CHARACTERIZATION OF A DOUBLE (AMYLOID PRECURSOR PROTEIN-TAU) TRANSGENIC: TAU PHOSPHORYLATION AND AGGREGATION

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Abstract—A double transgenic mouse expressing the amyloid precursor protein, bearing the Swedish mutations, and expressing tau protein containing three of the mutations present in frontotemporal dementia linked to chromosome 17 (FTDP-17), has been characterized. In the double transgenic mouse an increase in tau phosphorylation at serine S262 and S422 was observed compared with that found in simple transgenic mice. The phosphorylation at S262 was also found, in a much lower level, in the single transgenic mouse expressing amyloid precursor protein (APP), and it was absent in that overexpressing tau variant. Additionally, in the double transgenic mouse a slight increase in the amount of sarkosyl insoluble tau polymers was observed in comparison with that found in single transgenic tau mouse. Also, wider tau filaments were found in the double transgenic mouse compared with those found in the single transgenic mouse.

Our results suggest that β-amyloid peptide could facilitate the phosphorylation of tau at a site not directed by proline, such as serine 262, and that modification could facilitate tau aberrant aggregation. Also, they suggest that different types of tau filamentous polymers can occur in different mouse models for tauopathies, like those used for Alzheimer’s disease or FTDP-17. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: beta-amyloid aggregates, tau polymers, phosphorylation, Alzheimer’s disease, FTDP-17, kinases.

Alzheimer’s disease (AD) is characterized by the presence of two aberrant structures, senile plaques and neurofibrillary tangles, present in the brain of the patients (Alzheimer, 1907). Senile plaques are mainly composed of aggregates of a peptide, β-amyloid peptide (Aβ), that arises from the proteolytic cleavage of a precursor protein, APP (Glenner and Wong, 1984). In contrast, the main component of neurofibrillary tangles is tau protein in hyperphosphorylated form (Grundke-Iqbal et al., 1986). There is a hypothesis, the amyloid cascade hypothesis, suggesting that there is a link between the presence of Aβ and the phosphorylation and assembly of tau protein (Hardy and Selkoe, 2002). In this way, it has been suggested that the presence of β-amyloid could facilitate the phosphorylation of tau protein at both proline directed sites phosphorylated (PDP; Morishima-Kawashima et al., 1995) and non-proline directed sites phosphorylated (NPDP; Geula et al., 1998; Xie et al., 2002; Ferrari et al., 2003). In the first case Aβ could act as an antagonist of insulin receptor (Xie et al., 2002), facilitating the activation of GSK3, a PDP kinase; also, the role of Aβ in activation of NPD sites has been suggested (Geula et al., 1998). There are some indications supporting the amyloid cascade hypothesis. For example, Gotz et al., 2001 and Lewis et al., 2001 have found that the presence of amyloid peptide increases the formation of aberrant (sarkosyl insoluble) tau aggregates. On the other hand, studies in human AD cases following β-amyloid immunization, have shown a decreased amyloid burden, and decreased phosphorylated tau in neurites surrounding amyloid plaques, but not a reduction of neurofibrillary tangles (NFTs; Nicoll et al., 2003; Ferrer et al., 2004). These findings suggest that tau phosphorylation in dystrophic neurites of senile plaques, but not necessarily phosphorylation of tau in NFTs, is related with amyloid accumulation. Additionally, Rapoport et al., 2002 noted that the toxic effect of β-amyloid could not take place in cells lacking tau protein.

In contrast, in other tauopathies such as frontotemporal dementia linked to chromosome 17 (FTDP-17), the phosphorylation and formation of tau aggregates takes place independently of the presence of β-amyloid (Spillantini et al., 1998). Several mouse models expressing tau, bearing different mutations present in FTDP-17 patients, have been developed. In some of these models, but not in those expressing wild type tau, aberrant phosphotauggregates were found (Brion et al., 1999; Ishihara et al., 1999; Spitael et al., 1999; Probst et al., 2000; Gotz et al., 2001; Lewis et al., 2001; Lim et al., 2001; Allen et al., 2002;
In this work, we have tested whether the presence of Aβ, in aggregated form, may increase the phosphorylation and formation of sarkosyl insoluble tau aggregates. To do this we have characterized a double transgenic mouse generated from Tg2576, a mouse expressing APP containing the double mutation K670N and M671L, crossed with a mouse expressing the mutations G272V, P301L and R406W, found in FTDP-17. Our results indicate that the presence of Aβ aggregates significantly facilitates the phosphorylation of tau at an NPD site like that of Ser 262 and the formation of new sarkosyl insoluble aggregates.

**EXPERIMENTAL PROCEDURES**

**Transgenic mice**

A double Tg line was obtained by crossing Tg2576 and VLW lines. The progeny included double mutant APP (APPsw)/mutant tau (tauvlw) (hereafter termed APPsw-tauvlw), mutant APP (APPsw), mutant tau (tauvlw), and non-transgenic animals. Detailed characterization of APPsw and tauvlw lines have been previously published elsewhere (Mullan et al., 1992; Lim et al., 2001). In brief, the Tg2576 line overexpresses human APP containing the double Swedish mutation K670N-M671L (swe) under the control of the hamster prion protein promoter in a C57Bl6jxSJL hybrid background. Tg2576 mice develop cerebral Aβ deposits and spatial memory deficits in an age-dependent manner (Mullan et al., 1992). The tauvlw line overexpresses three mutations linked to frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17; G272V, P301L and R406W) driven by the mouse Thy-1 promoter in a C57Bl6jxCBA hybrid background (Lim et al., 2001). Tauvlw mice express human mutant tau at high levels in cortex and hippocampus with minimal expression in the spinal cord. Immunohistochemical analysis reveals high transgene expression in neuronal cell bodies and neurites in the cortex and the hippocampal formation. Ultrastructural analysis shows a pre-tangle appearance in neurons expressing mutant tau, with filaments of tau and increased numbers of lysosomes displaying aberrant morphology similar to those found in AD (Lim et al., 2001). Nine-, 16- and 25-month-old mice were used.

**Antibodies**

The antibodies employed to detect tau in soluble or aggregated form were BR134 and 7.51, which is directed against the microtubule-binding region (Novak et al., 1991; a kind gift from Dr. Wischik, UK); antibodies reacting with phospho residues 181, 202, 262 and 422, were purchased from Calbiochem (Germany). Antibody AT8 was from Innogenetics (Belgium), this antibody recognizes phosphorylated tau at residue 202 (Goedert et al., 1995). Antibody 12E8 (Athena Laboratories), which recognizes tau phosphorylated at serine 262, was a kind gift from Dr. Seubert
Table 1. Phosphorylation sites of tau in neurites surrounding amyloid plaques and in neurons in wild type mice (−/−), in APPsw (+/+), tau−/− (−/+), and double transgenic APPsw/tau−/− (+/+−) 16-month-old mice

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(Seubert et al., 1995). A monoclonal antibody directed against α-tubulin (Sigma) was used as an internal control for protein quantity.

Immunoblots analysis

Samples were run on 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell GmbH). The membrane was blocked by incubation with 5% skimmed milk in PBS and 0.1% Tween 20, followed by 1-h incubation at room temperature with the primary antibody in PBS. Primary antibody dilutions were 7.51 (1/100), AT8 (1/100) and 12E8 (1/1000). After three washes, the membrane was incubated with a horseradish peroxidase-anti-rabbit or anti-mouse Ig conjugate (DAKO), followed by several washes in PBS-Tween 20. The membrane was then incubated for 1 min in Western Lightning reagents (PerkingElmer Life Sciences). The labeled protein was subjected to densitometric analysis for quantification. The optical density of the stained protein bands, in arbitrary units, was measured. Statistical analysis was performed using Student’s t-test to determine tau phosphorylation level (at different sites) using ab AT8 or ab 12E8, the data were normalized to the total tau levels determined by the interaction of tau protein with ab 7.51.

Isolation of sarkosyl-insoluble tau aggregates

Preparation of sarkosyl-insoluble extracts from mouse brain of 16-month-old mice and electron microscopy of filaments was carried out as described previously (Greenberg and Davies, 1990). Rabbit anti-tau serum BR134 was used at a dilution of 1/30 for immunoelectron microscopy.

Ultrastructural analysis in frontal cortex and hippocampus was performed as indicated in Lim et al. (2001) in 25-month-old double transgenic mice and in single transgenic tau−/− mice.

Immunohistochemistry

Serial consecutive sections, 30 μm thick, were processed free-floating with the LSAB method (Dako LSAB+ kit) following the instructions of the supplier (Puig et al., 2004). Briefly, after blocking endogenous peroxidases, the sections were incubated with normal serum for 2 h and then incubated overnight at 4 °C with one of the primary antibodies. The phospho-specific tau Thr181 and tau Ser202, Ser262, Ser396 and Ser422 polyclonal antibodies were incubated at dilutions of 1:500 and 1:100, respectively. The antibody AT8 (direct against phosphoserines 199/202) was used at a dilution of 1/50. For Aβ immunohistochemistry, a slight variation of the protocol was introduced. Before blocking endogenous peroxidases, sections were incubated with 98% formic acid for 3 min to enhance antigenicity. The β Aβ40 and β Aβ42 rabbit polyclonal antibodies (kindly provided by Dr. M. Sarasa, Zaragoza) were used at a dilution of 1:500. The phosphorylation-dependent anti-SAPK/JNK (Thr183/Tyr185) rabbit polyclonal antibody (SAPK/JNK-P; Cell Signaling) was used at a dilution of 1:50. The phosphorylation-dependent rabbit polyclonal antibody to p-38 (Thr180/Tr182; p38-P; Calbiochem) was used at a dilution of 1:100. After washing, the sections were then incubated with link solution (LSAB) and with streptavidin–peroxidase solution for 15 min each at room temperature. The peroxidase reaction was visualized, as a dark blue precipitate, with NH4NiSO4 (0.05 M) in phosphate buffer (0.1 M), 0.05% 3,3’-diaminobenzidine, NH4Cl and 0.01% hydrogen peroxide. Blank sections stained only with the secondary antibodies were used as negative controls.

RESULTS

Tau phosphorylation in double transgenic mice

Control, transgenic APPsw, transgenic tau−/−, and double transgenic APPsw/tau−/− mice were analyzed. In this analysis, the phosphorylation of tau at one proline directed site, that recognized by antibody AT8, and at one non-proline directed site, that recognized by ab 12E8, were studied. Fig. 1A shows a slight increase in tau phosphorylation at the AT8 site in the different single APPsw and in the single tau−/− and double transgenic mice, compared with the control mouse, determined by Western blot, using equal amounts of protein (determined by the measurement of α-tubulin content). Fig. 1B shows the quantitation of that Western blot. Also, in single transgenic tau−/− and in the double transgenic APPsw/tau−/−, the phosphorylation of human tau (showing a lower mobility) can be found in phosphorylated form. When another antibody reacting with a PDP site, that reacting with serine 422, was tested, some differences were found among both the single and the double transgenic mice in the reaction with that antibody. In contrast, little, if any, reaction of tau protein was found in tau from wild type mice (Table 1).

Tau phosphorylation at one NPDP site was determined by the reaction with ab 12E8. In this case, a clear increase in tau phosphorylation was found when the control and the double transgenic APPsw/tau−/− mice were compared (Fig. 1C). Also, a slight increase in tau phosphorylation in transgenic APPsw, but less evident in transgenic tau−/−, was observed in different samples. The quantitation of the data is indicated in Fig. 1D. These results suggest that the presence of amyloid peptide may facilitate tau phosphorylation at this NPDP site. The age of the tested mice was 9 months. At this age it was previously shown a tau hy-
The formation of sarkosyl insoluble aggregates was further identified by electron microscopy. Mainly amorphous aggregates were found although Fig. 3 (A, B) shows the presence of some filamentous polymerized structures of about 10 nm that can react with tau antibodies from the double transgenic mouse. However, in the case of tau\(^{VW}\), the diameter of the isolated filamentous structures was much lower (about 2 nm; see Fig. 3C), as previously indicated (Lim et al., 2001).

Additionally, in double APP-tau transgenic mice ultrastructural studies were carried out on those AT8-immunoreactive neurons and processes surrounding amyloid deposits. In some AT8-positive processes a huge degeneration, but no filaments, was found (Fig. 4, inset left). However, in some AT8-positive healthy appearing neurons, filaments were found at the apical dendrite as well as in some areas of the neuronal soma (Fig. 4A, B). In addition, numerous AT8-negative degenerating processes were observed surrounding the amyloid deposits (not shown).

**Localization of A\(\beta\) aggregates and tau in hyperphosphorylated form**

Amyloid aggregates stained with antibodies to \(\beta A_{1-40}\) and \(\beta A_{1-42}\) were present in the hippocampus, amygdala and cerebral cortex in the APP and double APP-tau transgenic mice (Fig. 5). Occasional plaques were also observed in the subcortical white matter. No plaques were seen in tau transgenic and age-matched control mice. Phosphorylation-dependent tau antibodies Thr181, Ser202 (AT8), Ser262 (12E8) and Ser422 disclosed abnormal neurites containing hyperphosphorylated tau in the vicinity of the amyloid plaques in APP\(^{SW}\) mice, as previously reported. Abnormal neurites were not recognized with antibodies to phospho-tau Ser396. In contrast to neurites, neuronal somas containing phospho-tau in tau transgenic mice were stained only with phospho-tau antibodies Thr181 and Ser202, but not with antibodies Ser262, Ser396 and Ser422. These differences in tau phosphorylation sites between neurites and neuronal somas were reproduced in double APP\(^{SW}/tau^{VW}\) transgenic mice (Figs. 6 and 7; Table 1).

Interestingly, aged (25-month-old) double transgenic mice disclosed particular immunohistochemical features. In addition to fine punctuate tau-immunoreactive pro-
cesses, varicose and coarse neurites were found surrounding many amyloid deposits. Moreover, very strong AT8-immunoreactive neurons were seen in the entorhinal cortex, perirhinal fissure and, rarely, the isocortex and CA1 sector of the hippocampus (Fig. 7). These results suggest that the presence of Aβ aggregates may facilitate not only the tau phosphorylation at a non-proline directed site like serine 262, but also that at serine 202.

Colocalization of some tau kinases with tau aggregates

It has been already suggested a role for GSK3, in the tau modification observed in tau vlw (Pérez et al., 2003). Thus, we have tested for other additional kinases that may play a role in such modification using as criteria their colocalization with tau aggregates. These kinases are two members of MAPkinase family: SAPK/JNK-P and p38-P.

Sections stained with antibodies to SAPK/JNK-P and p38-P permitted the study of the expression of phosphorylated (presumably active) kinases in double transgenic
mice. Strong stress kinase immunoreactivity p38-P and SAPK/JNK-P immunoreactivity (Fig. 8A and C, respectively) was found surrounding cores of amyloid plaques. Consecutive sections disclosed SAPK/JNK-P and p38-P immunoreactivity in the majority of, if not all amyloid plaques. In contrast, p38-P immunoreactivity was seen in a small number of neurons in the entorhinal cortex (Fig. 8B) whereas SAPK/JNK-P was extremely rare, in similar neurons of the entorhinal cortex (Fig. 8D). No SAPK/JNK-P or p38-P immunoreactivity was seen in controls (data not shown).

**DISCUSSION**

Although a detailed study of kinases involved in tau phosphorylation in double transgenic mice is out of the scope of the present work, preliminary studies have shown stress kinase expression SAPK/JNK and p38-P surrounding amyloid plaques. Since the antibodies were directed to specific phosphorylation sites that activate stress kinases, the present results suggest activation of stress kinases as putative mediators of tau phosphorylation of neurites surrounding amyloid plaques. Similar findings have been reported for amyloid plaques in the single APP mutant (Puig et al., 2004). In contrast, active stress kinases were rarely encountered in neuronal bodies of target areas thus suggesting a discrete participation of stress kinases in the abnormal tau phosphorylation of neurons.

The possible effect of amyloid peptide aggregates on tau phosphorylation and aggregation has been analyzed by comparing these features in three different mice: one expressing a mutated form of APP, which overexpress β amyloid; a second one expressing a mutated form of tau protein that could result in the formation of tau polymers; and the double transgenic mouse that is generated after crossing the first two. In the APP transgenic mouse, an increase in the phosphorylation of a non-proline directed site was found, suggesting a role for amyloid peptide in this phosphorylation. Additionally, a slight increase, compared with the control mouse, was found in the APP transgenic mouse for a proline directed site, like that recognized by AT8, and, more evidently, in that site recognized by antibody 422, whose phosphorylation may be induced by Aβ (Ferrari et al., 2003).

In the tau transgenic mouse, as previously described (Lim et al., 2001), an increase in the phosphorylation at proline directed sites was observed at 9-month-old mice, and this increase can be correlated with the presence of aberrant tau aggregates, as also previously described (Lim et al., 2001). For the tau transgenic mouse the increased phosphorylation of that protein could be due mainly to an inhibition of phosphatase PP2A, as previously indicated (Gong et al., 1993; Goedert et al., 2000). On the other hand, the main phosphorylation could be due, at least in part to the action of GSK3 protein (Pérez et al., 2002, 2003). In the double transgenic mice, a main role for non-proline direct phosphorylation has been described (Santa-Maria et al., 2004). The consequence of that non-proline directed sites phosphorylated kinases in the double
transgenic mice correlates with an increase in tau aggregates that could be assembled by a possible mechanism different from those found in tauVlw. In this way, differences in the diameter of the filaments assembled in single (around 2 nm) and in double (around 10 nm) transgenic mice were observed. It is not known if the 2 nm filaments could be the precursors that facilitate the assembly of the 10 nm tau filaments.

Taking together our results we can suggest that in our double transgenic mouse two different, and independent, types of tau phosphorylation could occur. In one type amyloid peptide could facilitate non-proline directed phosphorylation. It is know that H9252 amyloid pathology is evident at 6 – 8 months in APPsw mice, and it could proceed to tau phosphorylation. Also, tau phosphorylation could occur at other different sites, independently of the presence of A\beta. This type of phosphorylation is mainly a proline directed modification. In the first type, it is likely that a mimicking of the effect postulate in the amyloid cascade hypothesis (Selkoe, 1989; Hardy and Selkoe, 2002) occurs, whereas in the other case, the effect could be that occurring in other type of tauopathies, such as FTDP-17, in which the A\beta is absent.

The immunohistochemical analysis described in this work supports the earlier hypothesis, since in those places where amyloid deposits are present, an increase in the phosphorylation at non-proline directed sites of tau protein is found, whereas at those places where no amyloid plaques were found, only tau phosphorylation at proline direct sites was observed. When amyloid peptide is present, a new phosphorylation at the non-proline directed site, identified by ab 12E8, occurs. In the tau transgenic mouse, in which amyloid peptide is not present, only an increase in proline directed sites occurs.

Additionally, our data partially concur with the data of Gotz et al., 2001, and Lewis et al., 2001, since we found an increase in tau aggregates when amyloid peptide is present, and our data further suggest a mechanism for such increase based on the phosphorylation of tau at its tubulin-binding region. This may result in an increase of tau that is not bound to microtubules and which, therefore, could have a greater capacity for self-assembly into polymers.

In contrast to this, tau phosphorylation at Ser 422, which does not occur in the wild type mouse, takes place in the APPsw transgenic mouse, and this phosphorylation could be related to the toxic effects of A\beta (Ferrari et al., 2003). This phosphorylation at serine 422 (that could be mediated by a member of MAP kinase family; Morishima-Kawashima et al., 1995) does not occur in the tauVlw transgenic mouse where aberrant 2 nm tau filaments are found, although recently Ferrari et al. (2003) have related this type of phosphorylation with tau aberrant polymerization into 10 nm tau filaments.

Finally, serine 422 is also phosphorylated in the double transgenic mouse, probably as a consequence of the modification found in APPsw transgenic mice.

In summary, our results suggest a role for A\beta in facilitating the phosphorylation of tau at NPDp sites and in a slight increase in the amount of sarkosyl-insoluble tau polymers. The relation between these two features has recently been analyzed (Santa-Maria et al., 2004). Also, our results indicate that tau phosphorylation precedes its assembly and it is compatible with a correlation between those features (Pérez et al., 2003; Alonso et al., 2001).

Additionally, these results also support the data indicating that injections of fibrillar A\beta in the cortex of monkey result in tau phosphorylation at serine 262 (Geula et al., 1998). Also, they are consistent with the fact that in models for tauopathies like that for FTDP-17 (Lim et al., 2001), fibrillar tau polymers can be assembled in the absence of phosphorylation of ser 262, but when an overexpression of A\beta takes place, that residue is modified and an increase of tau polymers is found, as shown in this work. Thus, amyloid peptide could facilitate tau polymerization.

However, it has been shown that soluble amyloid \beta-protein is increased in frontotemporal dementia with
tau gene mutations (Vitali et al., 2004) and that intraneuronal amyloid peptide could accumulate in the endosomal-lysosomal vesicles (Planel et al., 2004), which may facilitate tau self-assembly (Lim et al., 2001). Preliminary experimentation suggests that in the double transgenic mice there is an increased amyloid protein aggregation compared with that for single APP transgenic mice (Ribé et al., unpublished observations). In this way, not only can amyloid peptide facilitate tau polymerization; in addition, the expression of tauVLW may promote amyloid aggregation.

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