated Ser or Thr residue preceding a Pro. Pin1 is made of two regions: WW and catalytic domains. The WW domain is responsible for the Pin1 binding to phospho-Ser and phospho-Thr residues. It binds to the phosphorylated substrate through a pair of aromatic residues, Tyr23 and Trp34, which acts as a clamp for the proline moiety, and a β-hairpin loop Ser16 and Arg17, which interacts directly with the phosphorylated residue. Pin1 only binds to its substrate when the Ser16 of its WW domain is not phosphorylated [46]. The catalytic domain is responsible for the isomerase activity. It displays a cluster of basic amino-acids that could be implicated in the specificity of phosphorylated substrates. Both domains are necessary to Pin1 activity. Very recently, Pin1 was shown to be involved in AD [34,47]. Pin1 binds to phospho-Thr231 on Tau proteins [34,37,45]. This interaction may interfere indirectly with the phosphorylation of Tau, as it enhances the capacity of PP2A to dephosphorylate the protein. Pin1 is also found within degenerating neurons where it may also be associated to the large amounts of abnormally phosphorylated Tau proteins aggregated into filaments. Finally, Pin1 allows for the expression of cyclin D1 through c-*jun* activation and stabilization of β-catenin [48–50]. In a neuron, this may facilitate the coming out of G0 to G1 phase and thus, neuronal dedifferentiation and abnormal Tau phosphorylation [17,21,51]. Because Pin1 was found to be a key regulator of the mitotic transition, the earlier finding that Alzheimer' disease might be related to a reactivation of the cell cycle is in agreement with a functional role for Pin1 in AD [45] (Fig. 5).

9. Conclusion

In post-mitotic cells, reactivation of the cell cycle is an early step of apoptosis. In neurons, this reactivation of the cell cycle may be responsible for the abnormal Tau phosphorylation and aggregation, leading to neurofibrillary tangles in AD (Fig. 5). In fact, in neurons exhibiting neurofibrillary degeneration, both G1/S and G2/M markers are found suggesting that they bypass the classical apoptosis and an aberrant cell cycle occurs. This aberrant cell cycle leading to neurofibrillary degeneration may start as a classical neuronal apoptosis with the coming out of G0 to G1 phase and the appearance of specific markers such as cyclin D1 and E2F-responsive genes [14–18]. Induction of apoptosis also leads to the calpain activation and the formation of the p35 proteolytic product, p25 and a deregulated Cdk5 activity [39,42,43]. The complex Cdk5/p25 allows for the abnormal Tau phosphorylation with the appearance of mitotic epitopes [44]. The peptidyl prolyl *cis*/*trans* isomerase, Pin1 binds "mitotic phospho-Tau epitopes" allowing its nuclear depletion and thus does not bind to its classical substrates (cyclin D1, p53, etc.) [34,45,46,52]. This latter may be responsible for escaping the classical apoptosis in a subset of neurons. Altogether, these data suggest that the initiation of apoptosis in neuronal cells may also lead to neurofibrillary degeneration.

Acknowledgments

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