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Neuronal and glial tau-positive inclusions in diverse neurologic diseases share common phosphorylation characteristics

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Abstract Tau accumulating as paired helical filaments (PHF) in Alzheimer's disease brain is considered to be abnormally phosphorylated on distinct sites. To compare the phosphorylation state of tau-positive neuronal inclusions among diverse neurologic diseases, we have probed these lesions with three well-defined PHF/tau monoclonals, C5, M4 and tau 1, that most likely recognize three proline-directed phosphorylation sites in PHF-tau. In Alzheimer's disease brain all three monoclonals intensely immunostained intracellular neurofibrillary tangles, neuropil threads, senile plaque neurites, and "pretangle neurons" in a phosphorylation-dependent manner. They also stained, in the same manner, Pick bodies in Pick's disease, and neurofibrillary tangles and neuropil threads in various tangle-forming neurologic diseases. In most of these diseases (including Pick's disease, progressive supranuclear palsy, subacute sclerosing panencephalitis, and Alzheimer's disease) astrocytes and oligodendrocytes were found to contain tau-positive inclusions which showed the same immunocytochemical characteristics. Thus, the widely occurring tau-positive inclusions share common phosphorylation characteristics irrespective of underlying diseases or cell types.

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Introduction

Tau was known as a neuron-specific microtubule-associated protein that promotes tubulin assembly in vitro [35] and stabilizes microtubules in vivo [13]. Although tau was previously considered to be specific for axonal microtubules [3], recent evidence suggests that tau is also associated with dendritic microtubules in a distinct phosphorylated form [25], and even exists in astrocytes and oligodendrocytes [21, 25, 31]; therefore, tau can no longer be considered a neuron-specific protein.

Neurofibrillary tangles (NFT), originally described in Alzheimer's disease (AD), are bundles of unit fibrils with an unusual morphology: paired helical filaments (PHF). PHF are made up of two 10-nm filaments wound around each other, giving regular constrictions every 80 nm [15, 33]. At their early stage, PHF are composed solely of abnormally phosphorylated species of tau [18]. The abnormally phosphorylated sites are clustered in both amino- and carboxyl-terminal-flanking regions of the microtubule-binding domain [8]. Many of those sites appear to also be phosphorylated in fetal tau, as shown by shared immunoreactivities with several phosphorylation-dependent PHF polyclonal and monoclonal antibodies [9, 14].

NFT have thus far been documented in a wide range of neurologic disorders, including Down's syndrome, Pick's disease, progressive supranuclear palsy (PSP), parkinsonism-dementia complex of Guam (PDC), Hallervorden-Spatz disease (HSD), dementia pugilistica, subacute sclerosing panencephalitis (SSPE), and many other diseases [37]. They are composed of PHF or straight tubules and all are tau immunoreactive as shown by immunocytochemistry [5, 12, 19, 26–30, 32]. Furthermore, it has recently been shown that glial cells

in some of the above neurologic diseases occasionally contain tau-positive inclusions [22, 23, 38–40].

In AD, the abnormal phosphorylation of tau has been postulated to underlie its self-assembly into PHF, although there is little evidence that the phosphorylation precedes the PHF formation [1]. In fact, it has very recently been demonstrated that the microtubule-binding domain of tau itself can form filaments in vitro which closely resemble PHF [36]. We have been concerned about the role of the phosphorylation of tau in the formation of these inclusions and, thus, have undertaken the work to examine phosphorylation characteristics in the inclusions in a range of disorders, using three well-defined phosphorylation-dependent PHF/tau monoclonal antibodies: tau 1, M4, and C5. Here we show that various tau-positive inclusions in neuron and glia share similar phosphorylation characteristics. This suggests that the same mechanism, in particular the same protein kinase, is involved in the formation of tau-positive inclusions, irrespective of cell types or underlying diseases.

Materials and methods

Cases

Brains from patients with AD (five cases), Pick's disease (two cases), PSP (two cases), HSD (one case, 38-year-old male), SSPE (one case: 24-year-old male), PDC (one case), congenital muscular dystrophy of Fukuyama type (FCMD; one case, 24-year-old female) and nonneurologic diseases (four cases) were the basis for this study. One AD brain showed unusually severe cortical atrophy with innumerable extracellular (e-)NFT and astrocytic tau-positive inclusions which are composed of PHF [22]. Blocks from these brains were fixed in 10% neutral-buffered formalin for 1–3 weeks, dehydrated and embedded in paraffin. Sections, 6 μm thick, were obtained from the medial temporal lobe (including hippocampal formation and lateral occipitotemporal cortex) and frontal lobe except for PSP and FCMD cases from which blocks of the brain stem and diencephalon were available.

Antibodies

Monoclonals against PHF were raised as described elsewhere [9]. Briefly, BALB/c mice were immunized with Sarkosyl-insoluble pellets from AD brain together with complete Freund's adjuvant, followed by four boosters. Fusion of spleen cells with myeloma cells and screening of the hybridomas were performed as described previously [9]. Two PHF monoclonals, C5 and M4, were rigorously characterized [9]. Both monoclonals labeled PHF-tau and fetal tau on immunoblots, but these immunoreactivities were completely eliminated after dephosphorylation by alkaline phosphatase. The phosphorylated epitopes of M4 and C5 were shown to be located presumably around Thr-231 and Ser-396, respectively [9] (according to the numbering in the longest isoform of human tau [7]). The epitope of tau 1 was previously localized to residues 189–207 [16], and more recently to around Ser-199 and/or Ser-202 [2], the phosphorylation of which abolishes its immunoreactivity. The topography of the phosphorylated epitopes defined by these three monoclonals is schematically illustrated in Fig. 1. None of the monoclonals cross-reacts with neurofilaments or other proteins on the immunoblots. C5, M4 and tau 1 were used at the concentration of 2 μg/ml. Polyclonal antibodies to glial fibrillary acidic protein (GFAP; Dako, Sweden) and carbonic anhydrase II (CAII; The Binding Site Ltd., Birmingham, UK) were used as specific markers for astrocytes and oligodendrocytes, respectively [17].

Immunocytochemistry

Tissue sections were immunostained by the avidin-biotin method using diaminobenzidine (DAB) as chromogen, and were counterstained lightly with hematoxylin. Tau 1 immunoreactivity was examined on sections without or with prior alkaline phosphatase treatment which involves incubation of sections with 10 U/ml alkaline phosphatase (*E. coli*, Type III, Sigma) in 50 mM TRIS-HCl, pH 8.3 for 3 h at 37 °C. Double immunolabeling with each monoclonal and polyclonal antibody to GFAP or CAII was performed by combination of the peroxidase-antiperoxidase and the avidin-biotin methods using DAB and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) /nitroblue tetrazolium chloride (NBT) as chromogens.

Immunoelectron microscopy

Sections, 50 μm-thick, of the formalin-fixed frontal cortex from one case of Pick's disease and the subthalamic area including the

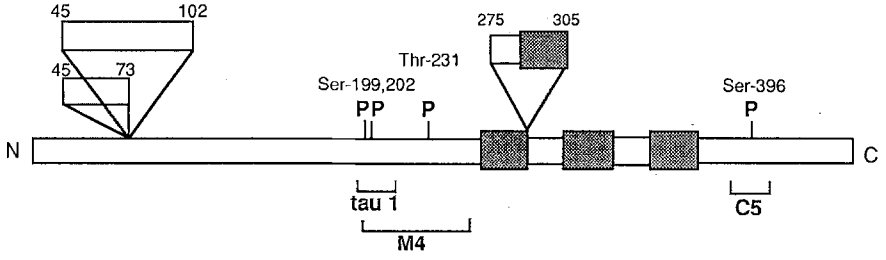

Fig. 1 A schematic illustration of the location of epitopes recognized by three phosphorylation-dependent PHF/tau monoclonals. The phosphorylated sites most likely recognized by the monoclonals are Ser-199 and/or Ser-202 (nonphosphorylated state) for tau 1 [2], Thr-231 for M4 [9], and Ser-396 for C5 [9]. Two kinds of inserts in the amino-terminal region and one insert in the carboxyl-terminal region are indicated. *Shaded boxes* represent microtubule-binding domains. The *numbering* is according to the longest human isoform (441 residues) [7]. (*PHF* paired helical filaments)

internal capsule from one case of PSP were cut on a vibratome. The sections were incubated with M4 or C5 at 4 °C for 48 h and visualized by the avidin-biotin method using DAB as chromogen. After postfixation with OsO_4 and dehydration, they were flat-embedded in Epon. Ultrathin sections were viewed without uranyl acetate and lead citrate staining in a JEOL 1200EX electron microscope.

Results

Neurofibrillary tangles and Pick bodies

In AD brains, C5, M4, and (only after dephosphorylation) tau 1 immunostained NFT, neuropil threads (NT) and senile plaque neurites (Fig. 2A–D). C5 stained NFT more intensely than NT or senile plaque neurites (Fig. 2A, C), whereas M4 (Fig. 2B, D) and tau 1 after dephosphorylation stained these lesions at equal intensity. These lesions were hardly stained with tau 1 without prior dephosphorylation (data not shown). NFT and NT in HSD (Fig. 3A–C), SSPE (Fig. 3D), PDC (Fig. 3E), FCMD (Fig. 3F), and PSP (Fig. 4F), as well as in nonneurologic aged individuals (data not shown), were similarly stained to those in AD by C5, M4 and with tau 1 after dephosphorylation. AD and HSD brains contained considerable numbers of e-NFT in the hippocampus and parahippocampal gyrus, which were positively but weakly stained with C5 (Figs. 2A, C; 3A), but barely stained with M4 (Figs. 2B, 3B). E-NFT were not labeled with tau 1 even after dephosphorylation (Fig. 3C). A subset of neurons in CA1 and CA2 portions of the hippocampus in AD and other disorders showed diffuse perikaryal and characteristic perinuclear stainings with tau 1 (after dephosphorylation, data not shown), M4 (Figs. 2B, 3E) and, less prominently, with C5 (Fig. 2A). In Pick's disease brains, most of the Pick bodies (PB) were strongly labeled with C5 (Fig. 3G); M4 also stained many PB but less intensely (Fig. 3H); after dephosphorylation, tau 1 stained PB to variable degrees (Fig. 3I).

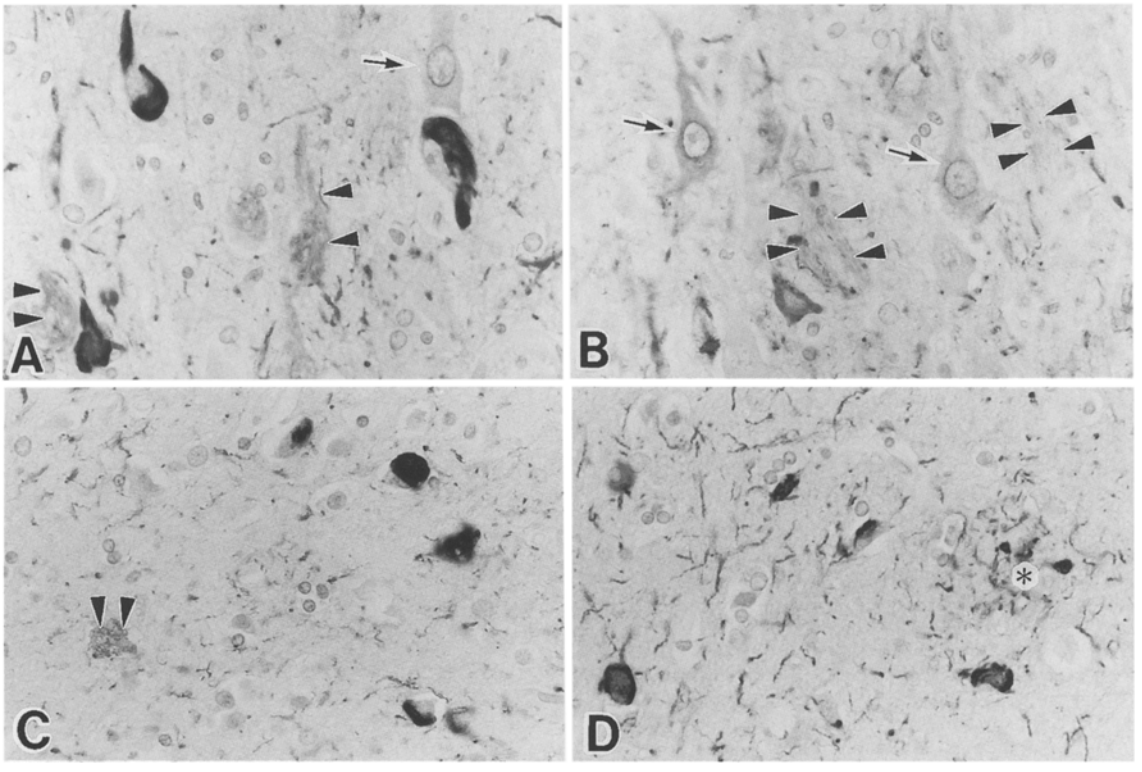

Fig. 2A–D AD cortex immunostained with C5 and M4. **A**, **B** CA1 portion of the hippocampus. **A** C5 immunolabels i-NFT strongly, but e-NFT (*arrowheads*) weakly. A neuronal perikaryon with perinuclear accentuation (*arrow*) is lightly immunolabeled ("pretangle neuron"). **B** M4 barely immunolabels e-NFT (*arrowheads*). An e-NFT in the *center* is outlined by the attached degenerating neurites. M4 stains "pretangle neurons" (*arrows*) more intensely than C5. **C, D** Temporal neocortex. **C** C5 labels i-NFT strongly, but NT less intensely. An e-NFT (*arrowheads*) is lightly stained. **D** M4 labels i-NFT, NT and senile plaque neurites (*asterisk*) equally. (*AD* Alzheimer's disease, *i-* intracellular, *e-* extracellular, *NFT* neurofibrillary tangles, *NT* neuropil threads) **A–D**×333

Glial tau-positive inclusions

Tau-positive inclusions were also found in glial cells in the affected areas from the cases of Pick's disease, PSP and SSPE, and in the hippocampus of two AD cases. In Pick's disease cortex (Fig. 4A, B), a part of the astrocytic perikarya and processes that were GFAP positive (Fig. 4B) were stained for C5 and M4 and for tau 1 after dephosphorylation (data not shown). Ultrastructurally, these astrocytes contained bundles of C5- and M4-reactive 15- to 20-nm fibrils which were in-

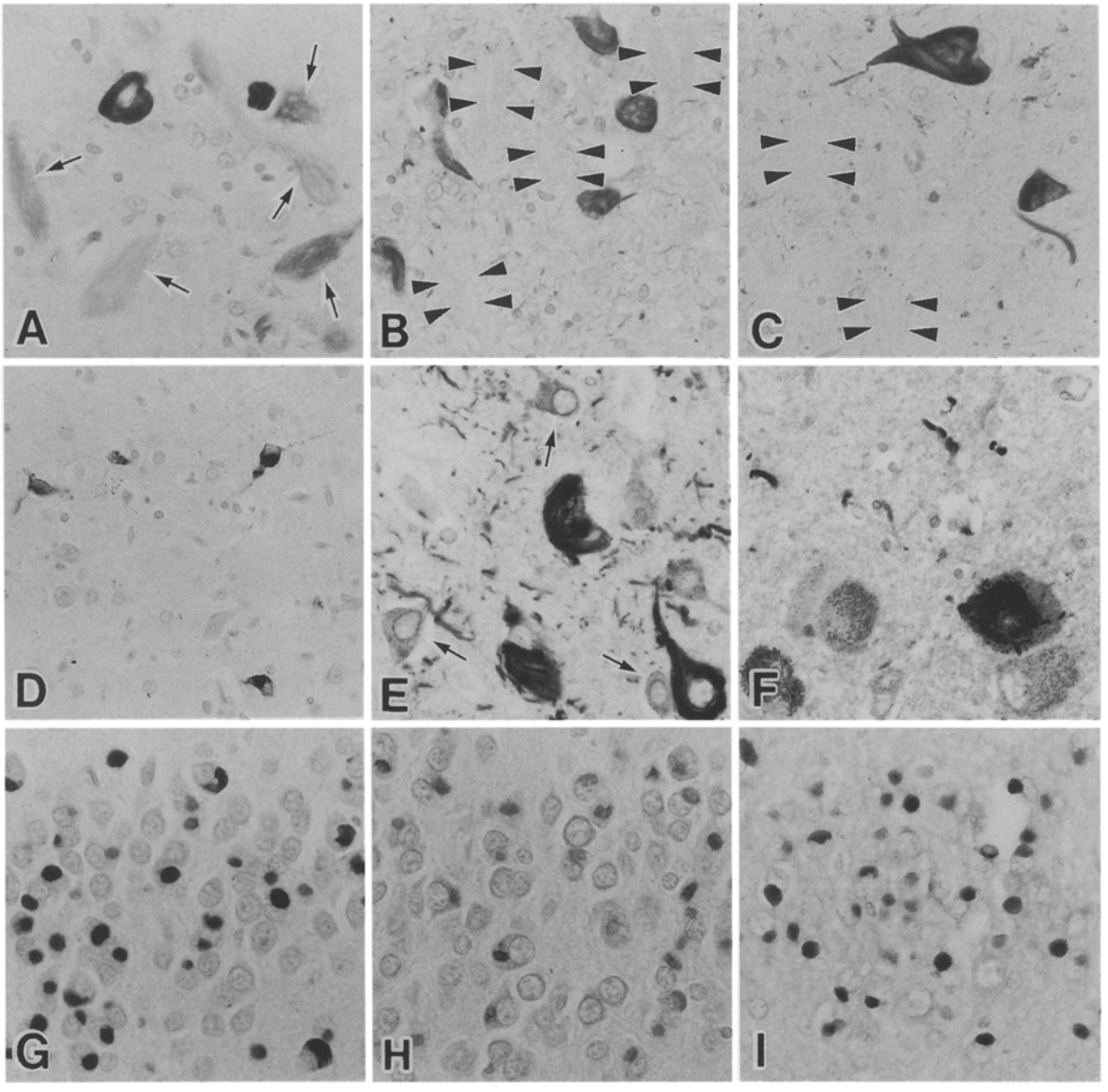

Fig. 3A–I Neurofibrillary pathology in brains affected with neurologic diseases other than AD. **A–C** Hallervorden-Spatz disease. CA1 portion of the hippocampus. **A** C5 labels i-NFT strongly but e-NFT (*arrows*) only weakly. **B** M4 staining; **C** tau 1 staining (after alkaline phosphatase treatment); M4 and tau 1 hardly immunostain e-NFT (*arrowheads*). **D** SSPE. Frontal cortex. C5 stains SSPE NFT. **E** Parkinsonism-dementia complex of Guam. CA4 portion of the hippocampus. M4 stains numerous NFT, NT and "pretangle neurons" (*arrows*). **F** Congenital muscular dystrophy of Fukuyama type. Locus ceruleus. M4 stains a NFT-bearing neuron (*right upper one*; others are melanin-containing neurons) and surrounding NT. **G–I** Pick's disease. Dentate gyrus of the hippocampus. **G** C5 strongly immunostains most PB. **H** M4 weakly stains most PB. **I** tau 1, after dephosphorylation, stains PB to variable degrees. (*SSPE* subacute sclerosing panencephalitis, *PB* Pick bodies) **A–C**, **E–I**×333, **D**×240

termingled with bundles of glial fibrils (Fig. 5A, B). These fibrils in astrocytes were quite similar to PB fibrils: straight tubules (Fig. 5C). In the subcortical white matter of an SSPE case, small round cells in the nerve fascicles (Fig. 4C), contained rod- or coil-like inclusions that were immunoreactive with all three monoclonals. These small cells were CAII positive and, thus, were concluded to be oligodendrocytes (Fig. 4D). In PSP brain, fibrillary inclusions in astrocytes (Fig. 4F) and coil- or thread-like inclusions in oligodendrocytes (Fig. 4E) were also positive for the three monoclonals. Ultrastructurally, the latter inclusions were composed of tightly packed straight tubules of 15–20 nm in diameter (Fig. 5D). In the hippocampi of two AD cases (an atypical case of juvenile onset [22] and a 74-year-old case of 14-year duration), where pyramidal neurons were almost completely lost and replaced by innumerable e-NFT, reactive astrocytes contained fibrillary inclusions positive for all three monoclonals (Fig. 4G).

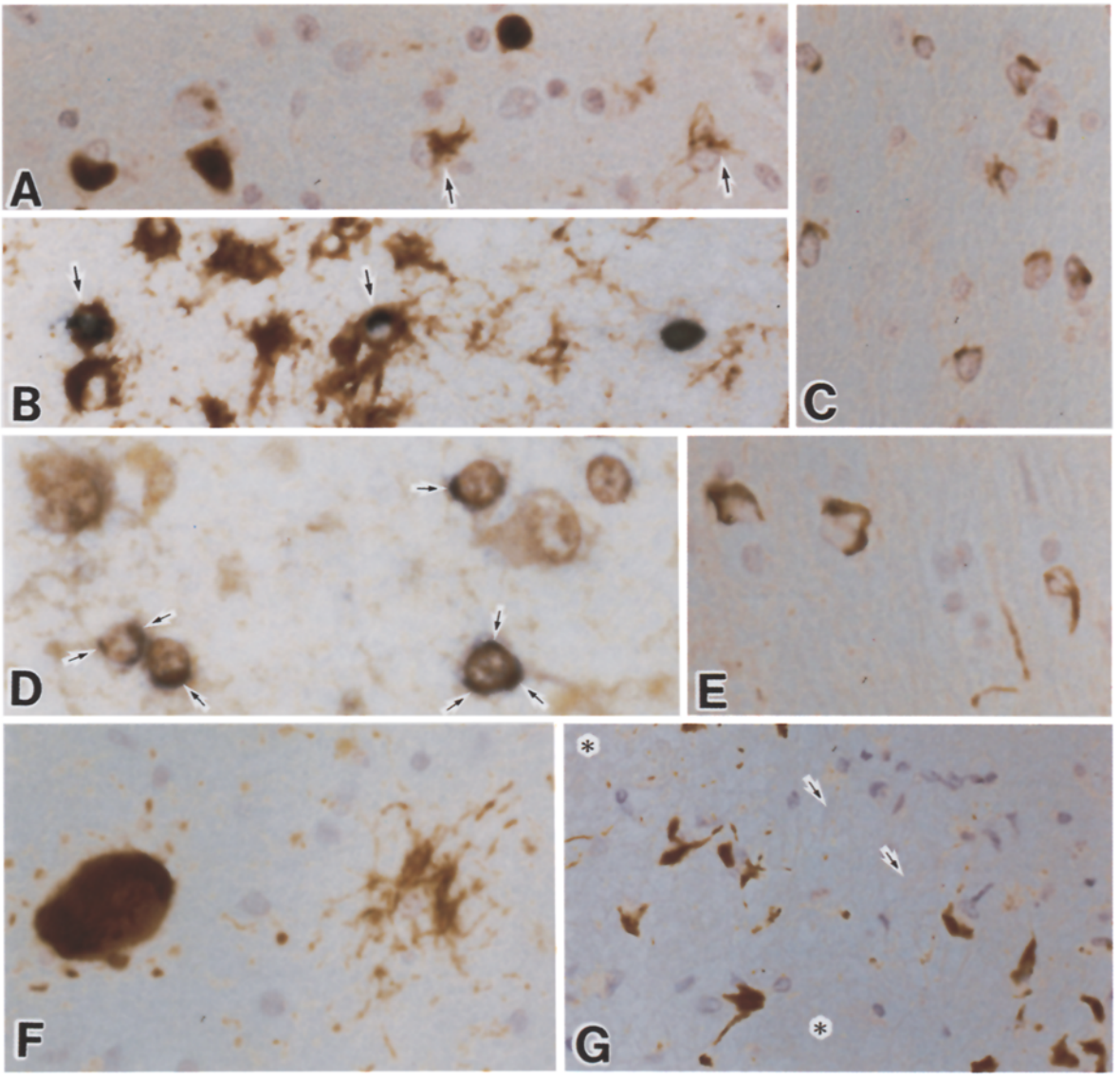

Fig. 4A–G Glial inclusions immunostained with C5 and M4. **A**, **B** Pick's disease. Frontal cortex. **A** C5 immunostains PB and fibrillary inclusions in astrocytes (*arrows*). **B** Double immunostaining for C5 (*dark blue*) and GFAP (*brown*). Two C5-positive deposits are located within GFAP-positive astrocytes (*arrows*). A C5-positive one in the right is a PB. **C**, **D** SSPE. Subcortical white matter of the frontal lobe. **C** M4 visualizes coil- or rod-like inclusions in interfascicular oligodendrocytes. **D** Double immunolabeling for C5 (*brown*) and CAII (*grayish blue*). C5-positive rods (*arrows*) are located in the CA II-positive cells. **E**, **F** PSP. **E** Internal capsule. Tau 1, after dephosphorylation, immunostains coiled inclusions in the cells, presumably interfascicular oligodendrocytes. **F** Red nucleus. C5 strongly stains a globose-type NFT (*left*) and fine processes of an astrocyte (*right*). **G** CA1 portion of the hippocampus from an atypical AD case [22]. Pyramidal neurons are completely lost and substituted for innumerable e-NFT (*arrows*), amyloid plaques (asterisks) and reactive astrocytes. Numerous astrocytes contain M4-positive inclusions. (*CA* carbonic anhydrase, *PSP* progressive supranuclear palsy) **A**, **C**×533; **B**×444; **D** ×1000; **E**, **F**×667; **G**×333

Discussion

The phosphorylation of PHF-tau is characterized by (i) its localization to amino- and carboxyl-terminal-flanking regions of the microtubule-binding domain [8]; (ii) many proline-directed sites which are also phosphorylated in fetal tau [34]; and (iii) several additional sites which are unique to PHF-tau [41] and may not be proline-directed (Morishima et al., unpublished data).

In this work we have employed three phosphorylation-dependent PHF/tau monoclonals: tau 1, M4 and C5. tau 1 probes for the nonphosphorylated state around Ser-199 and/or Ser-202 [2] (see Fig. 1). M4 probes most likely for the phosphorylation on Thr-231, and C5 presumably for that on Ser-396 [9]. All of the above sites are serine or threonine residues followed by proline, and are thus proline directed. It should be

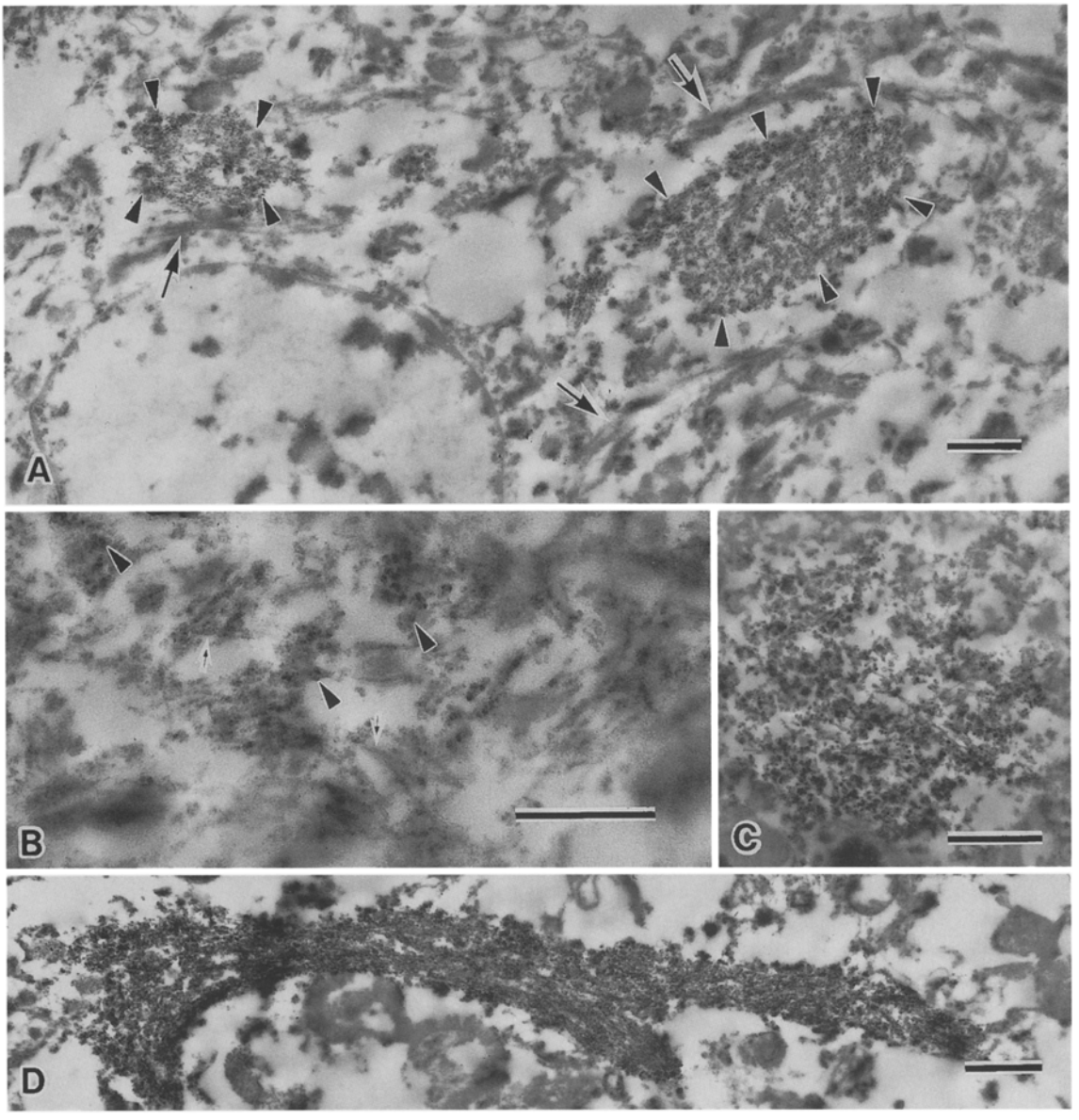

Fig. 5A–D Immunoelectron micrographs of M4-positive glial cells. **A–C** Pick's disease, Frontal cortex. **A** Bundles of M4-positive tubules (*arrowheads*) are intermingled with glial bundles (*arrows*) in an astrocyte. **B** A M4-positive astrocytic inclusion consists of straight tubules (*arrows*, longitudinal profiles; *arrowheads*, transverse profiles) of 15–20 nm in diameter. **C** A PB consists of straight tubules similar to those in astrocytes. **D** A thread-like inclusion within an oligodendrocyte-like cell in the internal capsule from a case of PSP (see Fig. 4E) is also composed of straight tubules of 15–20 nm in diameter. M4 staining. *Bars* **A**, **C**, **D**=1μm, **B**=500 nm

noted that the immunoreactivities with all three monoclonals do not necessarily indicate that all the phosphorylation sites are exactly the same among the lesions. In fact, there is some evidence suggesting that PSP NFT and NT are less phosphorylated than AD NFT and NT, as shown by less marked mobility shift of tau from PSP brain [6]. Nonetheless, strong immunoreactivities with all three monoclonals indicate the phosphorylation of distinct proline-directed sites and, thus, suggest the involvement of a proline-directed protein kinase(s).

This set of monoclonals, tau 1 (after dephosphorylation), M4 and C5, strongly labeled intracellular NFT, NT, and senile plaque neurites in AD brain. NFT and NT of other diverse neurologic diseases and PB were similarly labeled with the monoclonals. The ultrastructures of these inclusions are variable: AD NFT and NT are composed mainly of PHF and in part of straight tubules, while PSP NFT and PB fibrils consist chiefly of straight tubules. In this regard, perinuclear staining is unusual because immunoelectron microscopy has revealed that the tau immunoreactivities are located on ribosomes which contain no fibrils [24].

Recent reports have described the presence of tau-positive inclusions in glial cells [22, 23, 38–40] in some neurologic disorders where affected neurons tend to form NFT. Such tau-positive inclusions are composed of

PHF in astrocytes in AD [22] or of straight tubules of 15 or 20–25 nm in astrocytes and oligodendrocytes in PSP [23, 38, 39]. The present study has confirmed and further extended the observation to a wider range of disorders: astrocytes in AD, Pick's disease and PSP, and oligodendrocytes in SSPE and PSP. All of these glial inclusions share the same phosphorylation characteristics as those seen in NFT and NT. Currently, we do not know whether the presence of glial tau-positive inclusions is restricted to the NFT-forming neurologic diseases. In view of the frequent occurrence of these glial inclusions in PSP, it is possible that these inclusions are formed only in NFT-forming diseases. To explore this possibility, many diseases should be screened using the PHF/tau antibodies.

Our results, together with those of previous reports [22, 23, 38–40], strongly suggest that the formation of tau-positive inclusions is a rather common pathway irrespective of cell type, and that a common proline-directed protein kinase is involved, which includes mitogen-activated protein (MAP) kinases [4], cdc2-related kinases [10], and glycogen synthase kinase 3 [11, 20]. Since the astrocytes forming tau-positive inclusions are considered to be reactive species, a certain sustained external stimulus may be responsible for their formation. Furthermore, our observations validate the attempt using astrocytic culture to make an in vitro system for the tau-positive inclusion. If successful, it should allow us to elucidate the mechanism of the PHF formation in neurons, because their immunocytochemical identity implies the same pathogenesis.

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