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REGULAR PAPER

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Glycogen synthase kinase-3 is associated with neuronal and glial hyperphosphorylated tau deposits in Alzheimer's disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration

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Abstract Tau phosphorylation was examined in Alzheimer's disease (AD), Pick's disease (PiD), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) using phospho-specific tau antibodies recognizing the phosphorylated form of Ser202, Ser214 and Ser 396, and antibodies to non-phosphorylated glycogen synthase kinase-3α/β (GSK-3α/β), which regulates phosphorylation at these specific sites on tau and phosphorylated GSK-3βSer9 (GSK-3β-P); this antibody is directed to the inactive form of GSK-3β. Phospho-specific tau antibodies recognized disease-specific band patterns on Western blots of sarcosyl-insoluble fractions: four bands of 73, 68, 64 and 60 kDa in AD, two bands of 68 and 64 kDa in PSP and CBD, and two bands of 64 and 60 kDa in PiD. Moreover, anti-phospho-tau Ser202, Ser214 and Ser369 decorated neurons with neurofibrillary tangles, dystrophic neurites of senile plaques, neuropil threads, Pick bodies, astrocytes and oligodendrocytes with coiled bodies. No differences in the expression of GSK-3α/β were seen between neurons with and without neurofibrillary tangles. GSK-3α/β was enriched in sarcosyl-insoluble fractions, suggesting association of this kinase with tau hyperphosphorylation. In addition, strong expression of the phosphorylated form of GSK-3β was found in a subpopulation of neurons with neurofibrillary tangles, and in dystrophic neurites of senile plaques, neuropil threads, Pick bodies, tau-containing astrocytes and coiled bodies in AD, PiD, PSP and CBD. This was not due to cross-reactivity between GSK-3 and phospho-tau. Specific bands differing from those of phospho-tau were seen on Western blots of sarcosyl-insoluble fractions processed for GSK-3α/β and GSK-3β-P. Double-labeling immunohistochemistry discloses that GSK-3β-P co-localizes with abnormal tau in about 50% of neurons with neurofibrillary tangles, and in neuronal processes, astrocytes and oligodendrocytes in various tauopathies. The present results support a pivotal role for GSK-3 in tau phosphorylation in neurons and glial cells. Moreover, the elevated number of tau-containing cells stained with anti-GSK-3β-P antibodies suggests a partial inactivation of the kinase, or sequestration of the phosphorylated form, which may contribute to the regulation of the cascade of tau hyperphosphorylation in tauopathies, and to protect tau-containing cells from apoptosis.

Keywords Alzheimer's disease · Pick's disease · Progressive supranuclear palsy · Corticobasal degeneration · Tau

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Introduction

Abnormal tau hyperphosphorylation and deposition in the cytoplasm of neurons and glial cells is a major biochemical and structural characteristic in Alzheimer's disease (AD), Pick's disease (PiD), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD), which constitute the group of neurodegenerative tauopathies [4, 7, 9, 15, 18, 20, 38]. Tau proteins contain phosphorylation sites for a large number of protein kinases including protein kinase A (PKA), protein kinase C, cyclin-dependent kinase 5 (cdk5), mitogen-activated extracellular signal-regulated protein kinases (MAPK/ERK), stress-activated protein kinases (SAPK/JNK), p38 kinases, calcium/calmodulin-dependent kinase II (CaM kinase II), microtubule affinity-regulating kinase (MARK) and casein kinases [4, 20]. Several studies have shown the expression of cdk5, casein kinase 1 delta, MAPK/ERK, SAPK/JNK, p38, and, most importantly, the corresponding phosphorylated or active forms, in association with abnormal tau deposits in AD [10, 11, 19, 28, 40, 43, 44] and other

tauopathies [2, 10, 11, 30]. In addition, glycogen synthase kinase-3β (GSK-3β) has a pivotal role in tau phosphorylation in vitro [16, 24, 29, 31, 33, 34, 35, 39, 42]. Immunohistochemical studies have shown increased GSK-3β expression in association with neurofibrillary tangles in AD [27, 41], indicating that GSK-3β is implicated in tau pathology in AD. Moreover, increased GSK-3α/β and phosphorylated GSK-3βTyr216, which corresponds to an active form of the kinase, have been reported in association with granulovacuolar degeneration [21]. However, the expression of GSK-3β in other tauopathies is unknown.

The present study is focused on the expression of non-phosphorylated GSK-3α/β and phosphorylated GSK-3βSer9, which signals the inactive form of the kinase, and the relationship between kinase expression and abnormal tau deposition in AD, PiD, CBD and PSP. For this purpose Western blotting and immunohistochemistry were carried out with phospho-specific antibodies that recognize phosphoSer202, Ser214, and Ser 396, which are putative sites of tau phosphorylation by GSK-3β [13, 17, 38, 41]. Western blotting of sarcosyl-insoluble fractions and total hippocampal homogenates were processed for GSK-3α/β and GSK-3β-P to investigate the subcellular distribution of the active and inactive forms of the kinase in AD [5, 12]. Subsequently, single- and double-labeling immunohistochemistry for GSK-3α/β or GSK-3β-P, and phospho-tau was examined to determine GSK-3 and tau in individual neurons and glial cells. Finally, double labeling using in situ end-labeling of nuclear DNA fragmentation and GSK-3β-P immunohistochemistry was carried out to investigate the relationship between GSK-3β-P Ser9 and increased nuclear DNA vulnerability.

Material and methods

Cases

The following cases were included in the present study; AD: four men and six women categorized as stage III (three cases) and stage VI (seven cases) of Braak and Braak (age range 68–82 years, mean 72.1 years); PiD: one man aged 73 years and two women aged 71 and 82 years; CBD: two men aged 69 and 72 years, and two women aged 72 and 73 years; PSP: two men and four women (age range 63–81 years, mean 76.2 years); age-matched controls: four men and four women (age range 68–81 years, mean 73.5 years). No abnormalities were seen in controls with the exception of a few βA4 amyloid deposits in the entorhinal cortex and hippocampus in two cases. The delay between death and tissue processing was between 2 h and 7 h in both control and diseased brains.

General procedures

A complete neuropathological examination was carried out in every case in tissue that had been fixed in formalin for not less than 3 weeks; the tissue was then embedded in paraffin. Dewaxed sections, 7 μm thick, were stained with hematoxylin 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 or 200 kDa (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 a hybridoma produced by 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 (55–62 kDa). The pan-tau antibody recognizes phosphorylated tau in paraffin sections. Antibodies to glial fibrillary acidic protein (GFAP, DAKO, Dakopatts), βA4-amyloid (Boehringer Mannheim) and ubiquitin (DAKO) were used at dilutions of 1:250, 1:5 and 1:200, respectively.

For gel electrophoresis and Western blotting, fresh samples were obtained immediately at autopsy, frozen in liquid nitrogen and stored at –80°C until use. For specific immunohistochemical studies, fresh samples were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 48 h and embedded in paraffin.

Gel electrophoresis and Western blotting

A protocol for purifying paired helical filaments, described by Goedert et al. [14], was used. Frozen samples (of about 5 g) of the hippocampus in AD and PiD, striatum in CBD, and tectum in PSP were cut into pieces. The pieces 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, 0.1 mM 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 two supernatants (S1+S2) were then mixed and incubated with N-lauroylsarcosinate 1% for 1 h at room temperature while being shaken. Samples were then centrifuged for 1 h at 100,000 *g* in a Ti 70 Beckman rotor. Sarcosyl-insoluble pellets (P3) were re-suspended (0.2 ml/g starting material) in 50 mM TRIS-HCl pH 7.4. Protein concentrations were determined by the BCA method, and 10% SDS-polyacrylamide gel electrophoresis (PAGE) was run using a maxi-protean system (Bio-Rad); 100–200 μg 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 95°C for 5 min prior to gel loading. The proteins were then transferred to nitrocellulose membranes (Amersham) using an electrophoretic chamber system (Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). Nonspecific binding sites were blocked with TRIS-buffered saline solution pH 7.4 with 0.1% Tween-20 (TBST) containing 5% skimmed milk for 30 min and incubated with one of the primary antibodies for 1 h at room temperature. The rabbit polyclonal antibodies anti-phospho-tau Ser202, Ser214 and Ser396 (all from Calbiochem) were used at a dilution of 1:500.

Total homogenates and sarcosyl-insoluble fractions from the hippocampus of AD cases were run in parallel and processed for GSK-3. The anti-GSK-3α/β monoclonal antibody (StressGen) reacts with 51 and 47 kDa proteins corresponding to the expected molecular masses of GSK-3α and GSK-3β in total human hippocampal homogenates. The antibody was used at a dilution of 1:500. The anti-phospho-specific GSK-3β Ser9 (GSK-3β-P) antibody (Oncogene) is a rabbit polyclonal IgG antibody specific for the Ser9 phosphorylated form of GSK-3β. The antibody recognizes a band of about 55 kDa in total human hippocampal homogenates.

After washing, the membranes were incubated with the secondary antibody labeled with horseradish peroxidase (DAKO) diluted 1:1,000 for 1 h 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).

Phospho-tau, GSK-3α/β and GSK-3β-P immunohistochemistry

Immunohistochemistry was carried out following the ABC method. Dewaxed 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 4°C overnight with one of the primary antibodies. Phospho-specific tau rabbit polyclonal antibodies against Ser202, Ser214 and Ser396 (all of them from Calbiochem) were used. The antibodies were used at a dilution of 1:100. The anti-GSK-3α/β monoclonal antibody (StressGen) was used at a dilution of 1:500. The anti-phospho-specific GSK-3βSer9 antibody (Oncogene) was used at a dilution of 1:100. Following incubation with the primary antibody, the sections were incubated for 1 h with biotinylated anti-mouse or anti-rabbit IgG diluted 1:100, followed by ABC at a dilution of 1:100 for 1 h at room temperature. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. Some sections were counterstained with hematoxylin.

For double-labeling immunohistochemistry sections were incubated using a two-step protocol: initially with antibodies to GSK-3α/β or GSK-3β-P using visualization with diaminobenzidine and hydrogen peroxide, and subsequently with anti-pan-tau (Sigma) or with one of the phospho-specific tau antibodies. Different subclass-specific secondary antibodies were used in double-labeling studies utilizing two 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.

In situ end labeling of nuclear DNA fragmentation

This was carried out with a ApoptTag detection kit (Oncor) following the instructions of the supplier but omitting pre-incubation with proteinase-K. Double labeling with this method and GSK-3β-P was performed following a two-step protocol as previously. Cells with nuclear DNA fragments were recognized by a brown nuclear precipitate, whereas GSK-3β-P was recognized as a dark blue precipitate. Some sections were processed by inverting the order of ApopTag and immunohistochemistry.

Results

Gel electrophoresis and Western blotting to phospho-tau

Gel electrophoresis and Western blotting of sarcosyl-insoluble fractions showed a similar disease-specific band pattern with the different anti-phospho-tau antibodies (Fig. 1). AD was characterized by three bands of 68, 64 and 60 kDa preceded by a barely discernible small band of 73 kDa. PSP and CBD showed two bands of 68 and 64 kDa, whereas PiD was represented by two bands of 64 and 60 kDa.

Phospho-tau immunohistochemistry

The antibodies to phospho-tau Ser202, Ser214 and Ser396 stained neurons with neurofibrillary tangles, dystrophic neurites of senile plaques and neuropil threads in AD (Fig. 2A, D, H). Pick bodies in the entorhinal cortex and dentate gyrus were strongly stained with the same anti-phospho-tau antibodies (Fig. 2B, E, I). Antibodies to Ser202,

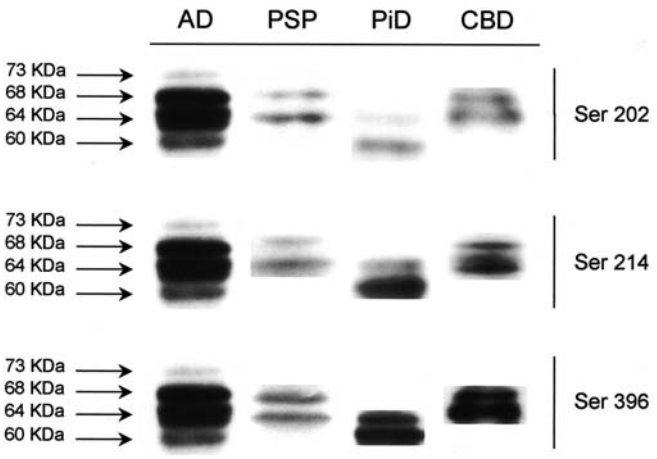


Fig. 1 Western blots of sarcosyl-insoluble fractions of AD, PSP, PiD and CBD using phospho-specific anti-Ser202, Ser214 and Ser369 antibodies. Four bands of 73, 68, 64 and 60 kDa are observed in AD, two bands of 68 and 64 kDa in PSP and CBD and two bands of 64 and 60 kDa in PiD (*AD* Alzheimer's disease, *PSP* progressive supranuclear palsy, *PiD* Pick's disease, *CBD* corticobasal degeneration)

Ser214 and Ser396 decorated neurofibrillary tangles and pre-tangles in PSP and CBD (Fig. 2C, J). The cytoplasm of many astrocytes and coiled bodies in oligodendrocytes in PiD, PSP and CBD showed phospho-tau immunoreactivity (Fig. 2F, G). The intensity of the immunoreaction was stronger with anti-Ser396 than with Ser202 and Ser214.

Control cases were not stained with anti-phospho-tau antibodies.

Gel electrophoresis and Western blotting to GSK-3α/β and GSK-3β-P in total homogenates and in sarcosyl-insoluble fractions in AD

Western blots of total brain homogenates in AD disclosed strong bands of 47 and 51 kDa corresponding to the molecular masses of GSK-3α and GSK-3β, as well as a weak band of 55 kDa corresponding to GSK-3β-P Ser9. Sarcosyl-insoluble fractions, recognized by a typical band pattern of phosphorylated tau, showed strong GSK-3 immunoreactivity. Strong bands of GSK-3α, GSK-3β and GSK-3β-P immunoreactivity were seen in these fractions (Fig. 3). These bands were not the result of cross-reactivity with phospho-tau as the molecular masses corresponding to GSK-3 isoforms largely differ from those of phospho-tau (Fig. 3).

Similar findings were observed in PiD, PSP and CBD (data not shown).

GSK-3α/β immunoreactivity

Weak GSK-3α/β immunoreactivity was found in the cytoplasm of neurons and glial cells in control cases. How-

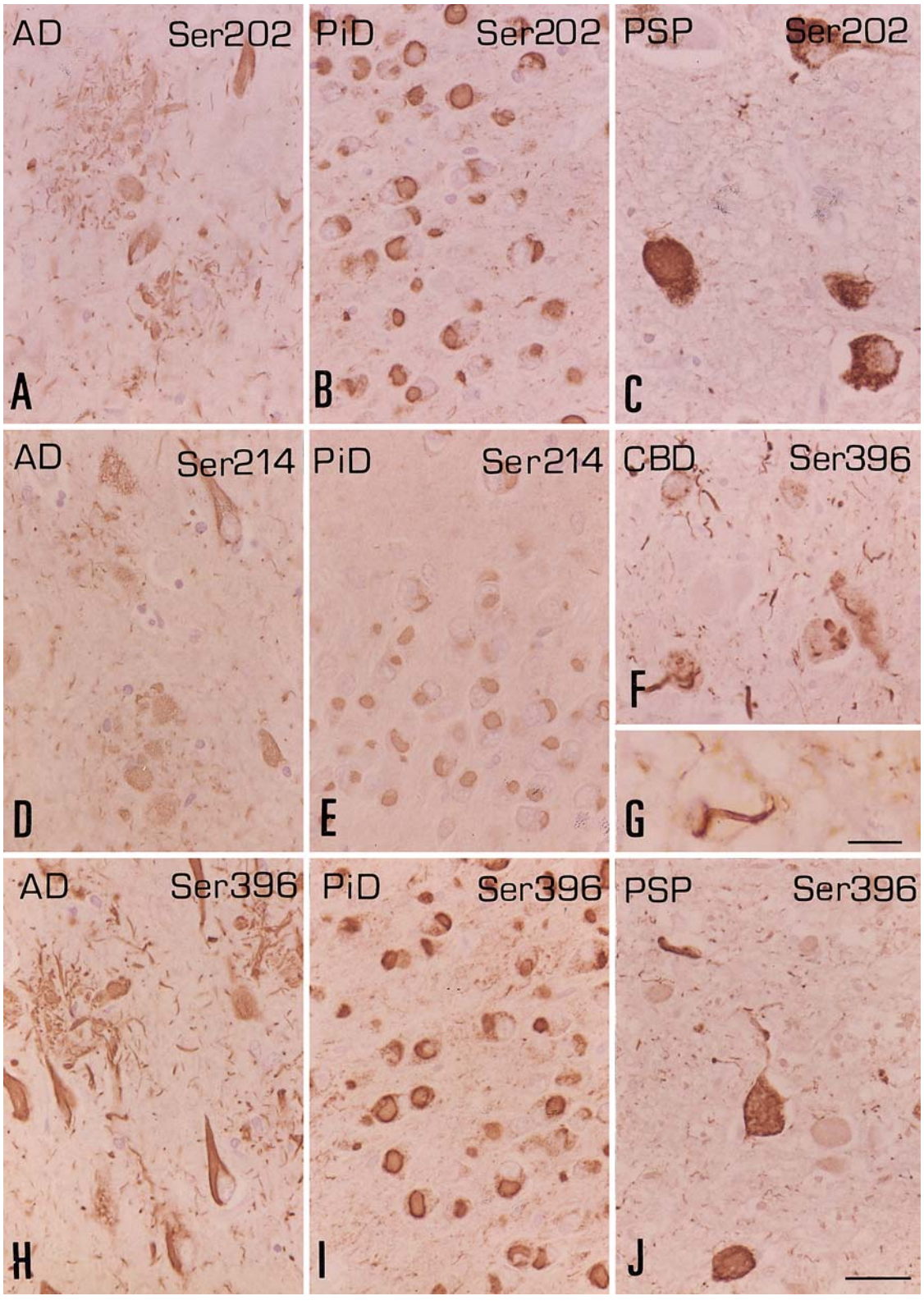


Fig. 2 Immunohistochemistry with phospho-specific tau Ser202 (**A**–**C**), Ser214 (**D**, **E**) and Ser396 (**F**–**J**) antibodies in AD (**A**, **D**, **H**), PiD (**B**, **E**, **I**), PSP (**C**, **J**) and CBD (**F**, **G**). Phospho-tau immunoreactivity is observed in neurons with neurofibrillary tangles, dystrophic neurites in senile plaques and neuropil threads in AD; Pick bodies in PiD; neurofibrillary tangles in PSP; and glial cells including coiled bodies in oligodendroglial cells in CBD. Paraffin sections slightly counterstained with hematoxylin. *Bars* **J** (also for **A**–**F**, **H**, **I**) 25 μm; **G** 10 μm

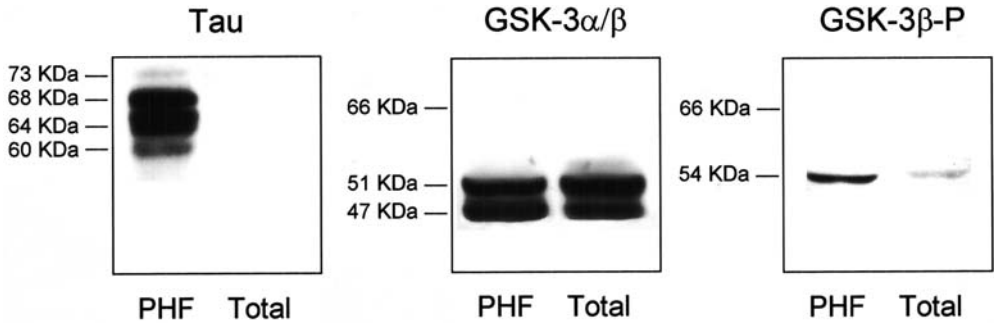


Fig. 3 Western blots of total brain homogenates and sarcosyl-insoluble fractions in AD. GSK-3α and GSK-3β are recognized by specific bands of 51 and 55 kDa, and 54 kDa, respectively, that differ in molecular mass from those of phospho-tau. Sarcosyl-insoluble fractions are enriched in GSK-3α/β and GSK-3β-P (*GSK* glycogen synthase kinase)

ever, increased GSK-3α/β immunoreactivity occurred in granulovacuolar degeneration in AD (Fig. 4A, B). Neurons with and without neurofibrillary tangles were stained equally with anti-GSK-3α/β antibodies, as revealed with double-labeling immunohistochemistry to GSK-3α/β and phospho-tau in AD, CBD and PSP (Fig. 4B, C, E, F). Similarly, neurons with and without Pick bodies were stained equally with anti-GSK-3α/β (Fig. 4D). Coiled bodies were not recognized with anti-GSK-3α/β (data not shown).

GSK-3β-P Ser9 immunoreactivity

No GSK-3β-P immunoreactivity was observed in controls. However, strong GSK-3β-P immunoreactivity was found in neurons with neurofibrillary tangles in AD, PSP and CBD, as well as in Pick bodies and astrocytes in PiD (Figs. 4G–I, 5A–C). Dystrophic neurites of senile plaques (Fig. 5A) and neuropil threads in AD, PSP and CBD (Fig. 5A, F) were GSK-3β-P immunoreactive. Similarly, the cytoplasm of many glial cells and coiled bodies in PiD, PSP and CBD was stained with anti-GSK-3β-P antibodies (Fig. 5D–G). Double-labeling immunohistochemistry to GSK-3β-P and tau disclosed co-localization ranging from 40% to 80% of tau-bearing neurons, coiled bodies and tau-containing astrocytes (Fig. 5H). GSK-3β-P was localized in dystrophic neurites of senile plaques and in neurofibrillary tangles, as well as in Pick bodies, indicating a physical relationship between GSK-3β-P and abnormal tau deposition.

Single and double labeling using in situ end-labeling of nuclear DNA fragmentation and GSK-3β-P immunohistochemistry

Variable numbers of neurons and glial cells were stained using in situ end-labeling of nuclear DNA fragmentation in control and diseased brains, although, on average, the number of stained cells was larger in AD, PiD, PSP and CBD than in controls.

Double-labeling disclosed no a single cell co-localizing nuclear DNA breaks and GSK-3β-P immunoreactivity (data not shown).

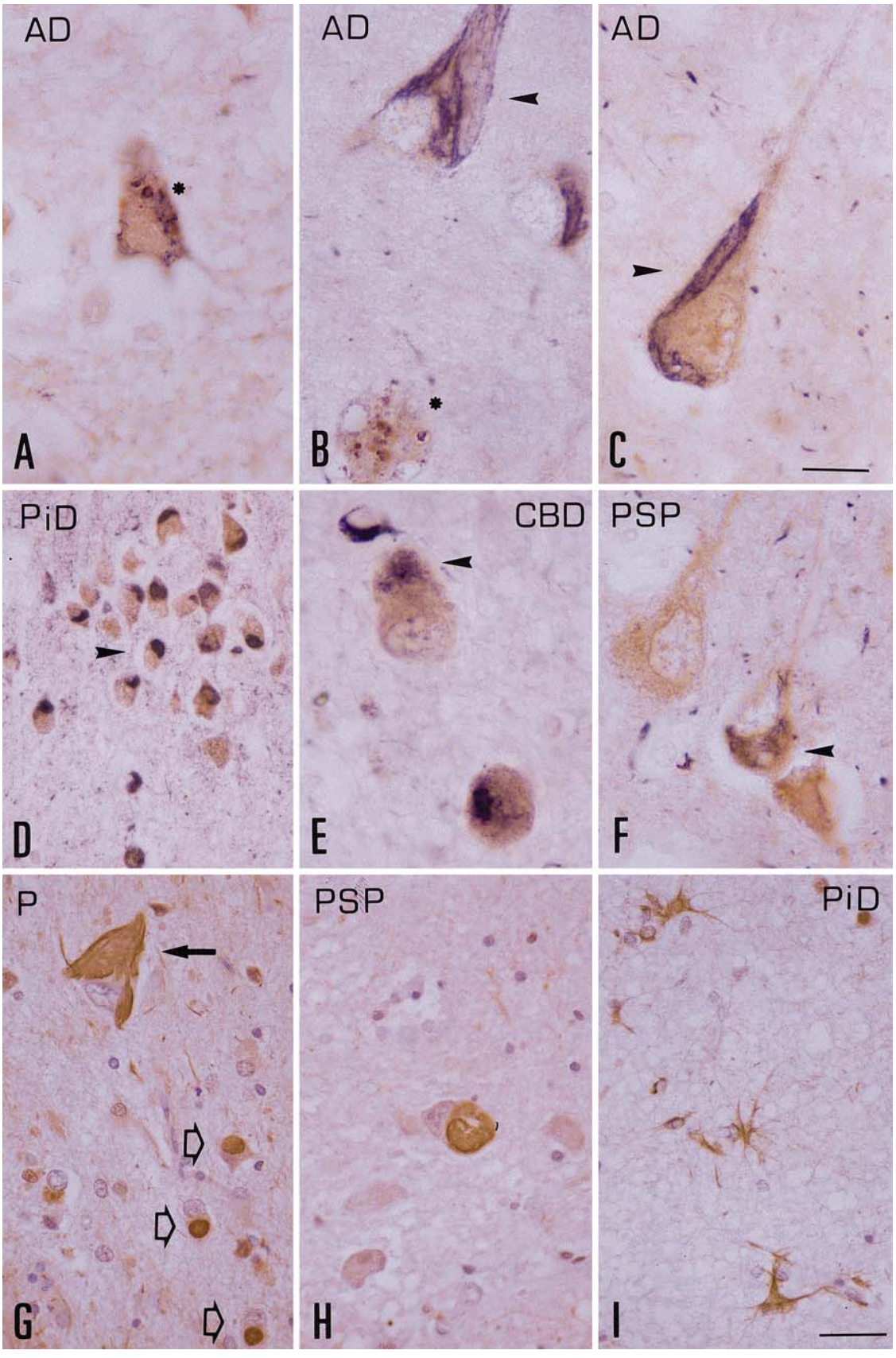
Discussion

Phospho-specific tau antibodies to Ser202, Ser214 and Ser396 decorate neurons with neurofibrillary tangles, dystrophic neurites of senile plaques, neuropil threads, Pick bodies, tau deposits in astrocytes and coiled bodies in oligodendrocytes in several tauopathies including AD, PiD, PSP and CBD. Gel electrophoresis and Western blotting of sarcosyl-insoluble fractions disclose disease-specific band patterns consisting of four bands of 73, 68, 64 and 60 kDa in AD, two bands of 68 and 64 kDa in PSP and CBD, and two bands of 64 and 60 kDa in PiD, in agreement with previous studies [3, 4, 20].

Several in vitro studies have shown that GSK-3β is expressed in neurons and that it may induce tau phosphorylation [16, 24, 29, 31, 33, 34, 35, 36, 39, 42]. GSK-3β-catalyzed phosphorylation of tau is modulated by other kinases [31, 33, 34, 42]. However, tau hyperphosphorylation and pre-tangle-like somatodendritic localization of tau has been produced in GSK-3β conditional transgenic mice [23]. Moreover, GSK inhibition prevents tau hyperphosphorylation [25]. These observations indicate that GSK-3β is a very potent activator of tau phosphorylation per se, and that over-expression of GSK-3 may induce tau phosphorylation. Increased GSK-3α/β expression has been previously reported in association with granulovacuolar degeneration in AD [21].

Previous immunohistochemical studies have shown increased GSK-3 expression in association with neurofibrillary tangles in AD [27, 41]. The present results support these observations showing GSK-3α/β particularly enriched in sarcosyl-insoluble fractions. Moreover, tau-bearing deposits in AD, PiD, PSP and CBD contain hyperphosphorylated tau at sites that are putative targets of GSK-3β. Ser202 and Ser396 may be phosphorylated by MAPKs, and Ser214 by PKA [4, 20, 22]. GSK-3β has the capacity to phosphorylate tau at Ser396 and, to a lesser degree, at Ser202 and Ser214 [13, 17, 39, 42]. Strong GSK-3α/β expression in sarcosyl-insoluble fractions could, in turn, phosphorylate tau, thus promoting the cascade of tau hyperphosphorylation in tauopathies.

In addition, strong immunoreactivity for the phosphorylated form of GSK-3βSer9 (GSK-3β-P) has been detected in dystrophic neurites of senile plaques, neuropil threads, Pick bodies, tau-containing astrocytes and oligodendrocytes with coiled bodies in AD, PiD, PSP and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◀ **Fig. 4** Weak GSK-3α/β immunoreactivity in AD (**A**–**C**), PiD (**D**), CBD (**E**) and PSP (**F**) is expressed equally in neurons with (*arrowheads*) and without abnormal tau deposits, as seen with double-labeling immunohistochemistry to phospho-tau (tauSer296) (*dark-blue precipitate*). Strong GSK-3α/β expression occurs in granulovacuolar degeneration (*asterisks* in **A** and **B**). Strong GSK-3-P Ser9 expression is found in neurofibrillary tangles (*arrow* in **G**) and Pick bodies (*white arrows*) in PiD associated with AD changes, in neurofibrillary tangles in PSP (**H**), and astrocytes in PiD (**I**). Paraffin sections slightly counterstained with hematoxylin. *Bars* **C** (also for **A**, **B**, **E**, **F**) 10 μm; **I** (also for **D**, **G**, **H**) 25 μm

CBD. GSK-3β is inactivated by phosphorylation at Ser9 [5, 12, 37], and the process of phosphorylation-inactivation may be triggered by PKB/AKT [1, 5], MAP kinases [32, 37] and PKA [8]. Previous studies have shown early expression of active MAPK/ERK and other stress kinases in association with hyperphosphorylated tau deposits in tauopathies [10, 11, 19, 28, 43, 44], thus suggesting MAPKs as putative inactivators of GSK-3β in these diseases.

Taken together, the present observations support a complex role for GSK-3 in tau phosphorylation in AD, PiD,

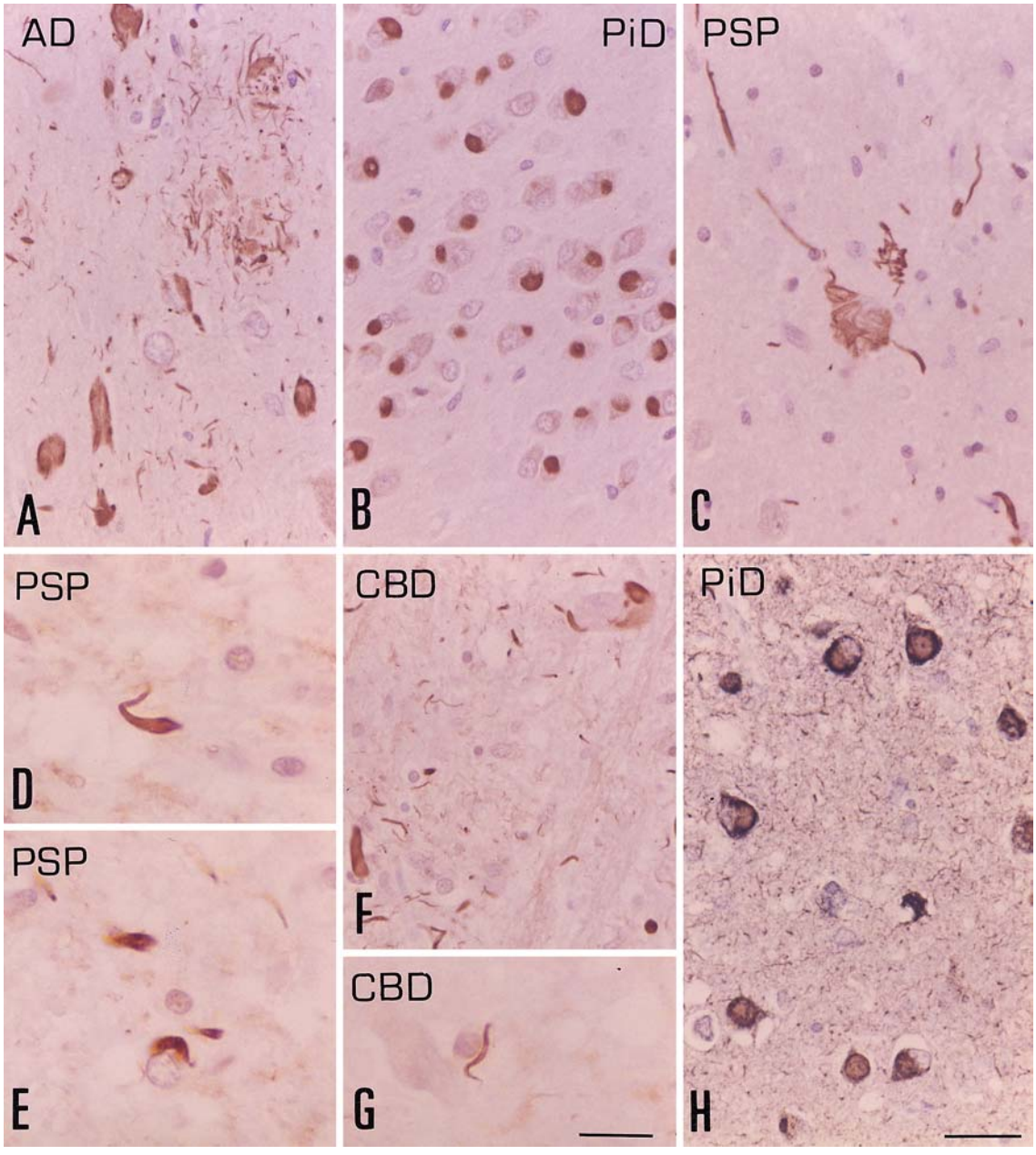

Fig. 5 Immunohistochemistry with phospho-specific GSK-3β Ser9 antibody (GSK-3β-P) in AD (**A**), PiD (**B**), PSP (**C**-**E**) and CBD (**F**, **G**). Strong GSK-3β-P immunoreactivity is found in neurons with neurofibrillary tangles, dystrophic neurites of senile plaques, neuropil threads, Pick bodies, astrocytes and oligodendrocytes with coiled bodies. Double-labeling immunohistochemistry shows co-localization of GSK-3β-P (*brown precipitate*) and pan-tau (*dark blue precipitate*) in the majority of tau-bearing neurons in the dentate gyrus in PiD. The pan-tau antibody recognizes only phosphorylated tau in paraffin sections. Paraffin sections slightly counterstained with hematoxylin. *Bars* **H** (also for **A**-**C**, **F**) 25 μm; **G** (also for **D**, **E**) 10 μm

PSP, and CBD, and suggest a similar scenario in other tauopathies. Elevated levels of GSK-3β-P in some neurons and glial cells in the different tauopathies is intriguing, as it would indicate inactivation of GSK-3β and sequestration of the inactive kinase at particular subcellular domains enriched with abnormal tau.

In addition, it has been demonstrated that GSK-3β-Pser9 has the capacity to inhibit apoptosis in certain paradigms [5, 6]. The present findings showed no association of nuclear DNA breaks and GSK-3β-P expression. It is possible that GSK-3β-P may prevent abnormal tau-containing cells from dying in AD and other tauopathies.

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