RESEARCH ARTICLE

Phosphorylated Protein Kinases Associated with Neuronal and Glial *Tau* Deposits in Argyrophilic Grain Disease

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Tau **phosphorylation was examined in argyrophilic grain disease (AGD) by using the phospho-specific *tau* antibodies Thr181, Ser202, Ser214, Ser 396 and Ser422, and antibodies to non-phosphorylated and phosphorylated mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK), stress-activated kinase (SAPK), c-Jun N-terminal kinase (JNK), p38 kinase (p-38), α-calcium/calmodulin-dependent kinase II (αCaM kinase II), and glycogen synthase kinase-3 (GSK-3), all of which regulate phosphorylation at specific sites of *tau*. This is the first study in which the role of protein kinases in *tau* phosphorylation has been examined in AGD.**

Hyperphosphorylated *tau* accumulated in grains and pre-tangles in the hippocampus, dentate gyrus, entorhinal and trans-entorhinal cortices, and amygdala in all cases. Ballooned neurons in the amygdala, entorhinal, insular and cingulate cortex, and claustrum contained αB-crystallyn and phosphorylated neurofilament epitopes. Some astrocytes and scattered oligodendrocytes containing coiled bodies were recognized with anti-*tau* antibodies. A few tangles were observed in the entorhinal cortex and hippocampus corresponding to Alzheimer's disease (AD) stages I-III of Braak and Braak. None of the present cases was associated with progressive supranuclear palsy or with α-synuclein pathology. Two bands of phospho-*tau* of 64 and 68 kDa were observed in Western blots of sarkosyl-insoluble fractions enriched with abnormal filaments in AGD, a pattern that contrasts with the 4-band pattern obtained in AD.

No modifications in the expression of non-phosphorylated MEK-1, ERK2 and GSK-3α/β, as revealed by immunohistochemistry, were seen in AGD, but sarkosyl-insoluble fractions were particularly enriched in JNK-1 and αCaM kinase II. Increased expression of the phosphorylated (P) forms of MAPK/ERK, SAPK/JNK, p38 and GSK-3β was found in grains and *tau*-containing cells in AGD. MAPK/ERK-P immunoreactivity was observed in pre-tangles and, diffusely, in the cytoplasm of ballooned neurons, but not in grains. Strong SAPK/JNK-P and P38-P, and moderate GSK-3b-P immunoreactivities selectively occured in grains, in neurons with pre-tangles and in the peripheral region of the cytoplasm of ballooned neurons. MAPK/ERK-P, SAPK/JNK-P, p38-P and GSK-3β-P were expressed in *tau*-containing astrocytes and in oligodendrocytes with coiled bodies. Western blots revealed kinase expression in sarkosyl-insoluble fractions but none of the phospho-kinase antibodies recognized hyper-phosphorylated *tau* protein.

These findings indicate complex, specific profiles of *tau* phosphorylation and concomitant activation of precise kinases that have the capacity to phosphorylate *tau* at specific sites in AGD. These kinases co-localize abnormal *tau* in selected structures and cells, including neurons with pre-tangles, ballooned neurons, astrocytes and oligodendrocytes.

Most of these kinases are involved in cell death and cell survival in certain experimental paradigms. However, double-labeling studies with the method of in situ end-labeling of nuclear DNA fragmentation and cleaved (active) caspase-3 immunohistochemistry show no expression of apoptosis and death markers in cells bearing phosphorylated kinases.

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Introduction

Braak and Braak described a demential disorder characterized by the presence of spindle-shaped argyrophilic grains loosely scattered throughout the neuropil in the CA1 area of the hippocampus and layer pre-β of the entorhinal cortex. Grains were also abundant in the adjacent temporal cortex, insular cortex, orbitofrontal cortex, amygdala and hypothalamic lateral tuberal nucleus. In addition, oligodendroglial coiled bodies were a constant accompanying feature (5, 6).

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	Age	Gender	Dementia	p-d	PFA	Formalin	Frozen	Braak stages	βA4
1	64	M	No	2	Yes	Yes	Yes	I	No
2	81	W	Yes	4	Yes	Yes	Yes	I	No
3	82	W	Yes	4	Yes	Yes	No	III	No
4	78	M	Yes	6	No	Yes	No	I	No
5	67	M	Yes	3	Yes	Yes	Yes	I	No
6	68	F	No	5	Yes	Yes	Yes	I	No
7	80	W	Yes	15	No	Yes	No	II	No
8	90	W	Yes	12	No	Yes	No	II	No
9	75	M	No	10	No	Yes	No	I	No
10	79	W	No	15	No	Yes	No	I	No
11	90	M	Yes	5	No	Yes	No	II	No

Table 1. Summary of cases examined in the present series. No isocortical *tau* pathology was seen in any case. Patients with PSP or with α-synuclein pathology were not included in this series. M: man, W: woman. p-d: post-mortem delay in hours. PFA: 4% paraformaldehyde fixation for 48 hours, paraffin embedding.

The terms argyrophilic grain disease (AGD), dementia with argyrophilic grains and dementia with grains have all been used to designate this form of dementia (38). Although grains may be encountered in brains from subjects without cognitive decline, they are quite common in association with dementia. An earlier study suggests that early sub-clinical lesions are limited to the anterior hippocampus, whereas the impairment of cognitive functions is related to the more widespread extension of grains in limbic areas (85). Recent studies further suggest that AGD is much more common in older subjects than was previously thought (7, 78, 82).

Neurofibrillary tangles corresponding to early Braak stages I-III in Alzheimer's disease (AD) are present in the majority of cases of AGD (7, 78, 82), while Aβ-amyloid deposits are either absent or present in only small amounts when compared to AD (80). In some cases, argyrophilic grains may also be encountered in combination with AD, dementia with tangles, progressive supranuclear palsy (PSP) and several α-synucleinopathies including Parkinson's disease, diffuse Lewy body disease and multiple system atrophy (35, 38, 49, 80).

The main protein constituent of grains and coiled bodies is the microtubule-associated protein *tau* in an abnormally hyper-phosphorylated state (79, 86). Moreover, abundant pre-tangle projection neurons have been described in limbic areas of AGD cases, and recent studies suggest that grains are preferentially localized in dendrites (35) and dendritic side branches of these pre-tangle neurons (81). By electron microscopy, it is seen that the grains consist of clustered filaments and straight tubules, but lack the paired helical filaments characteristic of AD (6, 35). Coiled bodies consist of accumulations of fibrils (35, 92). In addition to coiled bodies, *tau*-immunoreactive and αB-crystallin expressing neurons have been described as a constant feature of the amygdala in AGD cases (84). Moreover, along with coiled bodies, glial *tau* pathology in AGD is also found in astrocytes (4).

Abnormal *tau* phosphorylation and deposition in the cytoplasm of neurons and glial cells is a major biochemical and structural singularity in tauopathies, including AD, PSP, corticobasal degeneration (CBD) and Pick's disease (PiD) (9, 10, 21, 31, 41, 84). Yet individual tauopathies have clinical, neuropathological and biochemical particularities. Phosphorylation of *tau* occurs at different sites, and phosphorylated bands from homogenates of fractions enriched with paired helical filaments or with abnormal filaments show particular patterns. AD displays 4 bands of 72/74, 68, 64 and 60 kDa, whereas PSP and CBD are resolved into 2 bands of 68 and 64 kDa. PiD shows 2 bands of 64 and 60 kDa. Following dephosphorylation of the enriched fractions, all 6 *tau* isoforms are retrieved in AD. The isoforms encoded by exon 10, but not the isoforms from transcripts without exon 10, are recovered in PSP and CBD; therefore, PSP and CBD are 4-repeat tauopathies. Isoforms encoded by exon 10 are not detected following dephosphorylation in PiD (9, 10, 11, 30, 44, 65, 66). Recent studies have shown that AGD produces two bands of 68 and 64 kDa and that AGD is a 4-repeat tauopathy (79, 86).

Tau proteins contain phosphorylation sites for a large number of protein kinases, including protein kinase A (PKA), protein kinase C, cyclin-dependent kinase 5, glycogen synthase kinase-3β (GSK-3β), mitogen-activated extracellular signal-regulated protein kinases (MAPK/ERK), stress-activated protein kinases (SAPK/JNK), p38 kinases, calcium/calmodulin-dependent kinases (CaM kinase II), microtubule affinity-regulating kinase (MARK), and casein kinases. All these kinases have the ability to phosphorylate *tau* in vitro at specific sites (9, 10, 18, 29, 31, 40, 44, 46, 57, 58, 68).

Several studies have dealt with the expression and localization of certain kinases related with *tau* phosphorylation in the brain in AD (22, 24, 25, 42, 52-54, 62, 91, 95, 96). Less is known about the expression and localization of kinases in the brain in other tauopathies (2, 22, 24, 25, 62). The expression of kinases in AGD is unknown.

The present study is focused on the expression of non-phosphorylated MEK (up-stream activator of ERK), ERK, αCaM kinase II and GSK-3α/β, and phosphorylated MAPK/ERK, SAPK/JNK, p38 and GSK-3β, as well as on the relationship between *tau* deposition and kinase expression, and cell death in AGD.

Material and Methods

Cases. Eleven cases (5 men and 6 women), mean age 77.6 years (age range 64-90 years), were included in this series. Seven patients were afflicted by dementia, while the cognitive state was apparently preserved in 4 cases. The post-mortem delay (p-d) between death and tissue processing varied from 2 to 15 hours. Frozen samples were available in 4 cases, all of them with a very short p-d (between 2 and 5 hours). Fixation of selected brain regions with 4% paraformaldehyde followed by saccharose cryoprotection and freezing was carried out in 5 cases. A summary is found in Table 1.

Five age-matched cases with no neurological evidence of disease and with a p-d ranging from 2 to 6 hours were used as controls. Four cases with AD stage V and VI of Braak and Braak were examined in parallel for comparative purposes.

General procedures. A complete neuropathological examination was carried out in every case (diseased cases and controls) in formalin-fixed tissue for no less than 3 weeks; the tissue was then embedded in paraffin. Dewaxed sections, 7 μm thick, were stained with haematoxylin and eosin, luxol fast blue-Klüver Barrera, methenamine silver (PAM) and Gallyas, or processed for immunohistochemistry following the avidin-biotin-peroxidase method (ABC kit, Vectastain, Vector). Antibodies to phosphorylated neurofilaments of 170 kD or 200 kD (clones BF10 and RT97, Boehringer-Mannheim) were used at dilutions of 1:100 and 1:50, respectively. Antibodies to pan-*tau* (Sigma) were used at a dilution of 1:10. The pan-*tau* antibody is derived from the hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunized mouse. Purified bovine microtubule-associated proteins were used as immunogens. Several electrophoretic bands were encountered on SDS polyacrylamide gels (55 kDa-62 kDa). The pan-*tau* antibody recognizes phosphorylated *tau* in paraffin sections. Antibodies to glial fibrillary acidic protein (GFAP, Dako, Dakopats), βA4-amyloid (Boehringer-Mannheim), and ubiquitin (Dako) were used at dilutions of 1:250, 1:5, and 1:200, respectively.

Control brains showed no abnormalities excepting a few βA4-amyloid plaques in the hippocampus in two cases.

For gel electrophoresis and Western blotting, fresh samples from 4 cases (cases 1, 2, 5 and 6, Table 1) were immediately obtained at autopsy, frozen in liquid nitrogen, and stored at -80ºC until use.

For specific immunohistochemical studies, fresh samples from the hippocampus, entorhinal cortex and amygdala in five cases were fixed with 4% paraformaldehyde in PBS for 48 hours and embedded in paraffin. Specific immunohistochemical studies were carried out in four additional cases fixed in formalin for several days.

Filament isolation in the AGD hippocampus. Gel electrophoresis and Western blotting of total homogenates and fractions enriched with filaments. Fresh samples from the hippocampus were homogenized in buffer containing 100 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium decholate, 0.1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 mg/ml aprotinin, leupeptin, and pepstatin (Sigma). These were used as total hippocampal homogenates.

A protocol to purify paired helical filaments in AD, described by Goedert et al (30), was used in AGD. Frozen samples of the hippocampus (of about 5 g) were cut into pieces. They were gently homogenized in a glass tissue grinder in 10 vol (w/v) of cold suspension buffer consisting of 10 mM Tris-HCl (pH = 7.4), 0.8 M NaCl, 1 mM EGTA, 10% sucrose, 0.1mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin and 5 μg/ml pepstatin. The homogenates were first

centrifuged at 20 000 × g, and the supernatant (S1) was retained. The pellet (P1) was re-homogenized in 5 vol of homogenization buffer and re-centrifuged. The 2 supernatants (S1+S2) were then mixed and incubated with N-lauroylsarcosinate 1% for 1 hour at room temperature while shaking. Samples were then centrifuged for 1 hour at 100 000 × g in a Ti 70 Beckman rotor. Sarkosyl-insoluble pellets (P3) were re-suspended (0.2 ml per g of starting material) in 50 mM Tris-HCl (pH = 7.4). Protein concentrations were determined by the BCA method and 10% sodium dodecyl sulphate-poliacrylamide gel electrophoresis (SDS-PAGE) was run using a maxi-protean system (Bio-Rad). One hundred to 200 μg of protein was loaded in each lane with loading buffer containing 0.125 M Tris (pH=6.8), 20% glycerol, 10% mercaptoethanol, 4% SDS and 0.002% bromophenol blue. Samples were heated at 95ºC for 5 minutes prior to gel loading. Total hippocampal homogenates and fractions enriched with abnormal filaments were run in parallel. The proteins were then transferred to nitrocellulose membranes (Amersham) using an electrophoretic chamber system (Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). Non-specific binding sites were blocked with Tris-buffered saline solution pH=7.4 with 0.1% Tween-20 (TBST) containing 5% skimmed milk for 30 minutes, and incubated with one of the primary antibodies for 1 hour at room temperature. Control of protein content in each lane was carried out by the staining of selected gels with Coomassie blue and of the membranes with Ponceau (Sigma).

The monoclonal antibody to pan-*tau* (Sigma) was used at a dilution of 1:500. The rabbit polyclonal antibody to phospho-*tau*Thr181 was diluted 1:250. The rabbit polyclonal antibodies anti-phospho-*tau*Ser202, Ser214, Ser262, Ser396 and Ser422 (all from Calbiochem) were used at a dilution of 1:2500.

The anti-MEK-1 mouse monoclonal antibody (MI17020, Transduction Laboratories) is raised against amino acids 1-124 at the amino terminal region of human MEK-1. The antibody recognises a unique band of 45 kDa on Western blots of hippocampal homogenates. The antibody was used at a dilution of 1:500. The anti-ERK mouse monoclonal antibody (E17120, Transduction Laboratories) is raised against amino acids 219-358 at the carboxy terminal region of rat ERK2. The antibody recognises a unique band of 42 kDa on Western blots that corresponds to the expected molecular weight of ERK2. The antibody was used at a dilution of 1:2000. The rabbit polyclonal c-Jun N-terminal kinase-1 (JNK-1) (Santa Cruz Biotechnology), produced against the full length (amino acids 1-384) of human JNK-1, recognises a unique band of 46 kDa on Western blots. The antibody was used at a dilution of 1:200. The anti-αCaM kinase II monoclonal antibody (clone CBα-2, Zymed) is specific for α subunit of CaM kinase II. The antibody does not cross-react with other CaM kinase II isoforms. The antibody was used at a dilution of 1:150. The antibody recognises a unique band of 54 kDa corresponding to the molecular weight of αCaM kinase II. The anti-GSK-3α/β monoclonal antibody (StressGen) reacts with 51 and 47 kDa proteins corresponding to the expected molecular weight of GSK-3α and GSK-3β in human hippocampal homogenates. The antibody was used at a dilution of 1:500.

The anti-MAP kinase phospho-specific (MAPK/ERK-P) rabbit polyclonal antibody (Calbiochem) is raised against a synthetic phospho-tyrosine peptide corresponding to residues 196 to 209 of human p44 MAP kinase. The antibody detects phosphorylated Tyr204 of p44 and p42 MAP kinases (ERK1 and ERK2). The purified phospho-p38 MAP kinase (Thr180/Tyr182) (p38-P) rabbit polyclonal antibody (Cell Signaling) detects p38 MAP kinase only when activated by dual phosphorylation at Thr180 and Tyr182. The purified rabbit polyclonal phospho-SAPK/JNK (Thr183/Tyr185) antibody (SAPK/JNK-P) (Cell Signaling) is produced against a synthetic phospho-Thr183/Tyr185 peptide corresponding to the residues of human SAPK/JNK. The antibody detects SAPK/JNK only when activated by phosphorylation at Thr183/Tyr185.

The MAPK/ERK-P antibody recognizes 2 bands of 44 and 42 kDa corresponding to phosphorylated ERK1 and ERK2, respectively. The p38-P antibody recognizes one band of 41/43 kDa, whereas the SAPK/JNK-P antibody recognizes one band of 54 kDa. The antibodies to MAPK/ERK-P and SAPK/JNK-P were used at a dilution of 1:150. The antibody to p38-P was used at a dilution of 1:200.

The anti-phospho-specific GSK-3βSer9 antibody (GSK-3β-P, Oncogene) is a rabbit polyclonal IgG antibody specific for the Ser9 phosphorylated form of glycogen synthase kinase-3β. The antibody recognizes one band of about 50 kDa in hippocampal homogenates. The antibody was used at a dilution of 1:150.

After washing, the membranes were incubated with the secondary antibody labeled with horseradish peroxidase (DAKO) diluted 1:1000 for 1 hour at room temperature, washed again, and developed with the chemiluminescence ECL Western Blotting system (Amersham). Membranes were then exposed to autoradiographic films (Hyperfilm ECL, Amersham).

Samples of the hippocampus from 2 patients with sporadic AD, stage V and VI of Braak and Braak, with post-mortem delays of 2 and 3 hours, were processed in parallel for comparative purposes.

Electron microscopy of filament-enriched fractions. Sarkosyl-insoluble pellets (P3) were fixed with 2% glutaraldehyde in phosphate buffer for 6 hours, washed in phosphate buffer/saccharose and embedded in araldite. Ultra-thin sections were stained with uranyl acetate and lead citrate.

Electron microscopy of the sarkosyl-insoluble fraction (P3) in case 1 showed that pellets were enriched with filaments and straight tubules measuring between 5 and 20 nm. These characteristics were consistent with abnormal *tau*-containing filaments reported in several neurodegenerative diseases (15). No paired helical filaments were observed in this sample.

Phospho-tau and kinase immunohistochemistry. Immunohistochemistry was carried out following the ABC method. De-waxed sections were first boiled in citrate buffer and then stored overnight at room temperature. After blocking endogenous peroxidase, the sections were incubated with normal serum and then incubated at 4ºC overnight with one of the primary antibodies.

The following phospho-specific *tau* rabbit polyclonal antibodies were used: Thr181, Ser202, Ser214, Ser396 and Ser422 (all of them from Calbiochem). The antibodies were used at a dilution of 1:100, excepting anti-phospho-*tau*Thr181, which was used at a dilution of 1:250.

The anti-MEK-1 mouse monoclonal antibody (MI17020, Transduction Laboratories) was used at a dilution of 1:500. The anti-ERK mouse monoclonal antibody (E17120, Transduction Laboratories) was used at a dilution of 1:2000. The rabbit polyclonal c-Jun N-terminal kinase-1 (JNK-1) (Santa Cruz Biotechnology) was used at a dilution of 1:200. The anti-αCaM kinase II monoclonal antibody (clone CBα-2, Zymed) was used at a dilution of 1: 150. The anti-GSK-3α/β monoclonal antibody (StressGen) was used at a dilution of 1:500. The anti-MAPK/ERK-P rabbit polyclonal antibody (Calbiochem), the SAPK/JNK-P antibody (Cell Signaling) and the anti-phospho-specific GSK-3β-P antibody (Oncogene) were used at a dilution of 1:100. The p38-P rabbit polyclonal antibody (Cell Signaling) was used at a dilution of 1:200.

Following incubation with the primary antibody, the sections were incubated for 1 hour with biotinylated anti-mouse or anti-rabbit IgG diluted 1:100, followed by ABC at a dilution of 1:100 for 1 hour at room temperature. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. Tissue from control and diseased brains was processed in parallel to avoid day-to-day variations in the staining procedure. Some sections were counterstained with haematoxylin.

Double-labeling immunohistochemistry was conducted by incubating the sections following a 2-step protocol. The sections were first incubated with antibodies to one of the kinases, and the immunoreaction was visualized with diaminobenzidine and hydrogen peroxide as before. Subsequently, the sections were incubated with anti-pan-*tau* (Sigma) at a dilution of 1:10, anti-phospho-*tau* (Thr181 or Ser422), or anti-GFAP (Dako) at a dilution of 1:250. Some sections were first incubated with anti-pan-*tau* antibodies and then with antibodies to phospho-specific kinases. Different subclass-specific secondary antibodies were used in double-labeling studies utilizing 2 primary monoclonal or 2 primary polyclonal antibodies. The immunoreaction was visualized with 0.01% benzidine hydrochloride, 0.025% sodium nitroferricyanide in 0.0001% M sodium phosphate buffer (pH 6.0), and 0.005% hydrogen peroxide, or with NH_4NiSO_4 (0.05 M) in phosphate buffer (0.1 M), diaminobenzidine, NH_4Cl, and hydrogen peroxide. The first primary antibody was recognized as a brown homogeneous precipitate, whereas the second primary antibody was recognized as a dark blue precipitate.

Quantitative studies. Semi-quantitative studies were carried out in double-labeled immunohistochemical sections to gain understanding about the number of cells in a given region that co-localized phosphorylated *tau* and a particular phosphorylated kinase. Counts were made in every case at a magnification of ×400 covering three sections per region. The values for co-localization with the first primary antibody (ie, MAPK/ERK-P) were expressed as the percentage of the total number of cells recognized by the second primary antibody (ie, phosphorylated tau).

In situ end-labeling of nuclear DNA fragmentation and caspase-3 immunohistochemistry. Some sections were stained with the method of in situ end-labeling of nuclear DNA fragmentation with ApopTag: in situ apoptosis detection kit (Oncor) following the instructions of the supplier. The cleaved caspase-3 (17 kDa) rabbit polyclonal antibody (Cell Signaling) is produced with a synthetic peptide (KLH coupled) corresponding to

residues surrounding the cleavage site of human caspase-3, and it detects only the large fragment of activated caspase-3. It does not recognize full-length caspase-3 or other caspases. The antibody was used at a dilution of 1:100 following the ABC method.

Double-labeling with the method of in situ end-labeling of nuclear DNA fragmentation or cleaved caspase-3 and MAPK/ERK-P, SAPK/JNK-P, p38-P and GSK-3β-P immunohistochemistry was carried out following a 2-step protocol, as previously described.

Results

General neuropathological findings. All cases were characterized by the presence of large numbers of argyrophilic (Gallyas), pan-*tau*-immunoreactive grains in the hippocampus, entorhinal and trans-entorhinal cortices, and amygdala. Pre-tangle neurons were seen in the same regions. In the adjacent white matter, Gallyas-stained and pan-tau-immunoreactive coiled bodies were present in various amounts. Ballooned neurons, negative with Gallyas, were present in the amygdala, entorhinal, insular, and cingulate cortex, and claustrum. Pan-*tau*-immunoreactive, Gallyas-negative astrocytes were found in the amygdala and entorhinal cortex.

In addition, thorn-shaped astrocytes (found in many tauopathies) were present in the sub-pial and periventricular regions.

A few tangles were observed in the entorhinal cortex and hippocampus in all cases corresponding to early Braak and Braak stages I-III. βA4 amyloid deposits were absent in all cases as revealed by βA4-immunohistochemistry. None of the present cases was associated with PSP or with α-synuclein pathology (Table 1).

Tau profiles in fractions enriched with abnormal filaments. Fractions enriched with paired helical filaments in 2 cases of AD processed in parallel for comparative purposes showed 4 typical bands of 72/74, 68, 64 and 60 kDa following gel electrophoresis and blotting. A similar profile was seen with several phospho-specific anti-*tau* antibodies in AD. In contrast, only 2 bands of 68 and 64 kDa were seen in sarkosyl-insoluble fractions in AGD. The intensity of the bands was variable from one antibody to another despite identical protein loading and time of exposure. Lower signals were obtained with the Ser214 antibody (Figure 1). The pattern of 2 bands was similar in the four AGD cases examined. Interestingly, the intensity of the band of 64 kDa was higher than the upper band in every case when using the anti-Thr181 antibody. Total homogenates processed in parallel did not show phospho-*tau* (Figure 2).

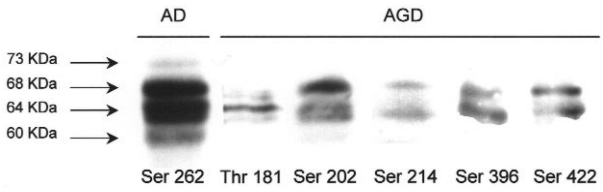

Figure 1. Gel electrophoresis and Western blotting of hippocampal fractions enriched with abnormal filaments, following the procedure of Goedert et al (29) to separate paired helical filaments in AD. Gels have been run using a Maxi-protean system, thus permitting the separation of bands ranging from 60 to 73 kDa of molecular weight. Homogenates of the hippocampus from AD have been processed in parallel for comparative purposes. AD shows four bands of 73, 68, 64, and 60 kDa. Samples of the AGD hippocampus show two bands of 68 and 64 kDa, excepting antibody Thr181 that shows a unique band of 64 kDa. The sample corresponds to case 1 (Table 1) with only a few neurofibrillary tangles in the entorhinal cortex. Total protein loading 100 μg, excepting Thr181 and Ser202, 200 μg.

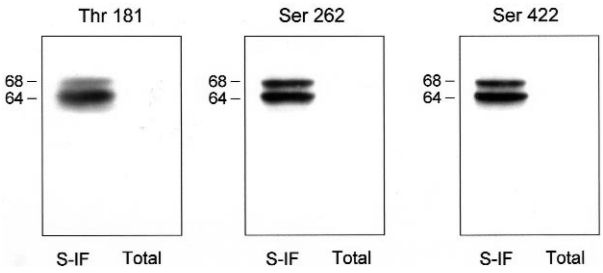

Figure 2. Western blots of sarkosyl-insoluble fractions (S-IF) and total hippocampal homogenates (Total) processed with anti-phospho-*tau* (Thr181, Ser262, Ser422). Two bands of phospho-*tau* of 68 and 64 kDa are found in sarkosyl-insoluble fractions, whereas no phosphor-*tau* is found in total homogenates. Note that the antibody Thr181 recognizes mainly the inner band of 64 kDa. AGD sample corresponds to case 5 in Table 1. Total protein loading 200 μg.

Tau immunohistochemistry with phospho-specific tau antibodies. *Grains, pre-tangles and tangles.* All phospho-specific tau antibodies used in the present study recognized grains, pre-tangles and tangles (Figure 3A-E). Antibodies to Thr181, Ser202 and Ser422 showed stronger staining of grains than antibodies to Ser214 and Ser396. Yet pre-tangles were heavily stained with antibodies to Thr181 and Ser396, whereas they were only moderately stained with antibodies to Ser202. A similar preferential staining was seen for neurons with pre-tangles and neurons with tangles.

Ballooned neurons in the amygdala. Phospho-specific anti-*tau* antibodies to Thr181, Ser202, Ser214, Ser396 and

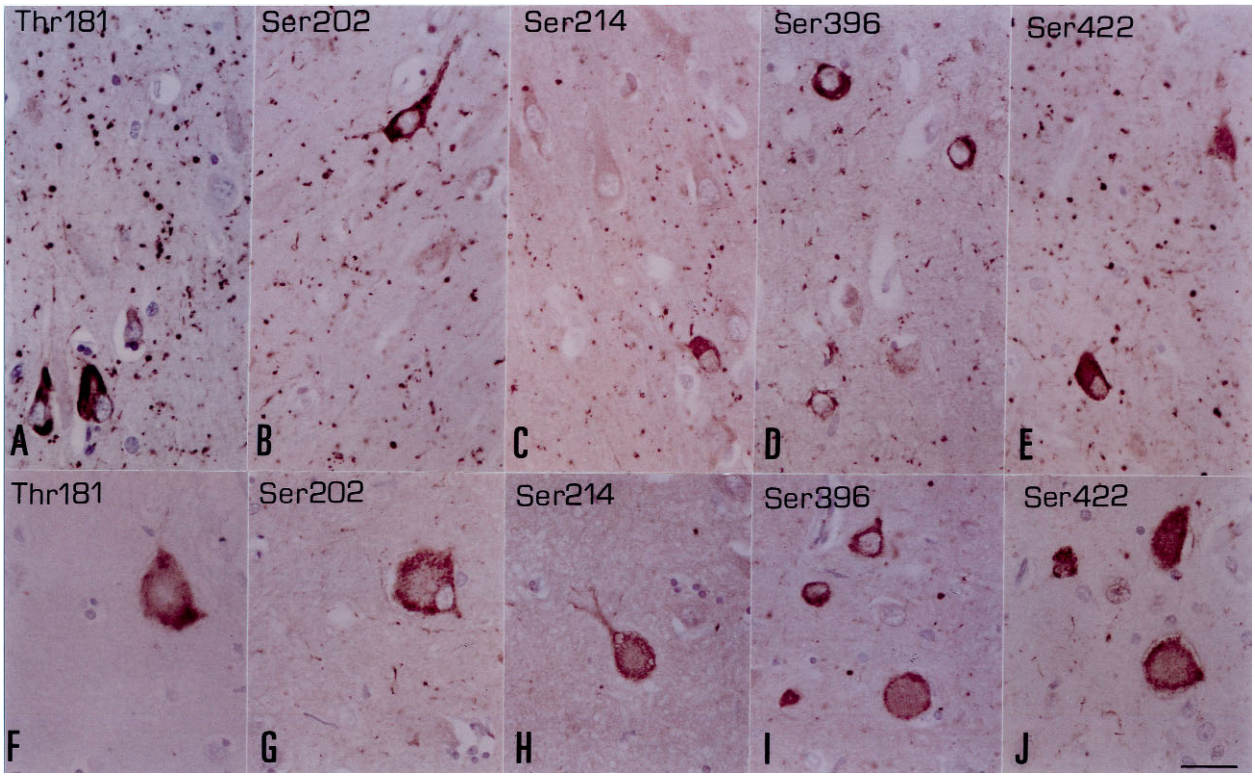

Figure 3. Phospho-*tau* immunoreactivity in the CA1 area of the hippocampus (**A-E**) and in ballooned neurons in the amygdala (**E-J**) in AGD. Grains are immunostained with different phospho-specific *tau* antibodies, although stronger immunoreactivity is obtained with antibodies to phospho-*tau*Thr181, Ser202, and Ser422 than with antibodies to Ser214. Phospho-*tau* is found in the periphery of the cytoplasm of ballooned neurons. Paraffin sections slightly counterstained with haematoxylin. Bar = 25 μm.

422 mainly stained the peripheral cytoplasm of ballooned neurons (Figure 3F-J).

Granular neurons of the dentate gyrus and glial cells. Individual neurons of the dentate gyrus were stained with anti-*tau* antibodies. Most positive neurons showed a diffuse or fine granular staining. Pick bodies were absent (Figure 4A, D, G, J, M).

Scattered astrocytes, mainly sub-pial and periventricular astrocytes (primarily tufted astrocytes), and coiled bodies in oligodendrocytes were variably stained with phospho-specific anti-*tau* antibodies. Astrocytes were moderately stained with antibodies to Thr181, Ser202 and Ser214, and strongly stained with antibodies to Ser422. Astrocytes were weakly stained with antibodies to tau phosphorylated at Ser214 (Figure 4B, E, H, K, N). Coiled bodies in oligodendrocytes were stained with all anti-tau antibodies (Figure 4C, F, I, L, O).

MAPK/ERK, SAPK/JNK and p38 kinase. No differences in the immunostaining between control and diseased brains were observed with anti-MEK1, anti-ERK2 and anti-JNK-1 antibodies (data not shown).

Grains, pre-tangles and tangles. MAPK/ERK-P in control-aged brain was restricted to weak diffuse expression in the somata of a few dispersed neurons. Yet strong MAPK/ERK-P-immunoreactive granules were found in the cytoplasm of scattered neurons in the hippocampus, entorhinal cortex and amygdala in AGD, while the grains were negative (Figure 5A). Weak SAPK/JNK-P immunoreactivity was found in the nuclei of neurons in control and diseased brains. Yet SAPK/JNK-P expression was found in the cytoplasm of a number of neurons, some of them bearing neurofibrillary tangles, in the hippocampus, entorhinal cortex and amygdala in AGD. In addition, strong SAPK/JNK-P immunoreactivity occurred in grains (Figure 5B-F). p38-P in control-aged brain was manifested as a weak diffuse immunoreactivity in the cytoplasm of individual neurons, but the majority of neurons were negative. Large numbers of neurons with diffuse cytoplasmic p38-P immunoreactivity and neurons with neurofibrillary tangles were observed; grains were also positive to p38-P (Figure 5G-I). Similar results were obtained in every case although phospho-kinase immunostaining decreased

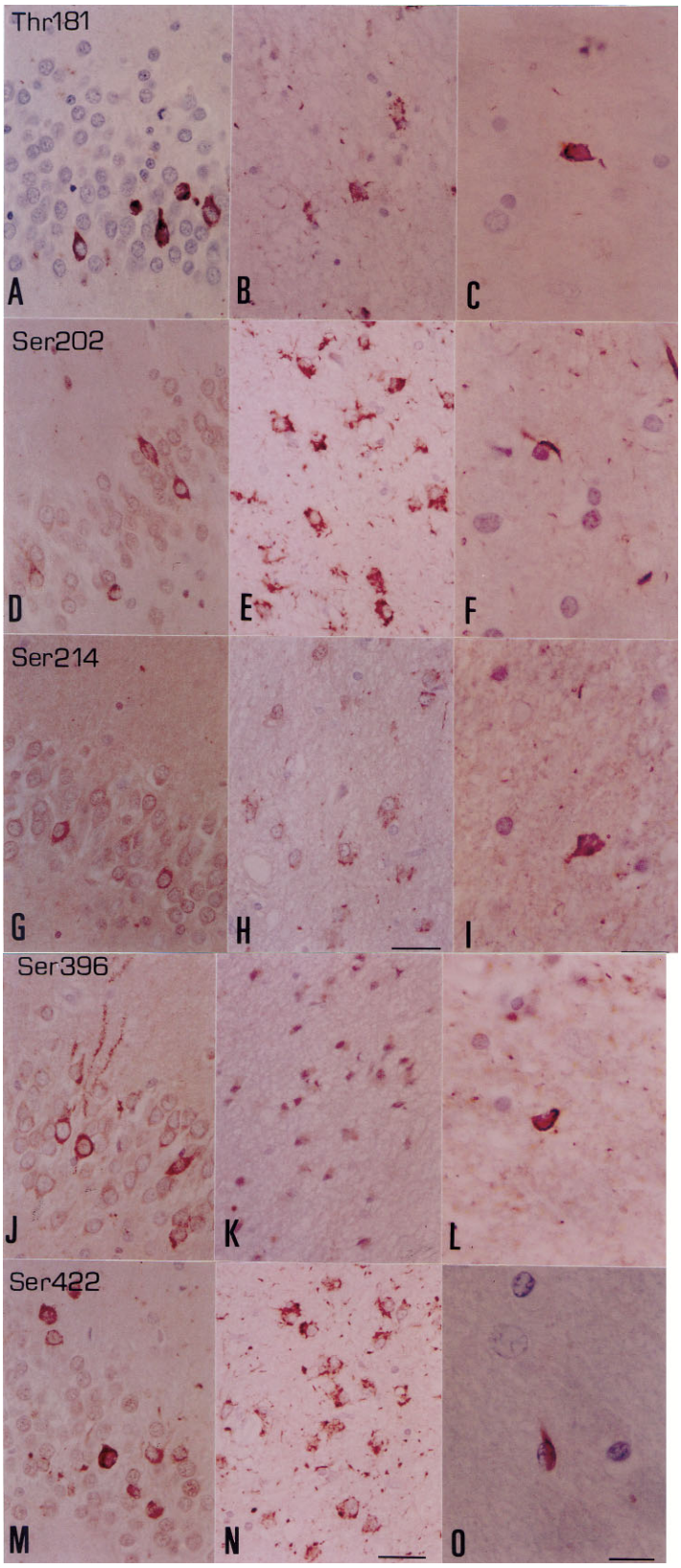

Figure 4. Phospho-*tau* immunoreactivity in neurons of the dentate gyrus (**A, D, G, J, M**), periventricular astrocytes (**B, E, H, K, N**), and oligodendrocytes with coiled bodies (**C, F, I, L, O**) as revealed with phospho-specific anti-*tau* antibodies to Thr181 (**A-C**), Ser202 (**D-F**), Ser 214 (**G-I**), Ser296 (**J-L**), and Ser 422 (**K-O**). Many neurons of the dentate gyrus contain phospho-*tau*, although none of them exhibits neurofibrillary tangle formation. Astrocytes are variably immunostained. Antibodies to Ser202 produce a very strong reaction, whereas astrocytes are only weakly stained with antibodies to Ser214 and Ser396. Paraffin sections slightly counterstained with haematoxylin. **A, B, D, E, G, H, J, K, M, N**, bars in **H** and **N** = 25 μm. **C, F, I, L, O,** bars in **I** and **O** =10 μm.

with post-mortem delay and with formalin, instead of paraformaldehyde, fixation.

Double-labeling immunohistochemistry disclosed pre-tangles rarely stained with MAPK/ERK-P antibodies, whereas SAPK/JNK-P and p-38-P decorated about 50% and 80%, respectively, of the neurons with pre-tangles in AGD (Figure 6). Similar to previous data in AD, tangles in AGD were differentially stained with anti-MAPK/ERK-P, SAPK/JNK-P and p38-P antibodies (23, 24). Only a small percentage of neurons with neurofibrillary tangles (about 20%) expressed MAPK/ERK-P, whereas about 30% and 50 to 70% of neurons with neurofibrillary tangles co-expressed SAPK/JNK-P and p38-P, respectively (data not shown).

Ballooned neurons in the amygdala. The cytoplasm of ballooned neurons in the amygdala was homogeneously stained with anti-αB-crystallin antibodies (Figure 7A, B) as described in detail elsewhere (79). Ballooned neurons were also diffusely stained with antibodies to phosphorylated neurofilament epitopes (Figure 8A). However, tau deposition was restricted to the peripheral regions of the soma (Figures 7C, 8B). MAPK/ERK-P expression was diffuse in the cytoplasm of ballooned neurons (Figure 7D), whereas SAPK/ JNK-P (Figure 7D-F) and p38-P (Figure 7G-I) immunoreactivity was predominantly peripheral.

Double labeling immunohistochemistry revealed co-localization of pan-tau and SAPK/JNK-P (Figure 8C-F) and p38-P (Figure 8G-I) in ballooned neurons.

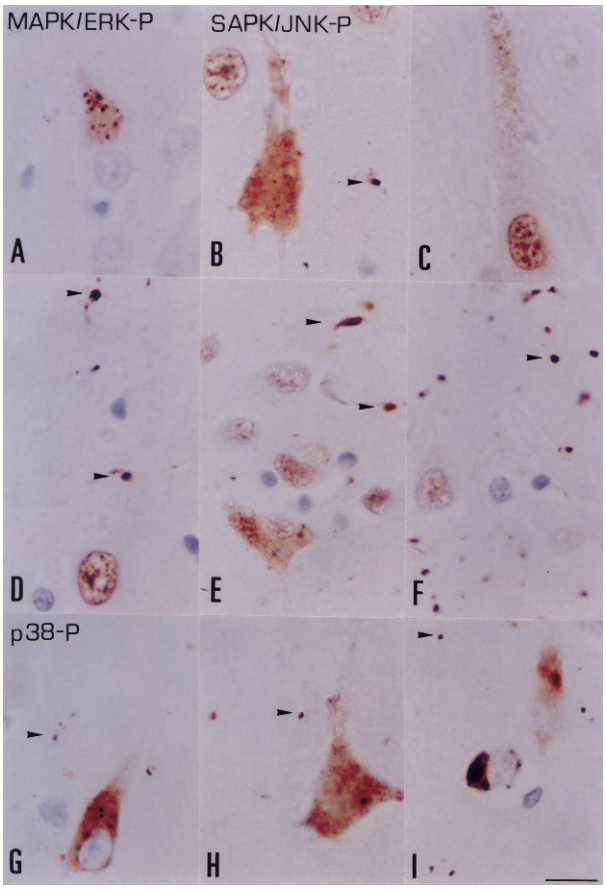

Figure 5. MAPK/ERK-P (**A**), SAPK/JNK-P (**B-F**) and p38-P (**G-I**) immunoreactivity in the hippocampus in AGD. MAPK/ERK-P-positive intracytoplasmic granules are observed in a few neurons, whereas diffuse cytoplasmic SAPK/JNK-P and p-38-P immunoreactivity is found in a number of neurons. In addition, SAPK/JNK-P and p38-P immunoreactivity is observed in grains (arrowheads). Note weak SAPK/JNK-P expression in the nuclei of neurons. Paraffin sections, slightly counterstained with haematoxylin, bar = 10 μm.

Glial cells. Phospho-specific antibodies to MAPK/ ERK, SAPK/JNK, and p38 kinases differentially stained sub-populations of glial cells, mainly astrocytes, and, rarely, oligodendrocytres with coiled bodies (Figure 9). The percentage of cells co-localizing MAPK/ERK-P, SAPK/JNK-P, and p38-P, along with pan-*tau*, was variable from one case to another and largely dependent on the post-mortem delay and fixation.

GSK-3. Antibodies to GSK-3α/β decorated the cytoplasm of neurons and glial cells. Tangles, pre-tangles, and grains were not distinguished by non-phosphorylated anti-GSK-3α/β antibodies (Figure 10A). Antibodies to phospho-specific GSK-3β (GSK-3β-P) recognized a

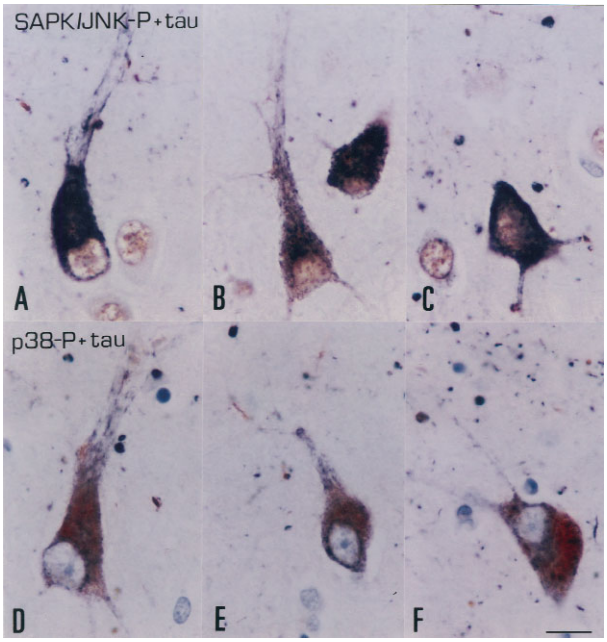

Figure 6. Co-localization of SAPK/JNK-P (**A-C**) or p-38-P (**D-F**), recognized as a brown precipitate, and pan-*tau*, recognized as a dark blue precipitate in the hippocampus in AGD. Neurons with pre-tangles show parallel expression of SAPK/JNK-P and p38-P. Grains are variably immunostained with anti-SAPK/JNK-P and p38-P antibodies. Paraffin sections, slightly counterstained with haematoxylin, bar = 10 μm.

small number of grains and pre-tangles and tangles (Figure 10B). Ballooned neurons were rarely stained. In addition, GSK-3β-P was present in astrocytes (Figure 10C), and in very few oligodendrocytes with coiled bodies (Figure 10D).

A summary of *tau* pathology and kinase expression in AGD is shown in Table 2. Details of Ser262 immunostaining are described in detail elsewhere (23).

Western blots of total homogenates and sarkosyl-insoluble fractions. Antibodies against MEK-1, ERK2, JNK-1, GSK-3α/β, and αCaM kinase II recognized specific bands in sarkosyl-insoluble fractions and total homogenates processed in parallel. Yet the intensity of the bands corresponding to JNK-1 and αCaM kinase II was markedly higher in sarkosyl-insoluble fractions than in total homogenates (Figure 11).

MAPK/ERK-P, SAPK/JNK-P, p38-P, and GSK-3 β-P were found in sarkosyl-insoluble fractions and in total homogenates. Yet while 2 bands of 42 and 44 kDa, corresponding to phospho-ERK1 and phospho-ERK2, were recovered in total homogenates, only one band of about 66 kDa was recovered in sarkosyl-insoluble frac-

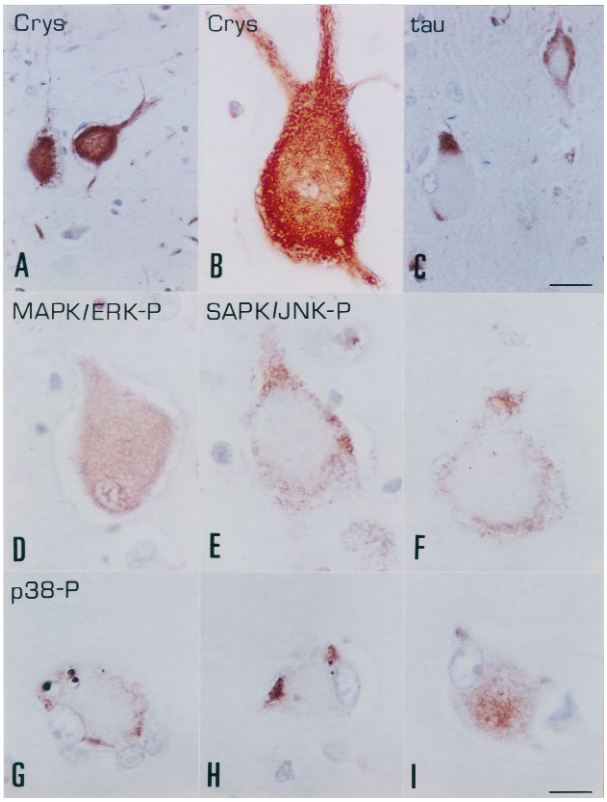

Figure 7. Ballooned neurons in the amygdala stained with anti-αB-crystallin (Crys) (**A, B**) and pan-tau (**C**). αB-crystallin occupies the whole cytoplasm, whereas *tau* immunoreactivity is restricted to the periphery of the ballooned cytoplasm. MAPK/ERK-P immunoreactivity occurs as a diffuse cytoplasmic immunoprecipitate (**D**). SAPK/JNK-P is observed in the periphery of the ballooned cytoplasm (**E, F**); p38-P is found as coarse granules (**G**), or as peripheral (**H**) or diffuse (**I**) immunoreactivity in individual neurons. Paraffin sections, slightly counterstained with haematoxylin. **A, C,** bar in **C** = 25 μm. **B, D-I,** bar in **I** = 10 μm.

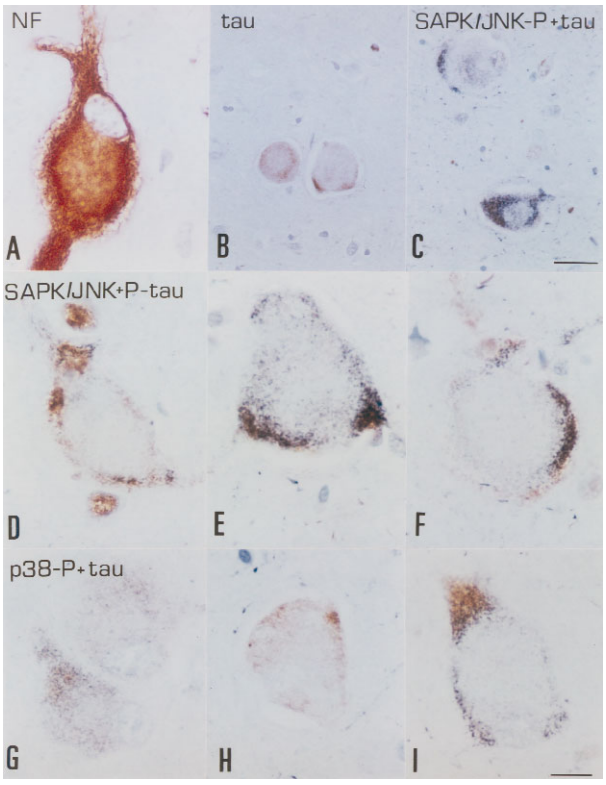

Figure 8. Ballooned neurons with diffuse phosphorylated neurofilament (NF: clone RT97) immunoreactivity (**A**) and peripheral *tau* deposition (**B**). MAPK/ERK co-localizes with *tau* in a number of neurons (**C**). SAPK/JNK-P (**D-F**) and p38-P (**G-I**) co-localize with *tau* in a percentage of ballooned neurons. Paraffin sections, slightly counterstained with haematoxylin. Antibodies to phospho-specific kinases are recognized as a brown precipitate, whereas *tau* is recognized as a dark blue precipitate in double-immunostained sections. **B, C,** bar in **C** = 25 mm. **A, D, I,** bar in **I** = 10 mm.

tions (Figure 11). None of the kinases labeled the sarkosyl-insoluble tau bands (Figure 11).

Caspase-3 immunohistochemistry and double-labeling of nuclear DNA fragmentation and MAPK/ERK-P, SAPK/JNK-P, p38-P, or GSK-3β-P immunohistochemistry. The method of in situ end-labeling of nuclear DNA fragmentation revealed positive neurons and glial cells in AGD, probably reflecting increased nuclear DNA vulnerability (34, 39, 75). Apoptotic figures were not seen in any case. Cleaved caspase-3 immunohistochemistry barely disclosed a single neural positive cell in AGD. However, the method was sensitive, as many active caspase-3-immunoreactive cells were seen in tissue sections from the cerebella of newborn irradiated rats that were processed in parallel, and in which apoptosis was commonly observed at 6 hours following ionizing radiation (data not shown).

Double-labeling of nuclear DNA fragmentation and kinase immunohistochemistry disclosed no relationship between cells bearing nuclear DNA breaks and cells with MAPK/ERK-P, SAPK/JNK-P, p38-P, or GSK-3β-P immunoreactivity. Double-labeling immunohistochemistry to phosphorylated kinases and active caspase-3 showed no co-localization (data not shown).

Discussion

The present results support AGD as a new tauopathy with particular neuropathological and biochemical traits. Hyperphosphorylated *tau* accumulates in grains, pre-tangles, ballooned neurons, and astrocytes, and coiled bodies in oligodendrocytes. Hyper-phosphory-

	Grains	Pre-tangles	Tangles	Ballooned N	Astrocytes	Coiled bodies
Thr181	+++	+++	+++	+ [p]	+	+
Ser202	+++	++	++	++ [p]	+	+
Ser214	++	+++	++	++ [d]	±	+
Ser396	++	+++	+++	+ [p]	+	+
Ser422	+++	+++	+++	++ [d]	++	+
MEK-1	-	-	-	-	-	-
ERK2	-	-	-	-	-	-
JNK-1	-	-	-	-	-	-
αCaMK II	-	-	-	-	-	-
GSK-3α/β	-	-	-	-	-	-
MAPK/ERK-P	-	+	+	++ [d]	+	+
SAPK/JNK-P	+++	++	++	+ [p]	++	+
P38-P	+++	+++	+++	+ [p]	++	+
GSK-3β-P	+	+	++	-	+	+

Table 2. Summary of the expression of phospho-specific *tau*, and non-phosphorylated MEK-1, ERK2, JNK-1, αCaMK II and GSK-3α/β, and phosphorylated kinases MAPK/ERK, SAPK/JNK, p38, and GSK-3β, in grains, pre-tangles, tangles, ballooned neurons in the amygdala, astrocytes, and coiled bodies in AGD. – negative or no modifications in relation to controls, ± mild or negative, + weak, ++ moderate, +++ strong expression. [d, p] diffuse or peripheral staining.

lated *tau* accumulation is also registered in Western blots of total hippocampal homogenates. A pattern of two bands of 64 and 68 kDa is encountered in Western blots of hippocampal homogenates enriched with abnormal filaments. This pattern of 2 bands is reminiscent of that found in PSP, CBD, and certain frontotemporal dementias linked to chromosome 17 associated with the P301L mutation, in which 4R-*tau* repeats (10+) are detected following de-phosphorylation (9, 44). This pattern differs from the 4-band (74/72, 68, 64 and 60 kDa) pattern typical of AD. None of the cases in the present series had associated PSP pathology. Dephosphorylation of phospho-*tau* in sarkosyl-insoluble fractions has shown a pattern of 4R-*tau* repeats, thus indicating AGD as a novel 4-repeat tauopathy (79, 86).

The most important finding of the present study is, however, the role of protein kinases in *tau* phosphorylation in AGD.

The family of MAP kinases is composed of several members including MAPK/ERKs, SAPK/JNKs, and p38 kinases (12, 50, 59). ERKs are activated through phosphorylation by MAPK/ERK kinases (MEKs), which in turn are activated by MEK kinases (MEKKs), The best characterized MAPK cascade is regulated by Ras, and consists of Raf isoforms, particularly Raf-1, MEK1/2, and ERK1/2 (12, 59). MAPK/ERKs phosphorylate *tau* and neurofilament proteins (17, 28, 43, 89).

JNKs are encoded by 3 genes, all of which are expressed in the brain (32). The JNKs are activated by phosphorylation through JNK kinases (JNKKs), also called MAP kinase kinases (MKKs) (37, 51). SAPKs are implicated in the phosphorylation of Lys-Ser-Pro motifs in neurofilaments and *tau* (29, 58).

The p38 kinases represent a family of 4 homologous kinases which derive from different genes (50). The p38 kinases are widely expressed in many tissues, including the brain, and are activated by dual phosphorylation. The phosphorylation is mediated by the MAP kinase kinases (MKKs) (16, 19, 45, 55, 90). p38 phosphorylates *tau* protein in vitro (29, 56, 57, 58).

CaM kinase II includes four subunits (α, β, γ, δ) that are encoded by 4 different genes (8, 60) and is activated through phosphorylation by CaM kinase kinase (72). CaM kinase II participates in the phosphorylation of proteins including *tau* (3, 69, 77).

GSK-3α/β is expressed in neurons, and it induces *tau* phosphorylation and the Alzheimer-like state of *tau* (33, 48). GSK-3β-catalyzed phosphorylation of *tau* is modulated by other kinases (64, 70, 71, 94). Recent studies have shown that GSK-3β rescues axonopathy in the central nervous system of human 4-repeat *tau* transgenic mice (74). More impressively, *tau* hyperphosphorylation and pre-tangle-like somatodendritic localization of *tau* have been produced in GSK-3β conditional transgenic mice (47).

GSK-3 is inactivated by phosphorylation at Ser9 (13, 26, 76), and the process of phosphorylation-inacti-

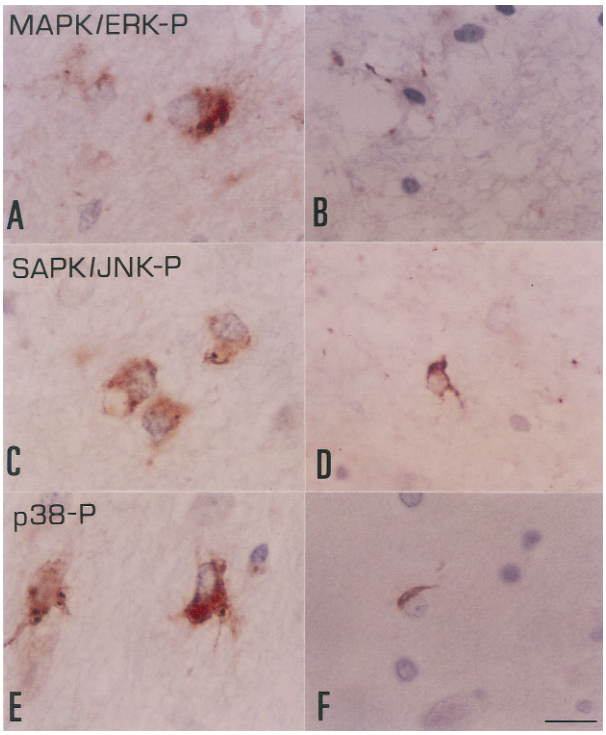

Figure 9. MAPK/ERK-P (**A, B**), SAPK/JNK-P (**C, D**) and p38-P (**E, F**) immunoreactivity in astrocytes (**A, C, E**) and oligodendrocytes (**B, D, F**) in AGD. Paraffin sections, slightly counterstained with haematoxylin, bar = 10 μm.

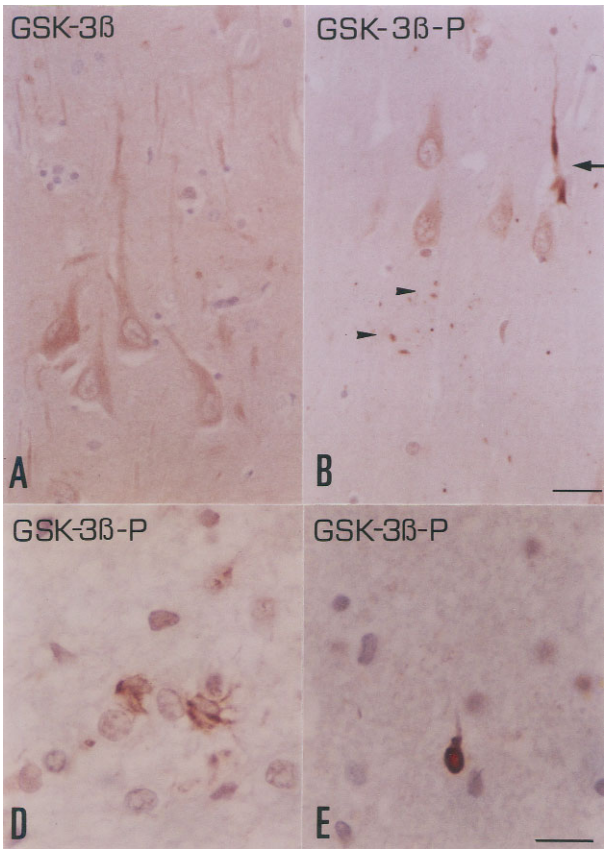

Figure 10. GSK-3α/β (**A**) and GSK-3β-P (**B-D**) immunoreactivity in AGD. GSK-3α/β is expressed in the majority of hippocampal neurons, but grains, pre-tangles, and tangles are not distinguished. GSK-3β-P immunoreactivity is observed in a minority of grains (arrowheads) and in tangles (arrow) in the AGD hippocampus (**B**). Periventricular astrocytes (**C**) and, rarely, coiled bodies (**D**) show GSK-3β-P immunoreactivity. Paraffin sections, slightly counterstained with haematoxylin. **A-C,** bar in **C** = 25 μm. **D**, bar = 10 μm.

vation may be triggered by AKT, MAP kinases, and PKA (13, 20, 67, 76).

Previous studies have shown strong MAPK/ERK-P immunoreactivity in neurons containing *tau* deposits in AD (23, 24, 42, 54). MAPK/ERK-P is also associated with *tau* deposits in neurons and glial cells in tauopathies, including PiD, PSP and CBD (2, 24). Strong SAPK/JNK-P and p38-P immunoreactivity occurs in neurons with neurofibrillary tangles and in dystrophic neurites of senile plaques in AD (24, 95, 96). SAPK/JNK-P and p38-P are also expressed in neurons with neurofibrillary degeneration and in neurons, astrocytes, and oligodendrocytes bearing tau deposits in other tauopathies (2, 24). CaM kinase II is associated with paired helical filaments (91). Finally, GSK-3β has been identified in neurofibrillary tangles in AD (52, 93), and with neuronal and glial hyper-phosphorylated *tau* deposits in AD and other tauopathies (23).

The present study has shown no modifications in the basal, non-phosphorylated expression of MEK-1, ERK2, JNK-1, GSK-3α/β, and αCaM kinase II in tissue sections processed for immunohistochemistry in AGD. Grains are not immunostained with antibodies to non-phosphorylated kinases. MEK-1, ERK2, and GSK-3α/β are distributed in sarkosyl-insoluble fractions and in total homogenates. Yet JNK-1 and αCaMK II are more abundant in the sarkosyl-insoluble fraction than in total homogenates, thus suggesting association with abnormal filaments in AGD. This distribution is similar to that previously described for paired helical filaments in Alzheimer's disease (91). Sarkosyl-insoluble fractions are also enriched in SAPK/JNK-P, p-38-P, and GSK-3β-P. One band of 66kDa MAP/ERK-P is recognized in sarkosyl-insoluble fractions, thus suggesting interactions of phospho-tau and phospho-MAPK/ERK. Yet none of the kinases labels the 2 bands of sarkosyl-insoluble tau, thus indicating that these antibodies do not recognize hyperphosphorylated tau protein.

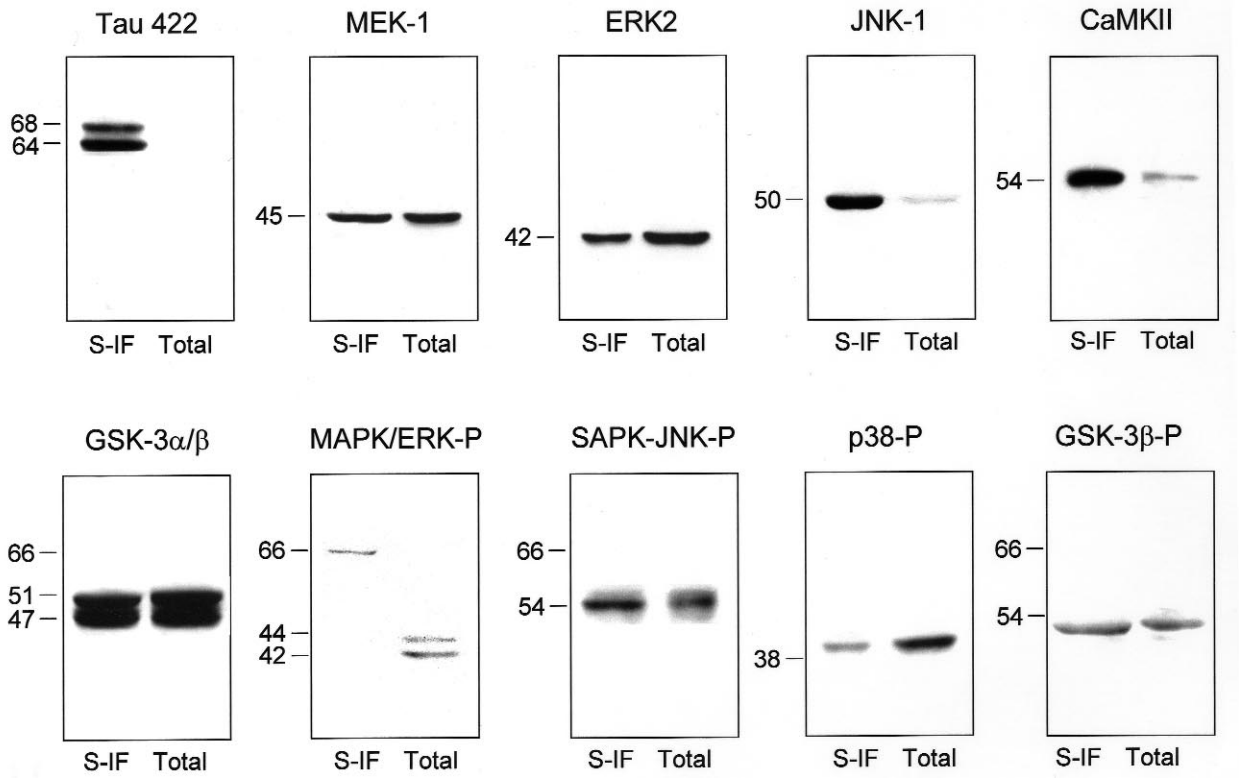

Figure 11. Western blots of sarkosyl-insoluble fractions (S-IF) and total hippocampal (Total) homogenates in AGD. The sample corresponds to case 5 (Table I). Phospho-*tau*422 recognizes 2 bands of 68 and 64 kDa in S-IF but not in total homogenates. MEK-1, ERK2, and GSK-3α/β are recovered in total homogenates and in sarkosyl-insoluble fractions. Yet the expression of JNK-1 and αCaM Kinase II (CaMK II) is higher in sarkosyl-insoluble fractions than in total homogenates. MAPK/ERK-P is represented by 2 bands of 42 and 44 kDa (corresponding to phospho-ERK1 and ERK2) in total homogenates. Yet a minor band of about 66 kDa is found in S-IF. SAPK/JNK-P is found in total and S-IF homogenates as a band of 54 kDa, whereas p-38-P, represented by a band of about 43 kDa, is mainly expressed in total homogenates, but is also seen in S-IF. GSK-3β-P (50 kDa) is found in S-IF and total homogenates. None of the antibodies to kinases labels the sarkosyl-insoluble *tau* bands in AGD. Total protein per lane 200 μg.

Increased expression in several phosphorylated kinases occurs in grains, neurons with pre-tangles, ballooned neurons, astrocytes, and coiled bodies in AGD.

Grains are recognized with antibodies to SAPK/JNK-P, p38-P, and, to a lesser extent, GSK-3β-P, but are negative with antibodies to MAPK/ERK-P. Yet neurons with pre-tangles are recognized with antibodies to MAPK/ERK-P. Ballooned neurons are diffusely stained with anti-MAPK/ERK-P. Similar characteristics are found in achromatic cortical neurons in CBD which are also filled with phosphorylated neurofilaments, such as ballooned neurons in AGD, and which exhibit diffuse cytoplasmic MAPK/ERK-P immunoreactivity (25). It is worth stressing that MAPK/ERK phosphorylates Lys-Ser-Pro repeats in neurofilament proteins in vitro (89), and therefore neurofilament phosphorylation by ERKs may be postulated in ballooned neurons on this basis.

More important is the putative role of kinases in the phosphorylation of specific sites in *tau* as derived from the examination of phospho-specific *tau* antibodies in AGD. Phosphorylation of *tau* at Thr181 can be conveyed by SAPK/JNK, p38, and MAPK/ERK (36, 57, 58). Phosphorylation at Ser202 is produced by SAPK/JNK, p38, MAPK/ERK, and GSK-3α/β (36, 57, 58, 88). Ser214, while mainly phosphorylated by protein kinase A (61), is also phosphorylated by GSK-3β (94). Ser396 is phosphorylated by SAPK/JNK, p38, MAPK/ERK, and GSK-3β (27, 48, 58, 59). Finally, Ser422 is mainly phosphorylated by members of the MAPK family, including MAPK/ERK, SAPK/JNK, and p38 (36. 57, 58).

Taken together, these results describe for the first time in AGD a scenario in which MAPK/ERK, SAPK/JNK, p38, αCaM kinase II, and GSK-3β phosphorylate *tau* at specific sites, and this phosphorylated *tau* is accumulated in neurons and glial cells. Yet it is dif-

ficult to discriminate a possible sequence of the activation of these kinases during the process of *tau* phosphorylation. The same phospho-specific *tau* antibodies and the majority of examined phospho-kinases, excepting MAPK/ERK, are expressed in grains, pre-tangles, and tangles. It has been suggested that phosphorylation at certain sites of *tau* mainly occurs at early stages of tangle formation in AD (1). However, a similar approach is difficult with the present panel of antibodies in AGD. Phosphorylation at Ser214 (and Ser262) strongly inhibits *tau* binding to microtubules and prevents their aggregation into paired helical filaments (61, 62). Yet there is no difference in the expression of phospho-*tau*Ser214 in pre-tangles and tangles in AGD.

In contrast to neurons, tufted astrocytes are barely immunostained with antibodies to Ser214 when compared with antibodies to Thr181, Ser202, Ser396, and Ser422. This indicates differences in *tau* phosphorylation between neurons and glial cells in AGD. Since MAPK/ERK-P, SAPK/JNK-P, p38-P, and GSK-3β-P are expressed in *tau*-bearing astrocytes, it can be assumed that other kinases play differential roles in neurons and glial cells. Whether this is associated with reduced PKA, a major kinase that phosphorylates *tau* at Ser214 in astrocytes, when compared with neurons, deserves further study. Yet CaM kinase II and casein kinase1 delta are differentially expressed in neurons and glial cells in several tauopathies (24, 62).

Elevated levels of GSK-3β-P in grains, pre-tangles and tangles in AGD is intriguing, as it would seem to indicate inactivation of GSK-3β and sequestration of the inactive kinase at particular sub-cellular domains enriched with abnormal *tau*.

Finally, the present results have shown no localization of MAPK/ERK-P, SAPK/JNK-P, p38-P, and GSK-3β-P in cells bearing nuclear DNA fragments, as seen with double labeling immunohistochemistry and the method of in situ end-labeling of nuclear DNA fragmentation. Furthermore, co-localization of MAPK/ERK-P, SAPK/JNK-P, p38-P, and GSK-3β, and active caspase-3, has not been found in a single cell in AGD. It has been demonstrated that GSK-3β-PSer9 has the capacity to inhibit apoptosis in certain paradigms (13, 14). Therefore, it may be suggested that GSK-3β-P may prevent abnormal *tau*-containing cells from dying. These results strongly support the idea, introduced in relation to other tauopathies (2, 24, 25), that the phospho-specific kinases examined here are not associated with cell death in AGD.

Acknowledgments

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