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Tau Phosphorylation by cdk5 and Fyn in Response to Amyloid Peptide Aβ_{25-35}: Involvement of Lipid Rafts

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Abstract. Alzheimer's disease (AD) is characterized by the accumulation of protein filaments, namely extracellular amyloid-β (Aβ) fibrils and intracellular neurofibrillary tangles, which are composed of aggregated hyperphosphorylated tau. Tau hyperphosphorylation is the product of deregulated Ser/Thr kinases such as cdk5 and GSK3β. In addition, tau hyperphosphorylation also occurs at Tyr residues. To find a link between Aβ and tau phosphorylation, we investigated the effects of short-term Aβ treatments on SHSY-5Y cells. We analyzed phosphorylated tau variants in lipid rafts and the possible role of Tyr18 and Ser396/404 tau phosphorylation in Aβ-induced signaling cascades. After 2 min of Aβ treatment, phospho-Tyr18-tau and its association with rafts increased. Phospho-Ser 396/404-tau became detectable in rafts after 10 min treatment, which temporally correlated with the detection of cdk5 and p35 activator in lipid rafts. To determine the role of cdk5 in tau phosphorylation at Ser396/404 in lipid rafts, we pre-incubated cells with cdk5 inhibitor roscovitine, and observed that the Aβ-induced tau phosphorylation at Ser 396/404 in rafts was abolished as well as cdk5/p35 association with rafts. These data suggest a role for cdk5 in the Aβ-promoted early events involving tau hyperphosphorylation, and their possible implications for AD pathogenesis.

Keywords: Alzheimer's disease, amyloid-β, cdk5, Fyn, lipid rafts, neuronal membrane, tau phosphorylation

INTRODUCTION

Microtubule associated protein tau is a neuronal protein that has several functions during neuronal development. Tau has been described in the cytosol [1] the nucleus [2,3], and the plasma membrane [4–7]. Tau undergoes posttranslational modifications such as phosphorylation, glycosylation and ubiquitination. Phosphorylation of tau on Ser/Thr is carried out by several kinases [8–11], such as cdk5 and GSK3β and largely account for the tau hyperphosphorylation observed in AD [8,10,12,13]. The phosphorylation of tau on Tyr is carried out by c-Abl [14] and Src family kinases Lck [15], Syk [16], Fyn and Src [17–19]. Fyn is activated by amyloid-β (Aβ)$_{25-35}$ and consequently, several proteins including tau, show an increased Tyr phosphorylation [14]. Tau has been detected in lipid rafts [20, 21], which are plasma membrane microdomains composed of cholesterol and sphingolipids that participate in signal transduction processes [22–25]. GSK3β was also detected in lipid rafts [26], but there are no reports of cdk5 in these membrane microdomains. In addition, high levels of hyperphosphorylated tau at Ser396/404 were found in lipid rafts from AD brains [27]. Due to the involvement of lipid rafts in AD [28,29] and its role in signal transduction mechanisms, we hypothesize that in lipid rafts, tau participates in signals transductions activated by the Aβ peptide. Changes in Ser396/404 phosphorylation of tau in response to Aβ have been

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described [30], as well as changes in Tyr phosphorylation [15,21]. In this study, we have used human neuroblastoma cell line SHSY-5Y to investigate changes in tau phosphorylation at Ser396/404 and Tyr18 in lipid rafts following stimulation with the amyloid fragment $A\beta_{25-35}$ with demonstrated neurotoxic activity. We found a correlation between the lipid raft association of cdk5/p35, cdk5 activity, and phospho-Ser396/404-tau. This result suggests a role for cdk5 in early events of tau phosphorylation in response to $A\beta_{25-35}$. This is an interesting finding, as the first evidence that $A\beta$ activation of the cdk5/p35 system also occurs at the neuronal membrane level, which could be an early event in the process leading to neuronal degeneration.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies used: tau5 (total tau) donated by Dr. L. Binder; 9G3 (phospho-tyr18-tau [18]; PHF-1 (phospho-Ser396/Ser404-tau) donated by Dr. P. Davies; Flotillin-1 (BD Transduction Laboratories, San Diego, CA); Transferrin Receptor, TfR (Zymed Laboratories). Rabbit polyclonal antibodies used: PY18 (phospho-tyr18-tau [18]; JM (total tau), gift of Dr. A. Takashima [31]. Antibodies against GSK3β, cdk5 C-8, p35-N20, and Fyn were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondaries peroxidase (HRP)-conjugated: anti mouse IgG and anti rabbit IgG were purchased from Jackson Immunoresearch Laboratories, Inc (West Grove, PA).

Cell culture

Human neuroblastoma cells, SHSY-5Y were grown in a mix 1:1 of MEM and F12 supplemented with 15mM Hepes, MEM Non-essential amino acids 100x, 10% fetal bovine serum and antibiotics pen-strep at 37^oC and 5% CO_2. All materials were purchased from GIBCO Laboratories Life Technologies, Inc. (Grand Island, NY).

Isolation of lipid rafts by Opti-prep gradients

Cell lysates were prepared from two 100mm dishes of SHSY-5Y. Cells were homogenized on ice, in final volume of 2 ml 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in MBS lysis buffer (25 mM MES, 150 mM NaCl, pH 6.5) with protease and phosphatase inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 100 μg/ml PMSF, 1mM sodium orthovanadate and 20 mM sodium fluoride). The lysate was incubated with DNaseI (final 20 μg/ml) for 1 h on ice. One volume of the homogenate was mixed with two volumes of Opti-prep (final Opti-prep concentration 40%) and 1.5 ml of this was placed at the bottom of the centrifugation tube. For step gradient, 1 ml of 30% Opti-prep (diluted with MBS) and 0.5 ml of 5% Opti-prep were layered on top. The tubes were centrifuged in a Beckman Optima MAX-E ultracentrifuge at 43,000 rpm ($\sim$150.000 xg) for 16h at 4°C (MLS50 swinging bucket rotor). Nine fractions of 200 μl each were collected from the top of the tube. 60 μl of each fraction were subjected to SDS-PAGE and Western blot using ECL Lighting Chemiluminescence Reagent Plus (Pierce, Rockford, IL). These blots were exposed at the same time and under the same conditions in order to compare presence or absence of proteins in lipid rafts, avoiding possible differences due to time of exposure. Thus, we begun with two 100 mm dishes of 70% confluent cells, and then we diluted the homogenate to prepare the gradients, and we determined that in those 60 μl loaded per well in each gel, the cell homogenate was diluted to a final concentration of around 5% or less.

$A\beta_{25-35}$, PP1 and roscovitine preparation and treatments

$A\beta$ peptide was dissolved in ultra pure distilled water at a final concentration of 10 mM, aliquoted, and stored at -20°C until use. For treatments, the stock solution was diluted directly into the media for a final concentration of 10 μM. The length of treatment ranged from 1 to 10 min. Some cells were pre-treated with 10 μM roscovitine for 1h at 37°C, prior to $A\beta$ incubation. Some cells were pre-treated with 1 μM PP1 analog (Calbiochem) for 1h at 37°C prior to $A\beta$ incubation. For roscovitine treatment, cells grown in 100 mm dishes were lysed and processed for lipid raft isolation; for PP1 incubation and analysis of total extracts, cells grown in 60 mm dishes were homogenized in 2% NP40 lysis buffer (25 mM Tris, 150 mM NaCl, pH 7.2 and 2% NP40 (Sigma-Aldrich, St. Louis, MO) with protease and phosphatases inhibitors as above. Samples from these cell homogenates were subjected to SDS-PAGE and Western blots. Half of the total homogenate was loaded per lane in each gel. We determined that out of the total volume loaded, the homogenate was diluted to a final protein concentration of around 42% or less. Quantification of blots was carried out by scanning the

photographic films of nitrocellulose membranes, and calculating signal intensity with Kodak digital Science densitometry program.

RESULTS

Effect of $A\beta_{25-35}$ on tau phosphorylation

In order to evaluate whether tau participates in Aβ-induced signal transduction, we treated SHSY-5Y cells with 10 μM $A\beta_{25-35}$ for varying times, then analyzed levels of tau phosphorylation at Tyr18 and Ser396/404. The $A\beta_{25-35}$ peptide has been described to produce neurotoxic effects quicker than the form $A\beta_{1-42}$ [32–34]. There is a basal level of tau phosphorylation at Tyr18, as observed in lane 1 in Fig. 1A and in the plot in Fig. 1B, may be due to regular interactions between Fyn and tau under control conditions. We observed an increase in Tyr18 phosphorylation of tau at 2 min followed by a decrease after 5 min treatment with the Aβ fragment (Fig. 1A, B). This event correlated with a previously reported increase in Fyn activation observed at 1 min after Aβ stimulation [15].

To investigate the possible participation of Fyn in this phosphorylation, we pre-incubated the cells with 1 μM PP1. This is a selective inhibitor of Src family kinases which, when used at low dosages, is more effective on Fyn activity [35]. Based on this fact and in previous results [15] we only worked with Fyn. Figure 1C shows that the Aβ-induced Tyr18 phosphorylation of tau is lost when cells were first pretreated with PP1. In contrast, there is an increase in phospho-Ser396/404-tau after 5 min (Fig. 1D). Due to the described association of tau with lipid rafts, we asked if these changes in tau phosphorylation were correlated with its subcellular compartmentalization.

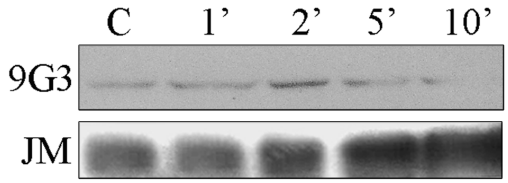
Tau distribution in lipid rafts: phosphorylation pattern and changes due to $A\beta_{25-35}$

To first ascertain the purity of the raft fractions, we assayed for both the presence of the lipid raft marker Flotillin-1 and the absence of the non-raft marker Transferrin Receptor (TfR) in Opti-prep gradient fractions. Flotillin-1 was present in fractions 3 to 5 while TfR was present only from fraction 6 to 9. We observed some differences in distribution of both markers from blot to blot in Fig. 2, and this is due to slight differences in the resulting Opti-prep concentration in the gradient. Because every experiment means a different gradient, some variations may result from the preparation of the gradient or from the centrifugation and fraction collections. That is why we decided to consider both markers and, more importantly, the Flotillin-1 enriched fractions were free of TfR. So because Flotillin-1 was present mainly in fractions 3 to 5 while TfR was present mainly from fraction 6 to 9, we considered fractions 3–5 as lipid rafts and 7–9 as non rafts.

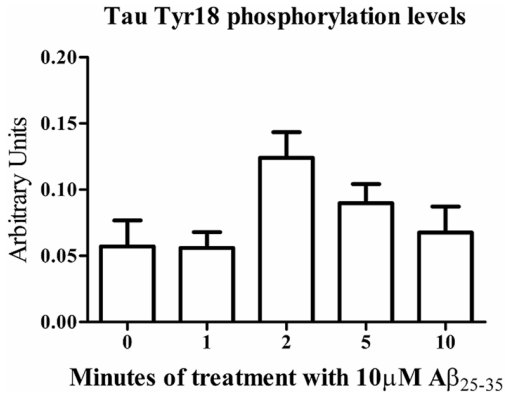
In the gradients analyzed, we found two tau subpopulations detected by JM polyclonal antibody, one in lipid raft fractions 3 to 5 and the other in non-raft fractions 7 to 9 (Fig. 2A). In these controls, phospho-Ser396/404-tau was only detected in the non-raft fractions. On the other hand, phospho-Tyr18-tau was detected in lipid raft fraction 5 and in non-raft fractions 7 to 9. When the cells were stimulated for 2 min with 10 μM $A\beta_{25-35}$, phospho-Tyr18-tau was then detected in the lipid raft fractions 4 and 5 (Fig. 2B), correlating with the Aβ-induced increase in phospho-Tyr18-tau observed in the total cell extract (Fig. 1A). At this time point of Aβ treatment, phospho-Ser396/404-tau was not detected in the lipid rafts fractions. However, after 10 min Aβ treatment, phospho-Tyr18-tau in rafts decreased while phospho-Ser396/404-tau appeared in lipid rafts fractions for the first time (Fig. 2C). Studies indicate that mobilization of both types of phospho-tau is dynamic in raft fractions, and that rafts are microdomains very sensitive to changes in the distribution of these tau variants. The signal of PHF-1 antibody in Fig. 2 is weaker as compared to Fig. 1 because we ran much less material in Fig. 2 (5% of total homogenate loaded as compared to around 42% in Fig. 1). The sensitivity of the lipid raft preparation limits the amount of starting material, if more cells are used to get a better signal, then lipid rafts fractions appeared contaminated with TfR.

Distribution cdk5 and GSK3β in lipid rafts: Effects of $A\beta_{25-35}$

To determine whether the kinases cdk5 and GSK3β might have a role in tau phosphorylation in response to Aβ, we analyzed their distribution in lipid rafts. Figure 2D shows the distribution of cdk5 and its activator p35, and GSK3β in lipid rafts of unstimulated SHSY-5Y cells. We detected GSK3β in lipid rafts, in agreement with a previous report [26]. We did not detect cdk5 or p35 in lipid rafts. After 2 min of incubation with Aβ peptide, cdk5 and p35 are still undetectable in raft fractions (Fig. 2E) while GSK3β has become absent. After 10 min of incubation, GSK3β relocat-



(a)



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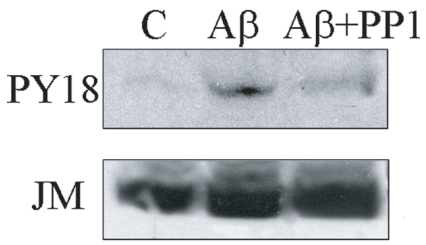

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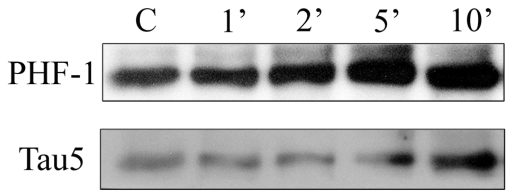

(d)

Fig. 1. Tau phosphorylation in response to 10 μM Aβ_{25-35} after 1, 2, 5 and 10 min of incubation. Total cell lysates from SHSY5Y cultures were analyzed by Western blots. (A) Tau phosphorylation at Tyr18 was detected with antibody 9G3. The blot was reanalyzed for total tau using the polyclonal antibody JM. There is a basal level of tau phosphorylation at Tyr18 observed in lane 1. Phosphorylation at Tyr18 peaks at 2min. (B) Quantitation of tau tyrosine phosphorylated at residue 18 in response to Aβ treatment ($n = 5$, five independent experiments) was obtained after normalizing 9G3 to JM signal (see Methods). (C) Inhibition of Fyn by 1 μM PP1 abolished the increase in tau phosphorylation at Tyr18 observed after 2 min Aβ_{25-35} treatment. (D) Tau phosphorylation at Ser396/404, detected by the monoclonal antibody PHF-1 was incrementally increased at 5 min Aβ treatment.

ed in lipid rafts, indicating a possible recharging of the GSK3β levels in membrane microdomains. At the same time, cdk5 and its activator p35 were detected for the first time in lipid rafts fractions (Fig. 2F). The association of cdk5 with lipid rafts temporally correlated with the detection of phospho-Ser396/404-tau. In or-

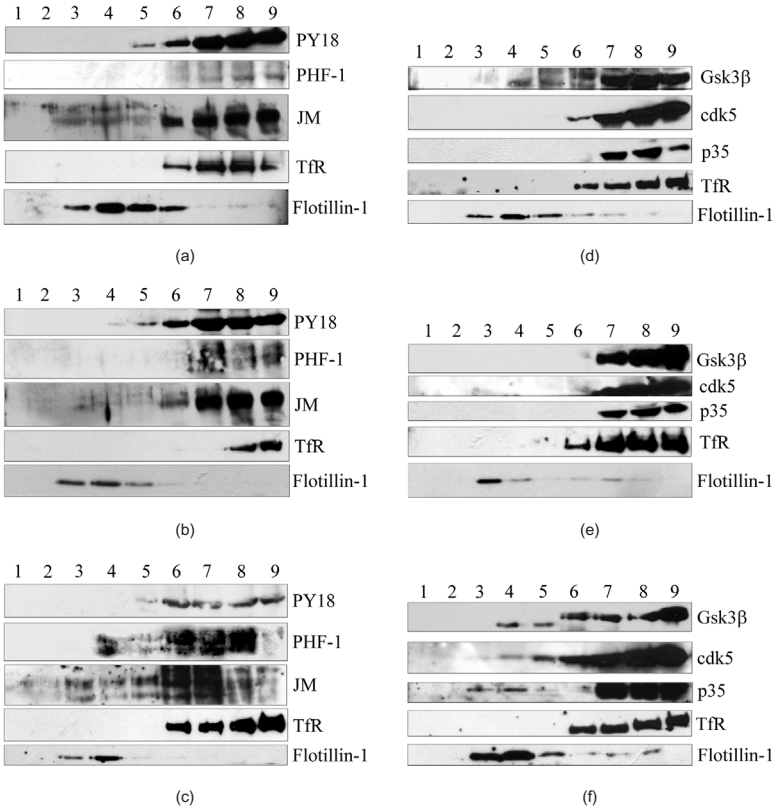

Fig. 2. Distribution of tau in SH-SY5Y lipid raft preparations after Aβ_{25-35} incubation. Tau phosphorylated at Tyr18 (PY18), tau phosphorylated at Ser 396/404 (PHF-1) and of total tau (JM) were detected in Opti-prep gradient fractions from (A) unstimulated, (B) 2 min Aβ, and (C) 10 min Aβ treatment of SH-SY5Y cells. In addition, the distributions of cdk5, p35 and GSK3β in the Aβ-treated cells are shown in (D) unstimulated, (E) 2 min Aβ, (F) 10 min Aβ-stimulated SH-SY5Y cells. Each set of gradient fractions was also probed with the lipid raft marker Flotillin-1 and the non-raft membrane marker Transferrin Receptor (TfR). Fraction 1 corresponds to the top of the gradient (5% Opti-prep) and fraction 9 the bottom of the gradient (40% Opti-prep). Fractions 3, 4 and 5 were considered as lipid raft fractions while fractions 7, 8 and 9 were non-raft fractions. The material loaded in each lane was 5% of total cell homogenate. Differences in markers distributions, Flotillin-1 and TfR are due to slight differences in the final Opti-prep concentration. All blots by the same antibody were exposed together and during the same length of exposure time.

der to determine if cdk5 was responsible for the change in tau phosphorylation in lipid rafts, we inhibited the kinase and analyzed tau phosphorylation. Cells were pre-incubated with 10 μM roscovitine, an inhibitor of cdk5, and then treated with Aβ for 10 min. When cdk5 was inhibited, its levels as well as p35 levels became undetectable in lipid rafts fractions (Fig. 3). For example, no signals at all was detected in fractions 4, 5 of lipid rafts. Similarly, phospho-Ser396/404-tau as well as total tau, were not detected in lipid rafts following roscovitine treatment (Fig. 3).

DISCUSSION

Our results show that while tau is mainly found in non-raft fractions, which contain cytosolic tau, a small fraction of tau is located in lipid rafts and undergoes changes in response to Aβ. In these studies we used Aβ_{25-35} on the basis of solid evidence that its aggregation capacity and neurotoxic effects are comparable with Aβ_{1-42} peptide [33,34]. Our initial characterization focused on phospho-Tyr18-tau, which was present mainly in non-raft fractions. Based on total cell lysates, we found that the levels of phospho-Tyr18 increased in response to Aβ after 2 min, an effect that was inhibited by low levels of PP1. This is consistent with the Aβ-induced activation of Fyn as previously reported [15] and the positioning of Fyn and tau in signal transduction pathways activated by Aβ [36,37]. Interestingly, we found Aβ-induced slight changes in Tyr18-tau in lipid raft fractions. Moreover, we detected significant changes in lipid raft tau with regards to phosphorylation at Ser396/404, a novel observation indicating tau

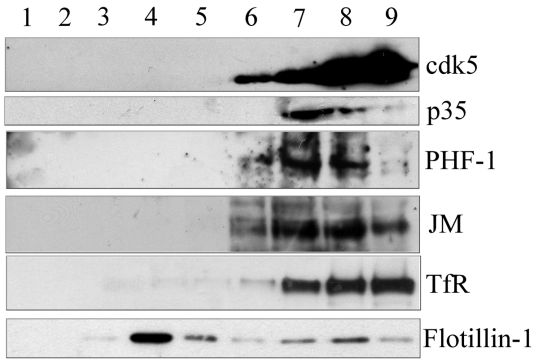

Fig. 3. Effect of roscovitine on cdk5/p35 and phospho-396/404-tau distribution in lipid rafts. Opti-prep gradient fractions collected from SH-SY5Y treated with roscovitine and $A\beta_{25-35}$ as described in Materials and Methods, were probed for cdk5, p35, phospho-396-404-tau (PHF-1) and total tau (JM). Lipid raft marker Flotillin-1 and non-raft membrane marker Transferrin receptor (TfR) are also shown. Fractions 3, 4 and 5 were considered as lipid raft, while fractions 7, 8 and 9 were considered as non-raft.

modification at the level of rafts. It has previously been described that phospho-Ser396/404-tau is primarily cytosolic as opposed to membrane associated [6,7]. We found that 10 min after Aβ-stimulation, this phosphorylated tau species appeared in lipid rafts, suggesting that either specific Ser/Thr kinases were acting on tau in lipid rafts or that there was some mobilization of this phosphorylated tau species from the cytosol to the membrane.

Because the major kinases that phosphorylate tau at Ser396/404 are cdk5 and GSK3β as involved in AD pathogenesis [8,9,11,38–41], we studied their behavior and membrane distributions in response to Aβ treatment. GSK3β has been found in lipid rafts [26] and in our system, we found that after 2 min of Aβ incubation, GSK3β disappeared from the lipid rafts domains to re-appeared at 10 min. A possible explanation is that by 2 min, GSK3β is activated by phosphorylation at its Tyr216, and relocated to the cytoplasm. After 10 min, a less phosphorylated form of the protein returns to lipid rafts, explaining the lower molecular weight of the GSK3β species. This scenario is supported by the finding that within 2 min of insulin treatment, GSK3β has an increased association with Fyn and becomes activated as well [42]. However, the behavior of GSK3β in lipid rafts did not reflect its activity with respect to tau phosphorylation at Ser396/404. Therefore, we investigated cdk5 shown to be overactivated in degenerating neurons of AD type [10]. The comparison between Aβ-treatments with and without roscovitine allowed us to observe a correlation between the presence of cdk5/p35 and phospho-Ser396/404-tau in lipid rafts. By inhibiting the activity of cdk5, the Aβ-induced appearance of phospho-Ser396/404-tau in lipid rafts at 10 min was abolished. Moreover, upon roscovitine treatment, total tau levels were decreased in lipid rafts, indicating that this drug affects the subcellular localization of tau and that the phosphorylation at Ser396/404 is associated with the Aβ-induced localization of tau to lipid rafts. Our results also show that roscovitine inhibits the localization of cdk5 to lipid rafts. The association of cdk5 with the lipid raft membrane seems to be mediated by its activator p35 [8,10,43], whose N-terminus can be myristoylated, a modification known to target proteins to lipid rafts [44]. In the presence of roscovitine, phospho-Ser396/404-tau exists in fractions 7 and 8 (Fig. 3). Even though further studies in this topic are needed, our explanation for the effect of roscovitine over tau in lipid rafts focus on tau interaction with cdk5. We can speculate that upon roscovitine action, cdk5 is unable to relocate to lipid rafts, then the cdk5 phosphorylated-tau at Ser396/404 occurring at 10 min Aβ treatment will be unable to relocate to lipid rafts. While GSK3β is found in non-raft fraction 9 (Fig. 2F), it may still be responsible for generating this tau subpopulation.

Our results show that Aβ rapidly affects tau phosphorylation patterns in lipid rafts and suggest that lipid rafts may serve as a propitious environment for the interaction between tau and cdk5, thereby facilitating tau phosphorylation at Ser396/404 during the very early response to Aβ. These findings implicate tau as a signal transduction protein responding to $A\beta_{25-35}$, giving tau a new role other than its role as a microtubule stabilizing protein [19]. For example, Aβ treatment affects actin polymerization at domains close to the cell membrane [45], and because tau can affect actin dynamics [44], tau might be employed by Aβ as an effector protein. The signaling pathways that connect the Aβ receptor to tau phosphorylation remain to be elucidated.

In addition, the residues Ser396/404 have been largely involved not only in pathological tau aggregation and PHFs formation, but also in fetal tau phosphorylation during brain development. This fetal tau phosphorylation resembles tau phosphorylation in AD [47]. Then, it is reasonable to think that changes in phospho-Ser396/404-tau in lipid rafts also occur during neuronal development. In summary, we have observed an increase in Tyr18-tau phosphorylation as fast as at 2 min after exposure to Aβ fragment, an event correlated with Fyn activation after Aβ stimulation. Moreover, we have detected for the first time that the cdk5/p35

complex is also involved in tau modification at the level of the neuronal membrane rafts. Studies suggest a role for cdk5/p35 complex in the Aβ-promoted early events involving tau hyperphosphorylation at the level of rafts, and their possible implications for AD pathogenesis.

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