Localization of active forms of C-jun kinase (JNK) and p38 kinase in Alzheimer's disease brains at different stages of neurofibrillary degeneration

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The principal protein component of paired helical filaments (PHFs) in Alzheimer disease is abnormally hyperphosphorylated tau (PHF-tau). The stress activated protein kinases JNK and p38 have been shown to phosphorylate tau at some sites only seen in PHF-tau. If JNK and p38 are involved in the abnormal hyperphosphorylation of tau, they should be activated in neurons undergoing neurofibrillary degeneration. In the present study, we determined the intracellular and regional distribution of the active forms of JNK and p38 kinase in entorhinal, hippocampal, and temporal cortices of brains staged for neurofibrillary changes according to Braak and Braak. Neurons with tangle-like inclusions positive for active forms of JNK and p38 kinase were found to appear first in the Pre-α layer of the entorhinal cortex, and then extend into other brain regions co-incident with the progressive sequence of neurofibrillary changes. The intraneuronal accumulation of active forms of JNK and p38 kinase apeared to precede the deposition of amyloid in the extracellular space. These data indicate that increased activation of the stress related kinases JNK and p38 occurs very early in the disease and might be involved in the intraneuronal protein phosphorylation/dephosphorylation imbalance that leads to neurofibrillary degeneration in Alzheimer disease.

Keywords: Alzheimer's disease, JNK, p38 kinase, neurofibrillary tangles

1. Introduction

In the Alzheimer disease (AD) brain, a large number of neurons are affected by the deposition of paired helical filaments (PHFs) in the form of neurofibrillary tangles (NFTs). On the basis of histological staining with silver, different stages of neurofibrillary pathology have been shown [1,3,9]. PHFs are composed mainly of abnormally hyperphosphorylated tau (PHF-tau) [13, 14,16,17]. Immunocytochemical studies with tau antibodies have identified early stages of tangle development in the AD brain. The earliest signs of neurofibrillary degeneration seem to be the appearance of hyperphosphorylated tau in neuronal cell bodies concomitant with the swelling of neuritic terminals. These silver-negative pretangle neurons have been observed at all stages of the disease [2,6,7,25,26]. The accumulation of PHF-tau in AD has been hypothesized to be the result of an altered tau protein kinase / phosphatase balance [14,18] with the equilibrium shifted towards phosphorylation in the AD brain. The activities of serine / threonine protein phosphatases (PP)-1 and PP-2A and tyrosine protein phosphatases are decreased in AD brain [11].

A large number of kinases can phosphorylate tau in vitro (for review, see [20]). However, sequential phosphorylations with selected kinases like PKA, CaMKII or cdk5 followed by GSK-3 are required to inhibit the binding of tau to microtubules [32,35]. Recent studies have shown elevated activities of cdc2 kinase and cdk5 in AD brain [23,34]. Furthermore, activated GSK-3

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and ERK have been localized to neurofibrillary tangles in AD hippocampus [26–28].

The stress-activated protein kinases C-jun amino-terminal kinase (JNK) and p38 kinase together with the p42/p44 extracellular signal-regulated protein kinase (ERK), belong to the family of mitogen activated protein (MAP) kinases [31]. These serine/threonine protein kinases are activated in response to a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus [4,10]. These kinases are distinguished by the sequence of the tripeptide dual phosphorylation motif that is required for activation, namely, Thr^{202}-Glu^{203}-Tyr^{204} for ERK, Thr^{183}-Pro^{184}-Tyr^{185} for JNK, and Thr^{180}-Gly^{181}-Tyr^{182} for p38.

A decrease in the phosphoserine/phosphothreonine phosphatase and phosphotyrosine phosphatase activities [11], and an increase in the markers of oxidative stress in AD brain [33] warranted an investigation of the stress activated protein kinases in the disease. We carried out an immunocytochemical investigation of the activated JNK and P38 kinase in AD brains staged for neurofibrillary degeneration by Braak staging [6] and found the presence of the activated JNK and p38 at very early stage of neurofibrillary degeneration. These kinases were detected first in the entorhinal cortex and then with progressive stages of the disease in other brain regions.

2. Materials and methods

2.1. Antibodies

Affinity-purified rabbit antibodies to active JNK (phospho JNK) and to active p38 kinase (phospho p38) were purchased from New England Biolabs, Inc., Beverly, MA. According to the supplier phospho-JNK antibody detects the phosphorylated threonine 183 and tyrosine 185 of the active form of p54/p46 JNK, but does not cross-react with either activated ERK1/2 or p38 kinase; phospho-p38 detects phosphorylated threonine 180 and tyrosine 182 of the active form of p38 kinase but does not cross react with the corresponding phosphorylated forms of either JNK or ERK1/2. The mouse monoclonal antibody (mAb) AT8 to phosphorylated serine 202 and threonine 205 of PHF-tau was obtained from Innogenetics (anti-human PHF-tau, Zwijndrecht, Belgium).

Table 1
Sex, age and neuropathological staging of accumulation of both amyloid deposits (A–C) and neurofibrillary (NF) changes (I–VI)

No.	Sex	Age	Amyloid deposits	NF changes
1	F	50	0	0
2	M	55	0	0
3	M	62	0	0
4	M	67	0	0
5	F	53	0	I
6	F	68	0	I–II
7	M	70	0	I–II
8	F	66	0	II
9	F	86	0	II
10	F	86	0	III
11	F	93	0	IV
12	M	85	A	IV
13	F	60	C	V
14	M	75	C	V
15	?	91	C	V
16	?	?	C	V

2.2. Materials

Tissue blocks from 16 cases were obtained at routine autopsy (Table 1). Blocks of temporal lobe including the entorhinal and temporal cortices, hippocampal formation and / or amygdala were fixed by immersion in a mixture of 4% paraformaldehyde and picric acid, pH 7.0 [6]. All tissue blocks were subsequently kept frozen until use. Tissues were sectioned at 50–100 μm.

2.3. Brain staging

Aldehyde fuchsin-Darrow red staining was used for topographic orientation [5]. Two sections were stained by the Gallyas silver-iodide technique for demonstration of neurofibrillary changes [9] and by immunocytochemistry with mAb AT8 for demonstration of PHF-tau pathology [7]. Demonstration of amyloid deposits was made by selective silver staining [8].

All cases were classified by applying the histopathological staging system for neurofibrillary changes and amyloid deposition, as described previously [6]. This staging procedure permits the differentiation of six stages (Table 1) with increasing severity of neurofibrillary changes, mainly in the entorhinal cortex / hippocampal formation. The transentorhinal stage I was defined by the selective involvement of NFTs and numerous dendritic neuropil threads (NTs) in projecting cells residing within the transentorhinal region. Accentuated transentorhinal pathology and a very mild involvement of the entorhinal Pre-α and Ammon's horn sector is seen in the transentorhinal stage II. The limbic stages, III/IV, showed severe changes in the entorhinal region and hippocampal formation in addition to the

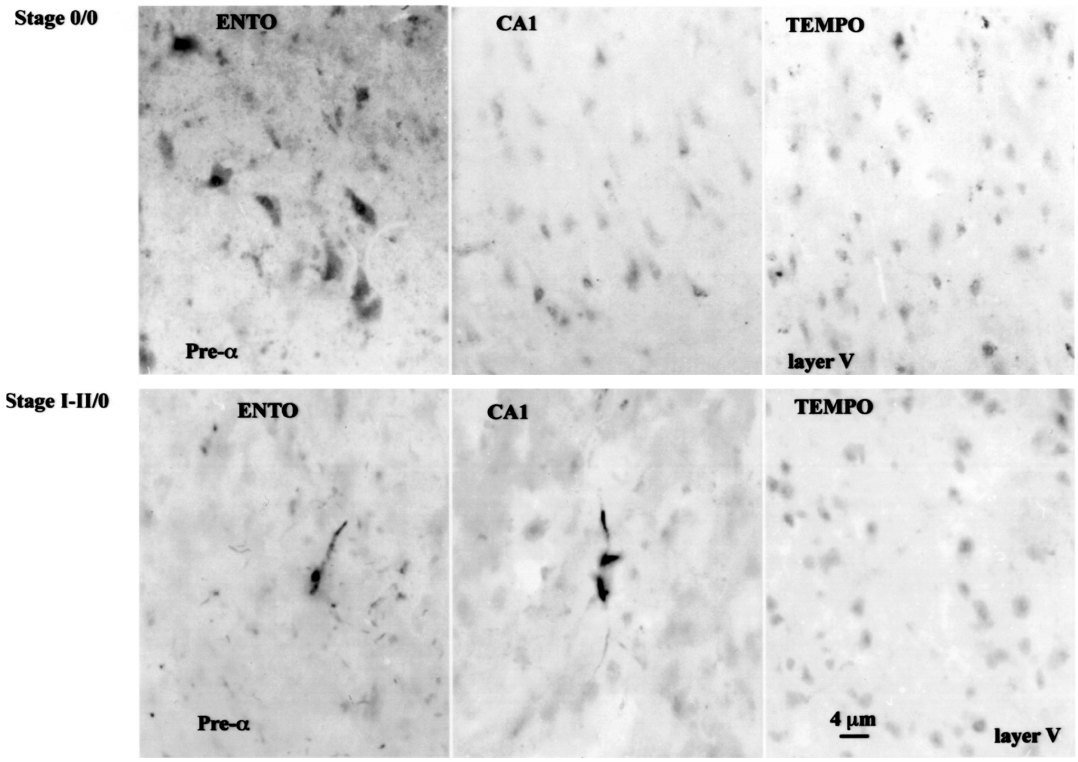

Fig. 1. Immunoreactivities of the active form of JNK/SAPK in brains at stage 0/0 and I–II. ENTO, entorhinal cortex; CA1, hippocampal CA1 sector; TEMPO, temporal cortex. Stage 0/0, case 1. Stage I–II; case 6.

transentorhinal regions, with changes extending within the limbic system. The isocortical stages, V/VI, were characterized by severe widespread destruction of limbic regions and in addition involvement of the isocortical association areas.

Tissues were also classified with respect to the extent of amyloid deposition [6]. In this classification, the term amyloid refers to plaque-like deposits with or without a neuritic component. Stage 0 was characterized by the total absence of amyloid deposits. Stage A showed a few plaques in the basal isocortex. Stage B showed many plaques in the basal isocortex and allocortex. Stage C showed large numbers of plaques in all parts of the cortex.

2.4. Immunocytochemistry

Immunostaining of free floating frozen sections was performed using procedures described previously [7] with some modifications. Briefly, incubations were performed for 40–44 h at 4°C with mAb AT8, at a dilution of $1:2000$, or rabbit antibodies to the active forms of JNK and p38 kinase at $1:100$. Sections were then incubated with biotinylated anti-mouse IgM or anti-rabbit IgG at a dilution of $1:200$ for 2h, and visualized with the avidin-biotin-peroxidase complex kit (Vector, Burlingame, CA) with 3-3′-diaminobenzidine-4 HCl/H_20_2 (DAB, Sigma, St. Louis, MO) as substrate. Double immunofluorescent staining was also used, with CY^{TM}3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to stain bound active JNK and p38 kinase antibodies, and CY^{TM}2- conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to stain bound mAb AT8.

3. Results

3.1. Distribution of active forms of JNK and P38 kinase in brains with stage 0 neurofibrillary changes

In the non-pathological stage 0/0 control cases without PHF tau immunoreactivity and no amyloid depositions, pale staining in the neuronal cytoplasm and relatively strong staining in nuclei for active JNK was found. Pyramidal neurons in layer Pre-α of the en-

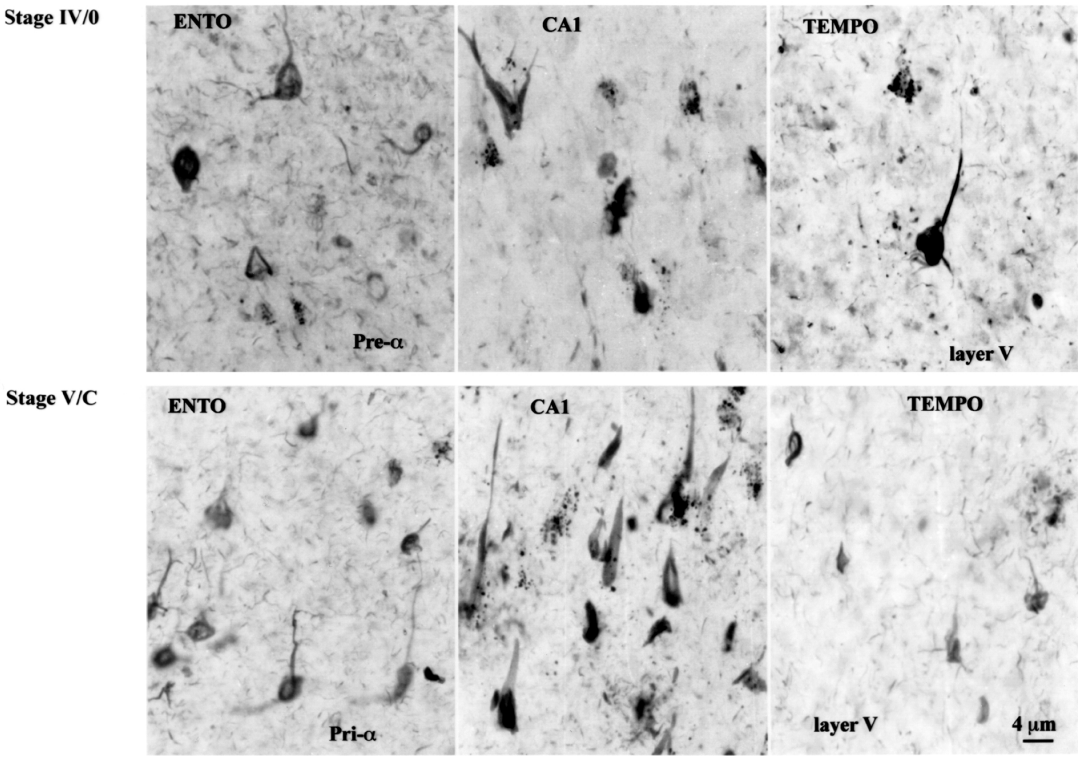

Fig. 2. Immunoreactivities of the active form of JNK/SAPK in brains at stage IV/0 and V/C. ENTO, entorhinal cortex; CA1, hippocampal CA1 sector; TEMPO, temporal cortex. Stage IV/0, case 11. Stage V; case 13.

torhinal cortex (Fig. 1, Stage 0/0) were more intensely stained than the pyramidal neurons of the CA1 area of the hippocampus and of layer V of the temporal cortex (Fig. 1, Stage 0/0). With antibody to active p38 kinase, only faint staining in the cytoplasm of Pre-α layer pyramidal neurons of the entorhinal cortex was observed, whereas the staining in CA1 and temporal cortex was negative (Fig. 3, Stage 0/0). AT8 staining was not observed in any of these cases.

3.2. Distribution of active forms of JNK and P38 kinase in brains with stage I–II neurofibrillary changes

In brains with transentorhinal stages I and II, a few neurons which contained irregular fibrous strands positive for active JNK were found in the Pre-α layer of the entorhinal cortex and the CA1 sector of the hippocampus (Fig. 1, Stage, I–II), while pyramidal neurons of the temporal cortex (Fig. 1, TEMPO of Stage I–II) were similar to the controls (stage 0). In addition to these inclusions, weakly stained thick and thin fibers, dots, and rods, were also detected in the Pre-α layer of the entorhinal cortex and the CA1 sector of the hippocampus (Fig. 1, Stage I–II).

In the case of active P38 kinase some of the immunopositive inclusions observed resembled classical neurofibrillary tangles. They appeared in the Pre-α layer of the entorhinal cortex and the hippocampal CA1 sector, but not in the temporal cortex (Fig. 3, Stage II/0).

A few neurons with various sizes and numbers of intensely stained, coarse granules and diffuse material that were positive for active P38 kinase were seen in the hippocampal CA1 sector (Fig. 3, Stage II/0). This pattern of neurons was not seen in sections stained with the antibody to active JNK. Some fibers in the neuropil were also positive for active P38 kinase. However, compared to JNK, their number was less (data not shown).

3.3. Distribution of active forms of JNK and p38 kinase in brains with stage III–IV neurofibrillary changes

As compared with transentorhinal stages I and II, the brains with the limbic stages III and IV showed increased numbers of JNK or P38 kinase positive tangle-

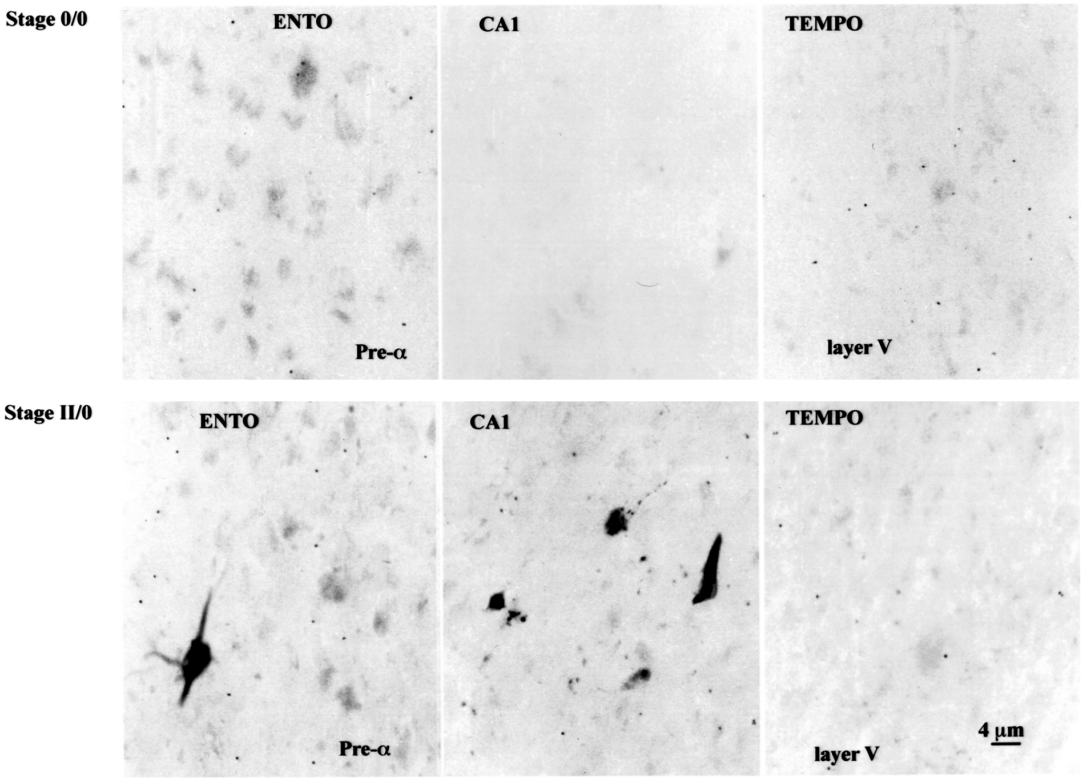

Fig. 3. Immunoreactivities of the active form of p38 in brains at stage 0/0 and II/0. ENTO, entorhinal cortex; CA1, hippocampal CA1 sector; TEMPO, temporal cortex. Stage 0/0, case 4. Stage II/0; case 8.

like structures in the Pre-α layer of the entorhinal cortex, and the CA1 sector of the hippocampus (Fig. 2, Stage IV/0, Fig. 4, Stage IV/A). This staining pattern was not dependent upon the presence of amyloid. Active JNK or P38 kinase-stained tangles appeared also in layers Pre-β, Pre-γ, Pri-α Pri-β, Pri-γ of the entorhinal cortex (data not shown) and layers III (data not shown) and V of the temporal cortex (Fig. 2, Stage IV/0; Fig. 4, Stage IV/A). A number of neurons with coarse granules positive for active JNK or active P38 kinase were seen in the Pre-α layer of the entorhinal cortex, the CA1 sector of the hippocampus, and III (data not shown) and layer V of the temporal cortex (Fig. 2, Stage IV/0; Fig. 4, Stage IV/A). A larger number of fibers in the neuropil was positive for active JNK as compared with active P38 kinase.

Double immunofluorescent staining showed that active P38 kinase and JNK were co-localized with tau abnormally hyperphosphorylated at Ser-202/Ser-205 (AT8 site) but also stained a number of neurons that were negative with AT8 (data not shown).

3.4. Distribution of active forms of JNK and p38 kinase in brains with stage V/VI neurofibrillary changes

In the isocortical stages V and VI, large numbers of active JNK or P38 kinase positive tangles, and neurons containing intensely stained coarse granules and fibers in the neuropil were seen as compared to the limbic stages III and IV. These changes were most prominent in layers Pre-α to Pre-γ, and Pri-α to Pri-γ of the entorhinal cortex, the hippocampal CA1 sector and layers III (data not shown) and V of the temporal cortex (Figs 2, and 4, Stage V/C). These changes were especially prominent in the CA1 sector of the hippocampus (Figs 2 and 4, Stage V/C).

4. Discussion

A number of kinases have been shown to phosphorylate tau at some of the same sites at which PHF tau is phosphorylated. However, it appears that only upon sequential phosphorylation with GSK-3 a number of ad-

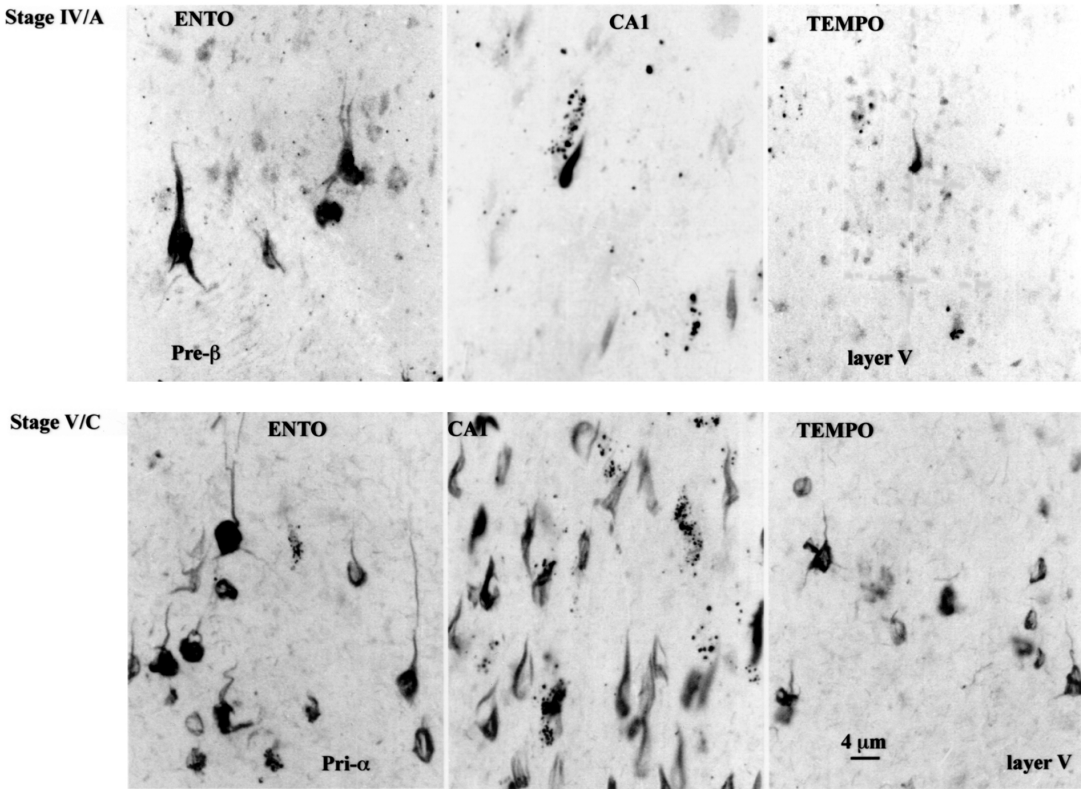

Fig. 4. Immunoreactivities of the active form of p38 in brains at stage IV/A and V/C. ENTO, entorhinal cortex; CA1, hippocampal CA1 sector; TEMPO, temporal cortex. Stage IV/0, case 12. Stage V; case 13.

ditional sites are phosphorylated and the binding of tau to microtubules and its ability to promote microtubule assembly are inhibited [32,35].

Amongst others the stress-activated JNK and p38 kinase have the ability to phosphorylate tau in vitro at several sites seen in PHF-tau [29,30]. The association of active p38 with neurofibrillary tangles and the activation of MAP kinase pathways had also been reported recently [37,38]. Furthermore, the accumulation of activated p38 kinase has been recently shown in neurons affected by NFTs [15]. The present study was concerned with elucidating whether the occurrence of the activated stress kinases JNK and p38 in situ, might correspond to the different stages of neurofibrillary pathology seen in AD. We studied by immunohistochemistry the distribution of both activated JNK and p38 in the entorhinal cortex, hippocampus and temporal cortex from a series of AD brains at different stages of neurofibrillary changes, as defined by the Braak staging [6].

Based on the immunoreactivities of antibodies to active forms of JNK and p38 kinase, structures positive for these kinases were observed in the earliest to late stages of the disease. We also found similar structures with antibodies to activated MAP kinase (Pei, et al., in preparation). In the non pathological control cases only JNK but not p38 kinase seemed to be activated. The co-labeling of active JNK or active p38 kinase with NFT was seen in the later stages of the disease. This type of immunostaining which has also been found to be preferentially associated with active but not with inactive GSK-3 [26], active MAP kinase [27,28], cdc2 [34], cdk5 [24,36] and PKA [19] is reminiscent of classical NFTs, as visualized by silver impregnation, or by immunocytochemistry with mAbs Tau-1 or AT8 [2,7]. Taken together, these data suggest that the development of NFT in neurons is progressive, and that the different immunostained structures may represent different stages of neurons undergoing neurofibrillary degeneration.

We also found that the appearance and number of JNK or p38 kinase positive neurons paralleled the development of AD-related neurofibrillary degeneration in both sequence and topography [6]. These data suggest that JNK and p38 kinase amongst others may be involved in the hyperphosphorylation of PHF-tau, particularly at an early stage. It therefore appears that

the aberrant neuronal accumulation of activated JNK and p38 kinase coincides with the progression of NFT development, and suggests that these two kinases may play a role in pre-phosphorylation of tau which may make it a more favorable substrate for subsequent phosphorylation by other protein kinases like GSK-3.

JNK and p38 kinase can be activated by dual phosphorylation on a specific domain following exposure to inflammatory cytokines, UV radiation, heat and osmotic shocks. Furthermore, the activation of p38 kinase has been observed in microglia upon contact with Aβ [22]. This finding has been used to support the hypothesis that the pronounced gliosis around amyloid plaques may render neighboring neurons subject to inflammatory cytokines in AD brain [15,29,30]. However, in the present study we observed that the activation of JNK and p38 kinase occurred in stages I and II–III brains which did not show deposition of $beta$-amyloid suggesting that amyloid might not be necessary to induce inflammatory reactions in the vicinity of the affected neurons. It, therefore, might be more likely that other factors than β-amyloid might have induced the activation of stress kinases at these early stages. A major likely causative factor for the activation of JNK and p38 and other tau kinases of the AD brain, is the reduced activity of both PP-2A/PP1 and tyrosine phosphatases which will keep the tau kinases in phosphorylated/activated state [11,12] and, in the case of PP2A/PP1, also result in hyperphosphorylated tau.

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