

Up-Regulation of Inhibitors of Protein Phosphatase-2A in Alzheimer's Disease

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The activity of protein phosphatase-2A (PP2A) is compromised and is believed to be a cause of the abnormal hyperphosphorylation of tau in Alzheimer's disease (AD) brain. We investigated in AD the role of the two known endogenous PP2A inhibitors, called I_1^{PP2A} and I_2^{PP2A} , which regulate the intracellular activity of PP2A in mammalian tissues. We found a significant increase in the neocortical levels of I_1^{PP2A} and I_2^{PP2A} in AD as compared to control cases by *in situ* hybridization. The immunohistochemical studies revealed that I_2^{PP2A} was translocated from neuronal nuclei to cytoplasm in AD. The 39-kd full-length I_2^{PP2A} was selectively cleaved into an ~20-kd fragment in AD brain cytosol. Digestion of the recombinant human I_2^{PP2A} with AD brain extract showed an increase in the generation of the ~20 kd and other fragments of the inhibitor as compared to control brain extract. Double-immunohistochemical studies revealed co-localization of PP2A with PP2A inhibitors in neuronal cytoplasm and co-localization of the inhibitors with abnormally hyperphosphorylated tau. These studies suggest the possible involvement of I_1^{PP2A} and I_2^{PP2A} in the abnormal hyperphosphorylation of tau in AD. (*Am J Pathol* 2005, 166:1761–1771)

Neurofibrillary degeneration of the abnormally hyperphosphorylated tau is one of the hallmarks of Alzheimer's disease (AD) and tauopathies.^{1–3} Unlike normal microtubule-associated protein (MAP) tau, which stimulates assembly and stabilizes microtubules,⁴ the hyperphosphorylated tau sequesters normal tau, MAP1 and MAP2 and inhibits assembly, and depolymerizes microtubules.^{5–7}

The activities of protein phosphatase (PP) 2A and PP1 are compromised in AD brain,^{8,9} and the inhibition of PP2A activity by okadaic acid produces in metabolically active brain slices from adult rats the abnormal hyperphosphorylation of tau that inhibits its binding and the

promotion of microtubule assembly *in vitro*.¹⁰ Injection of calyculin A, a potent and specific inhibitor of PP2A and PP1, into rat hippocampus bilaterally induces the tau hyperphosphorylation and develops deficit in spatial memory retention in Morris water maze.¹¹ In the CA3 area of the AD hippocampus the expression of mRNAs of the catalytic subunit PP2A C α and the regulatory subunits PR55 γ and PR61 ϵ of PP2A, a trimeric holoenzyme, are reduced.¹² The expression of PP2Ac gene^{13,14} and of PP2A AB α C protein subunits have been found to be down-regulated in the affected area of AD brain.¹⁵ Furthermore, the level of methyltransferase, which promotes PP2A activity by methylating its catalytic subunit PP2Ac and the association of the PP2Ac with the scaffolding subunit A and regulatory subunit B α to form the trimeric holoenzyme in the brain are decreased in AD.¹⁶ Thus, it is strongly suspected that PP2A, which also regulates the activity of PP1 through dephosphorylation of PP1 inhibitors, I-1/DARPP-32, might be involved in the abnormal hyperphosphorylation of tau and that the abnormally phosphorylated tau can indeed be responsible for the AD neurofibrillary degeneration.

The intracellular PP2A activity is regulated by two inhibitor proteins, called I_1^{PP2A} and I_2^{PP2A} , in mammalian tissues.^{17–19} They inhibit PP2A in a noncompetitive manner with the substrate and exhibit apparent K_i values in the nanomolar range.¹⁷ I_1^{PP2A} has been found to be the same protein as PHAP I,²⁰ LANP,²¹ pp32,²² and mapmodulin.²³ Proteins homologous to I_2^{PP2A} have also been isolated and described as human SET,^{19,24} PHAP II,²⁰ and template-activating factor-1 β (TAF-1 β).²⁵ Except for mapmodulin,²³ it has been reported that these proteins could inhibit the PP2A activity *in vitro*.

In this study we show 1) an increase in the mRNA expressions of I_1^{PP2A} and I_2^{PP2A} in temporal and in ento-

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rhinal cortices of AD as compared to age-matched control cases; 2) a shift in the intracellular distribution of I₂^{PP2A} from its primarily nuclear location to the cytoplasm in neurons and as well as a cleavage of the full-length 39-kd protein to a ~20-kd fragment in AD brains; 3) an increase in the I₂^{PP2A} cleavage activity in AD brain; and 4) a co-localization of the two inhibitors with PP2A in neuronal cytoplasm and with the abnormally hyperphosphorylated tau in neurons with early- to middle-stage neurofibrillary degeneration.

Materials and Methods

Case Demographics and Tissue

Postmortem human hippocampus, temporal cortex (middle temporal gyrus), and cerebellum were dissected from seven age- and postmortem-matched cases of AD and seven cases of controls without neurological disease. The AD cases were diagnosed clinically by National Institute of Neurological and Communicative Diseases and Stroke—Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria²⁶ and after death histopathologically by The Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) criteria.²⁷ The case demographics including postmortem intervals are reported in Table 1. For histochemical studies, serial sections (40 μm thickness) were cut and stored in an anti-freezing solution²⁸ at –20°C until used. Paraffin-embedded sections (5 μm thickness) were used only for double-fluorescent immunohistochemistry between PP2A catalytic subunit and PP2A inhibitors.

In Situ Hybridization

Five cases from AD and five cases from control group were examined (Table 1). Generation of probes for I₁^{PP2A} and I₂^{PP2A} and *in situ* hybridization were performed as previously described.²⁸ Digoxigenin-labeled cRNA

probes (anti-sense and sense probe) were made by *in vitro* transcription using the human I₁^{PP2A} or I₂^{PP2A} cDNA²⁹ subcloned into pGEM-T vector (Promega, Madison, WI) as template in the presence of digoxigenin-labeled dUTP. For control study, pTRI-GAPDH-human (Ambion, Austin, TX) was used for template. Sections (40 μm) were postfixed for 20 minutes in 4% formaldehyde, followed by a 5-minute wash in 0.1 mol/L phosphate buffer, pH 7.2. Sections were treated with 0.001% proteinase K (Promega), and subsequently for 10 minutes in 0.1 mol/L triethanolamine and 0.225% acetic acid anhydrous solution. After washing with 0.1 mol/L phosphate buffer, sections were dehydrated through a series of increasing concentrations of ethanol and air-dried.

The sections were prehybridized for 30 minutes at 50°C in hybridization buffer (10% sodium dextran sulfate, 20 mmol/L Tris-HCl, pH 8.0, 0.3 mol/L NaCl, 0.2% sarcosyl, 0.02% heat-denatured salmon sperm DNA, 1× Denhardt’s solution, 50% formamide), and then hybridized overnight at 50°C in hybridization solution with 100 ng/ml of cRNA probes. After rinsing in 5× standard saline citrate at 60°C, the sections were washed in 50% formamide/2× standard saline citrate at 60°C for 30 minutes (high stringency wash). The sections were subjected to 30 minutes of RNase digestion at 37°C with 1 μg/ml of RNase A (Roche, Indianapolis, IN) in 10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L ethylenediamine tetraacetic acid, 0.5 mol/L NaCl, and then washed at high stringency. For detection of digoxigenin-labeled cRNA probes, anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) was reacted at a dilution of 1:500 and color was developed by incubation with 4-nitro blue tetrazolium chloride and 5-bromo-4 chloro-3 indolylphosphate solution (Roche).

Quantitative Analysis of *in Situ* Hybridization

To determine and compare the mRNA expression of PP2A inhibitors between AD and control, three images at ×20 magnification were captured from the entorhinal cortex, temporal cortex, and cerebellum. The intensity of the signals in stained neurons was determined by the program Simple PCI (C Imaging System, Cranberry Township, PA) and normalized per pixel in the circumscribed area. *In situ* hybridizations were performed on serial sections for I₁^{PP2A}, I₂^{PP2A}, and GAPDH. The levels of I₁^{PP2A} and I₂^{PP2A} mRNA intensities were normalized to the level of GAPDH mRNA intensity in the corresponding tissue. Mean values for each individual were analyzed by *t*-test between the AD and control cases for I₁^{PP2A} and I₂^{PP2A}. Differences with *P* < 0.05 were considered significant. All quantification and analysis were performed blind to the disease status.

Antibodies

The following antibodies were used: anti-I₁^{PP2A} (R-42089), a rabbit affinity-purified polyclonal antibody to a synthetic peptide corresponding to amino acid residues 10 to 23 of I₁^{PP2A}

Table 1. Profiles of Alzheimer’s Disease and Control Cases

		Age (years)	Sex	PMI (hour)	Braak score
AD	01–02*	89	F	3.0	V
	01–05*	80	F	2.2	VI
	01–07*	85	F	1.7	V
	01–10*	78	F	1.8	VI
	01–11*	95	F	3.2	VI
	01–12	86	M	2.3	VI
	01–13	91	F	3.0	V
Control	00–34*	85	M	3.2	II
	00–49*	86	F	2.5	III
	01–31*	81	M	2.8	III
	01–37*	88	F	3.0	II
	01–46*	90	F	3.0	III
	01–51	88	F	3.5	III
	02–01	88	F	3.0	IV

PMI, post mortem interval.

*These cases were used only for *in situ* hybridization and immunohistochemistry.

(rat/human); anti-I₂^{PP2A} (R-42187), a rabbit affinity-purified polyclonal antibody to a synthetic peptide corresponding to amino acid residues 18 to 29 of human I₂^{PP2A}; anti-I₂^{PP2A} (R1482), a rabbit affinity-purified polyclonal antibody to a synthetic peptide corresponding to amino acid residues 161 to 177 of human I₂^{PP2A}; anti-I₁^{PP2A} (5G6), a monoclonal antibody (mAb) to human recombinant I₁^{PP2A}; mAb to PP2A catalytic α subunit (BD Science, San Diego, CA); several phospho-dependent antibodies to tau phosphorylated at various sites: mAb PHF-1 to tau pSer396/pSer404;^{30,31} mAb M4 to pThr231/pSer 235;³² mAb 12E8 to tau pSer262/pSer356;³³ mAb β -actin (Sigma, St. Louis, MO); and rabbit polyclonal antibody to topoisomerase I (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Immunohistochemistry

Both frozen sections and paraffin-embedded sections were used. In the case of paraffin sections (5 μ m), they were dewaxed and antigen retrieval was performed by microwave irradiation in 10 mmol/L sodium citrate buffer, pH 6.0, 5 minutes at 850 W, twice. After washing in Tris-buffered saline, frozen sections were treated with 0.3% H₂O₂ plus 5% bovine serum albumin in Tris-buffered saline for 30 minutes. After this treatment, frozen sections and paraffin sections were subjected to the following same protocol. Sections were blocked with 5% normal goat serum in bovine serum albumin/Tris-buffered saline for 10 minutes and then treated with primary antibodies. Polyclonal antibodies to I₁^{PP2A} and I₂^{PP2A28} were used at the concentration of 5 μ g/ml. In the case of double-labeled immunohistochemical staining, PHF-1 diluted to 1/200, or M4 diluted to 1/1000, or 12E8 diluted to 1/250, or 5 μ g/ml of mouse antibody to PP2A catalytic subunit was mixed with I₁^{PP2A} or I₂^{PP2A} antibody at this step. After overnight incubation at 4°C, sections were washed with Tris-buffered saline and incubated with biotinylated anti-rabbit IgG diluted to 1/200 for 1 hour at room temperature. After washing, the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine were used to visualize the I₁^{PP2A} and I₂^{PP2A} antibody staining. In the case of double-immunohistochemical staining for PP2A inhibitors and phosphorylated tau, the sections were developed with Oregon green goat anti-mouse IgG (Molecular Probes, Eugene, OR) diluted to 1/1000. For double-fluorescent immunohistochemistry of PP2A inhibitors and PP2A catalytic subunit, Cy-3 goat anti-rabbit F (ab')₂ (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted to 1/1000 and biotinylated anti-mouse IgG diluted to 1/200 were used as secondary antibodies and avidin fetal calf serum (Vector Laboratories) was used for green fluorescent visualization. Finally sections were subjected to Sudan Black B staining to inhibit the lipofuscin auto-fluorescence.

Counts of Neurons with Redistributed I₂^{PP2A}

A total of more than 150 neurons with I₂^{PP2A} immunoreactivity were counted in five to seven random fields at

$\times 20$ magnification (Zeiss Axioscope) which covered $\sim 80\%$ of each temporal cortex section. The immunopositive neurons were divided into two groups based on the positive or negative nuclear immunoreactivity. The ratio of neurons with negative nuclei to those with positive nuclei were calculated for each AD and control case. Mean values for each brain were analyzed by *t*-test between the five AD and five control cases. Differences with *P* < 0.05 were considered significant.

Preparation of Nuclear and Cytosolic Fractions from AD and Control Brains

For preparation of nuclear and cytosolic fractions, temporal cortex from seven cases of each group was homogenized at 4°C in 10 vol of 0.32 mol/L sucrose, 1 mmol/L KH₂PO₄, pH 6.5, 1 mmol/L MgCl₂, 1 mmol/L AEBSF, 2 μ g/ml of leupeptin, 2 μ g/ml of aprotinin, 1 μ g/ml of pepstatin A, and 0.1 mmol/L EGTA using a glass-Teflon homogenizer with 20 strokes. The homogenate was centrifuged at 850 $\times g$ for 10 minutes at 4°C and the supernatant (S1) was saved. The pellet was resuspended with 5 vol of the same buffer, then centrifuged as above. The pellet was separated from supernatant (S2) and resuspended with the same buffer (N1). S1 and S2 were pooled and centrifuged at 100,000 $\times g$ for 20 minutes at 4°C to obtain supernatant (S3). N1 was used as the nuclear fraction and S3 was used as the cytosol fraction for Western blots.

Western Blots

Nuclear and cytosol fractions from AD and control brains (40 μ g/gel lane for I₁^{PP2A} blots, and 75 μ g/gel lane for I₂^{PP2A} blots) were subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The protein bands were transferred on Immobilon-P membrane (Millipore, Bedford, MA) and detected by incubation with 2.5 μ g/ml of 5G6 mouse mAb to I₁^{PP2A} or 2.5 μ g/ml of R1482 rabbit polyclonal antibody to I₂^{PP2A}, followed by incubation with anti-mouse or -rabbit horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratory), development with ECL Western blotting detection reagents (Amersham Biosciences Corp., Piscataway, NJ) for 1 minute and visualization by exposing to Hyperfilm ECL (Amersham Biosciences Corp). The ECL films of the blots were scanned and analyzed using TINA 2.0 software (Raytest, Straubenhardt, Germany). I₁^{PP2A} and I₂^{PP2A} signal intensities were normalized to signal intensity of topoisomerase I for nuclear fraction and of β -actin for cytosol fraction. Mean values for each individual were analyzed by *t*-test between the seven AD and seven control cases for I₁^{PP2A} and I₂^{PP2A}. Differences with *P* < 0.05 were considered significant.

In Vitro Digestion of Recombinant I_2^{PP2A} with Brain Extract

Three cases of control (case numbers 0034, 0049, 0201; see Table 1) and three cases of AD brains (case numbers 0102, 0105, 0110; see Table 1) were studied. Middle temporal gyrus of each case was homogenized at 4°C in 5 vol of 25 mmol/L Tris (pH 7.2) without any proteinase inhibitors using glass-Teflon homogenizer, 20 up and down strokes. The homogenate was centrifuged at $16,100 \times g$ for 10 minutes at 4°C, then the supernatant was collected. This supernatant (20% brain extract) was used as a source of the I_2^{PP2A} hydrolase activity.

Recombinant human brain I_2^{PP2A} , 10 μ g,²⁹ was incubated with or without 700 μ g of 20% brain extract at 37°C for 0, 7.5, 15, 30, 60, and 90 minutes, and 24 hours. At 24 hours a second dose of the same brain extract was added and incubation continued for another 15, 30, and 60 minutes. The incubated samples containing 100 ng of recombinant I_2^{PP2A} plus 7 μ g of brain extract per gel lane was subjected to 10% SDS-PAGE, followed by transfer of the separated proteins to Immobilon-P membrane and development of the Western blots with antibody R1482 to I_2^{PP2A} .

Mass Spectrometric Analysis of I_2^{PP2A} and Its Cleavage Products

For mass spectrometry, 7 μ g of recombinant I_2^{PP2A} was digested with 7 μ g of AD brain extract at 37°C for 90 minutes. Reaction mixture was subjected to 10% SDS-PAGE. The brain extract incubated in the absence of the recombinant I_2^{PP2A} was used as a control. Several bands were cut out from Coomassie Brilliant Blue-stained gel and analyzed by matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry at the Stanford PAN Facility (Palo Alto, CA).

Results

Increase in the mRNA Levels of I_1^{PP2A} and I_2^{PP2A} in AD Temporal and Entorhinal Cortices

To study the role of PP2A inhibitors in neurofibrillary pathology, the distribution and expression of mRNAs of these inhibitors were determined by *in situ* hybridization histochemistry in AD and control brains. The expression of the two inhibitors was found mainly in neurons with high intensity in temporal cortex, entorhinal area of hippocampus, and granular cell layer of cerebellum (Figure 1; a to c). A strong hybridization signal was detected in pyramidal cell layers of CA1 to CA4 and granular cell layer of dentate gyrus in the hippocampus (data not shown). In the cerebellum Purkinje cells, and basket and stellate cells in molecular layer also showed strong hybridization (data not shown). No hybridization signals were detected in the control experiments using sense RNA probes, indicating the specificity of the two anti-sense RNA probes (data not shown).

A comparison of the levels of I_1^{PP2A} and I_2^{PP2A} mRNAs between AD and control cases revealed a disease-associated increase of neuronal mRNA in temporal and entorhinal cortices. To account for any variability among the cases, and accurately evaluate differences between AD and control, all quantitative analyses were normalized to the intensity of GAPDH mRNA for each serial tissue section. The relative expression of I_1^{PP2A} and I_2^{PP2A} mRNAs after normalization with GAPDH mRNA was ~25% ($P < 0.001$) and 10% ($P < 0.05$) higher in AD temporal and entorhinal cortices, respectively (Figure 1; a, b, d, e) than in the corresponding areas of the control cases. In granule cell layers and Purkinje cells in the cerebellum, no significant differences were found in the levels of I_1^{PP2A} and I_2^{PP2A} mRNAs between AD and control cases (Figure 1, c and f).

Redistribution of I_2^{PP2A} from Neuronal Nucleus to Cytoplasm in AD Brain

Subcellular localization of I_1^{PP2A} and I_2^{PP2A} were reported previously in various cultured cells. I_1^{PP2A} , which is homologous to PHAP-I/LANP/mapmodulin/pp32, is localized in both cytoplasm and nucleus^{20,21,23,34} and I_2^{PP2A} , which is the same as SET/TAF-1, is mainly localized in the nucleus.^{24,35,36} TAF-1 has been previously shown to be cleaved and the N-terminal cleaved half, which like the full-length protein has PP2A inhibitory activity, is localized in the cytoplasm.³⁶

We investigated the subcellular distribution of both I_1^{PP2A} and I_2^{PP2A} in AD and control brains immunohistochemically. Consistent with the previous studies, subcellular localization of I_1^{PP2A} was in the cytoplasm and/or nucleus and of I_2^{PP2A} was mainly in the nucleus in human temporal cortex from control brains (Figure 2a). Although the subcellular localization of I_1^{PP2A} was similar in AD and control brains, I_2^{PP2A} was, with increased frequency, translocated from the nucleus to the cytoplasm in AD temporal cortex (Figure 2a). The number of cells that showed the translocation in AD temporal cortex were counted and compared to the translocated neurons in control. The ratio of neurons with immunonegative nuclei to those with immunopositive nuclei for I_2^{PP2A} was more than sixfold greater in AD than control brains (Figure 2b; $P < 0.05$). The same shift from the nuclear to cytoplasmic compartment was also seen in AD hippocampus (Figure 2c). In cerebellum, both PP2A inhibitors in AD had similar subcellular localization compared to that in control cases (Figure 2d).

Cleavage of I_2^{PP2A} in AD Temporal Cortex

To biochemically confirm the immunohistochemical results, Western blots were performed using nuclear and cytosol fractions prepared from AD and control temporal cortices. The levels of I_1^{PP2A} were found to be increased both in nuclear and cytosol fractions in AD but reached significance ($P < 0.05$) only in the former (Figure 3; a to c). On the other hand, consistent with immunohistochem-

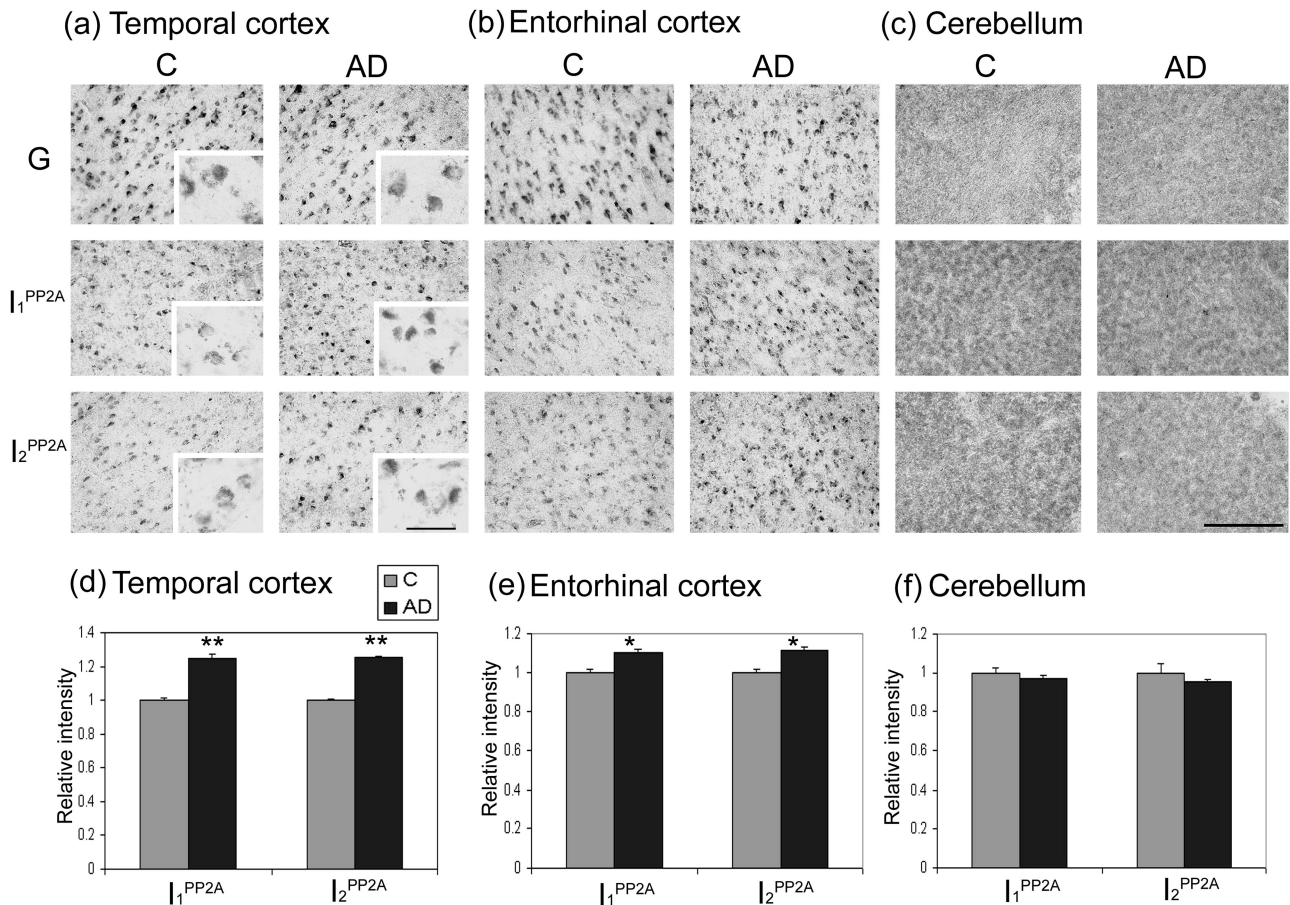


Figure 1. Expression of I_1^{PP2A} and I_2^{PP2A} mRNAs in AD and control brains. **a-c:** Expression of I_1^{PP2A} and I_2^{PP2A} mRNAs in temporal cortex, entorhinal cortex, and cerebellum from AD and control (C) brains determined by digoxigenin-labeled *in situ* hybridization. I_1^{PP2A} and I_2^{PP2A} signals from five AD and five control cases were quantitated using PCI software and normalized by GAPDH (G) signal. **d-f:** Mean \pm SEM of normalized data relative to control. Differences between AD and control cases were analyzed statistically by *t*-test. The levels of both I_1^{PP2A} and I_2^{PP2A} mRNAs were elevated in AD temporal cortex (** $P < 0.001$) and entorhinal cortex (* $P < 0.05$). Scale bars: 200 μ m; 50 μ m (insets).

ical findings, the signal of I_2^{PP2A} in nuclear fraction was reduced ($P < 0.05$) in AD compared to control (Figure 3, a and d). In the cytosol, the 39-kd I_2^{PP2A} was cleaved and the levels of the fragments were higher in AD than in controls. The signal of I_2^{PP2A} at 39 kd in the cytosolic fraction was decreased in AD (Figure 3, a and e; $P < 0.05$). A major cleavage product, the ~20-kd I_2^{PP2A} polypeptide, which was seen in cytosolic and not in nuclear fraction, appeared in few control and most AD cases (Figure 3a). In addition to the ~20-kd fragment, weak signals of ~56-kd, ~34-kd, and ~25-kd I_2^{PP2A} immunoreactivities were also seen in both control and AD cytosols. The level of 20-kd polypeptide was significantly higher in AD cytosol compared to that in control (Figure 3f; $P < 0.05$). The combined levels of the I_2^{PP2A} -39-kd and -20-kd polypeptides and as well as of the 39-kd I_2^{PP2A} plus all of its cleavage product polypeptides were increased ($P < 0.05$) in AD compared to control (Figure 3, a and g).

We also performed the same study using nuclear and cytosol fraction from cerebellum. Expression levels of both PP2A inhibitors in AD were not significantly different between AD and control cases (Figure 3; h to l). In addition, only background level of ~20-kd I_2^{PP2A} was

seen in the cerebellum (Figure 3h), suggesting that this cleavage of I_2^{PP2A} was selective to areas of the brain that develop neurofibrillary pathology.

Cleavage of Recombinant I_2^{PP2A} by Brain Extract

To confirm whether ~56-kd, ~34-kd, ~25-kd, and ~20-kd polypeptides of I_2^{PP2A} are generated from I_2^{PP2A} full-length protein (277 amino acids), recombinant I_2^{PP2A} was digested by brain extracts from AD and control cases. Digestion of recombinant I_2^{PP2A} by both AD and control brain extracts resulted in at least five to six strong signals (Figure 4a). In AD cases, the 39-kd signal was reduced in a time-dependent manner with a simultaneous appearance of ~56-, ~34-, and ~25-kd signals within 7.5 minutes of digestion. This was followed by the appearance of a ~22-kd fragment during 15 to 30 minutes of digestion.

On the other hand, in control cases the appearance of these products was apparently delayed and the ~22-kd fragment was not seen during digestion. We did not detect any ~28-kd fragment in AD cases, but an ~56-kd

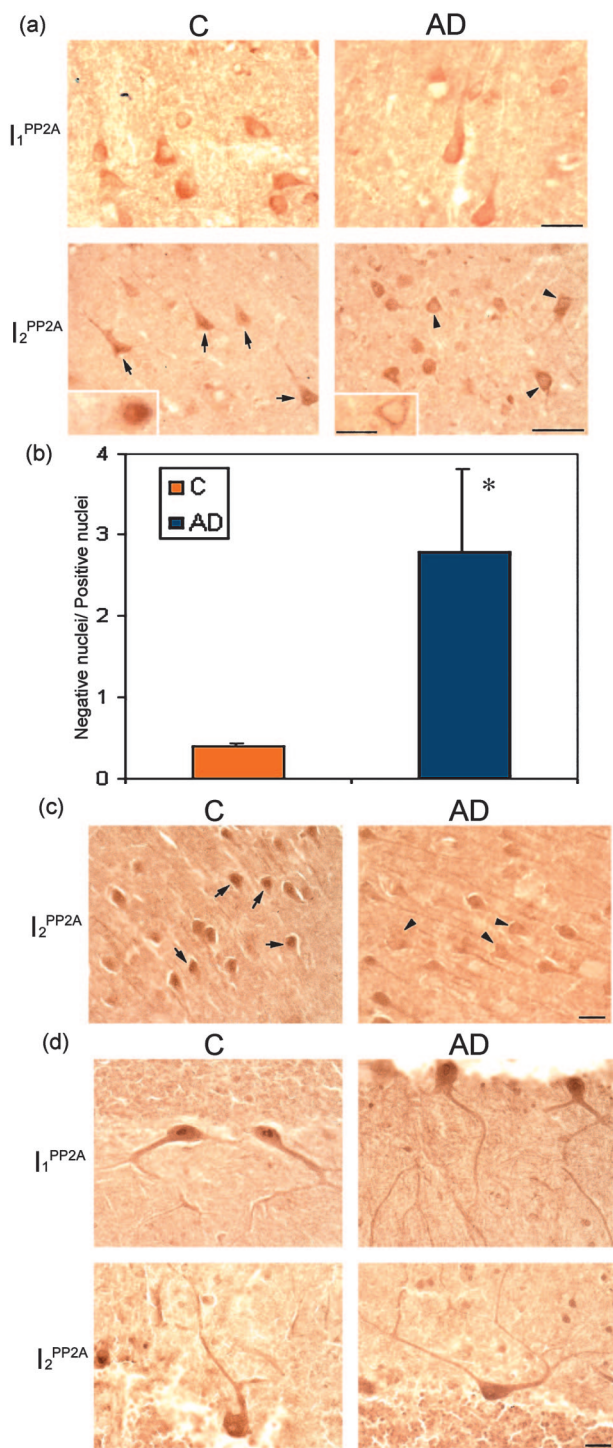


Figure 2. Subcellular localization of I_1^{PP2A} and I_2^{PP2A} in AD and control brains. I_1^{PP2A} and I_2^{PP2A} expression was studied by immunohistochemistry using affinity-purified polyclonal antibodies in temporal cortex (TC), hippocampus, and cerebellum. **a:** I_1^{PP2A} was expressed in the cytosol and/or nucleus in both AD and control (C) brains in TC. I_2^{PP2A} was predominantly expressed in the nucleus (arrows) in TC from control brain, but was translocated from nucleus to cytosol (arrowheads) in AD brain. **b:** The ratio (mean \pm SEM) of neurons with immunonegative nuclei to those with immunopositive nuclei. In AD the number of neurons in temporal cortex showing the translocation of I_2^{PP2A} from nucleus to cytoplasmic localization increased markedly ($*P < 0.05$). **c:** I_2^{PP2A} in AD hippocampus (CA1) was also translocated from nucleus (arrows) to cytoplasm (arrowheads) as compared to control. **d:** Subcellular localizations of I_1^{PP2A} and I_2^{PP2A} were similar between AD and control in the cerebellum. Scale bars: 25 μ m [a (I_1^{PP2A} and insets), b, d]; 50 μ m [a (I_2^{PP2A})].

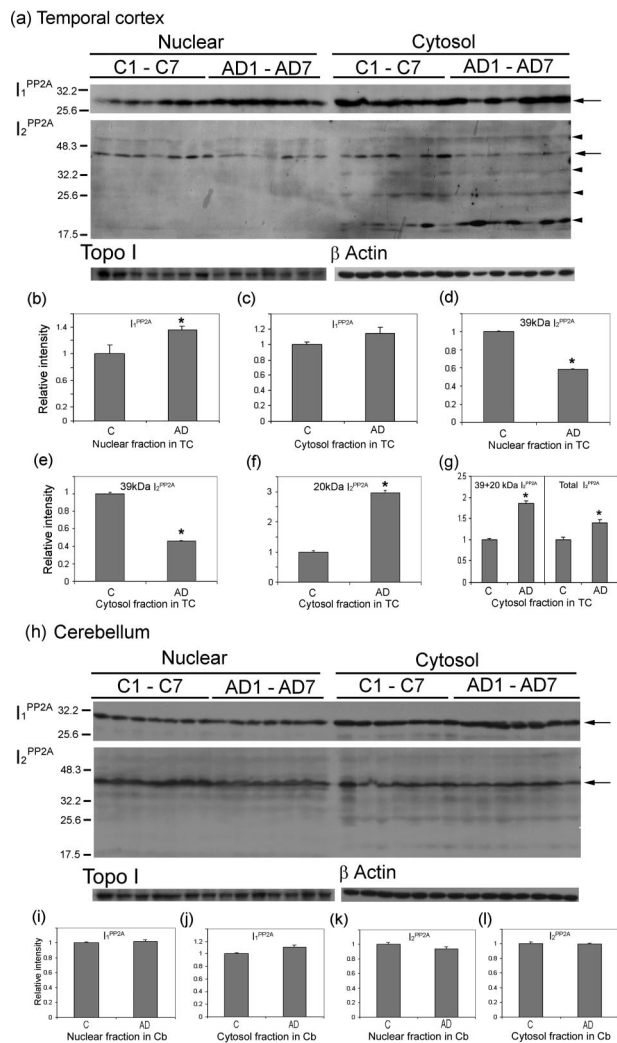


Figure 3. Cleavage and distribution of I_2^{PP2A} in nuclear and cytosolic fractions in AD and control brains. Subcellular fractions from temporal cortex (TC) and cerebellum (Cb) of control (C) and AD cases were analyzed by Western blots. I_1^{PP2A} and I_2^{PP2A} signals were quantitated using TINA 2.0 software and normalized by topoisomerase I (Topo I) for nuclear fraction and β -actin for cytosol fraction. **a-c:** In temporal cortex, the level of I_1^{PP2A} in AD was increased significantly in nuclear fraction (**a, b**; $*P < 0.05$) and insignificantly in the cytosol (**a, c**) as compared to control cases. In contrast, the level of I_2^{PP2A} in the nuclear fraction was decreased in AD as compared to the control cases (**a, d**; $*P < 0.05$). The 39-kDa I_2^{PP2A} in the cytosol fraction was decreased in AD (**a, e**; $*P < 0.05$), but an ~20-kDa fragment of I_2^{PP2A} was significantly increased in AD as compared to controls (**a, f**; $*P < 0.05$). Total relative intensity of both 39-kDa plus 20-kDa I_2^{PP2A} and as well as of the 39-kDa plus all its cleavage product polypeptides (total I_2^{PP2A}) were increased in AD as compared to control (**a, g**; $*P < 0.05$). **h:** In cerebellum, expression levels of both I_1^{PP2A} and I_2^{PP2A} were similar in AD and control cases (**h-i**), and only background level of 20-kDa fragment of I_2^{PP2A} was seen in cerebellum. **b-g, i-l:** Mean \pm SEM of normalized data relative to control. The left of **a** and **h** indicates the molecular weight markers, and on the right of **a** and **h**, full-length I_1^{PP2A} (30 kDa) and I_2^{PP2A} (39 kDa) is indicated by arrows, and the cleavage products of I_2^{PP2A} by arrowheads (from top to bottom, the ~56-, ~34-, ~25-, and ~20-kDa polypeptides).

signal could be seen in both AD and control cases, which was probably a dimer of the ~28-kDa fragment, suggesting that the ~28-kDa fragment in AD cases formed the dimer (within 7.5 minutes) earlier than in control (Figure 4a). Identically treated brain extract alone did not generate these signals (data not shown). These *in vitro* digestion conditions used were not sufficient to generate the

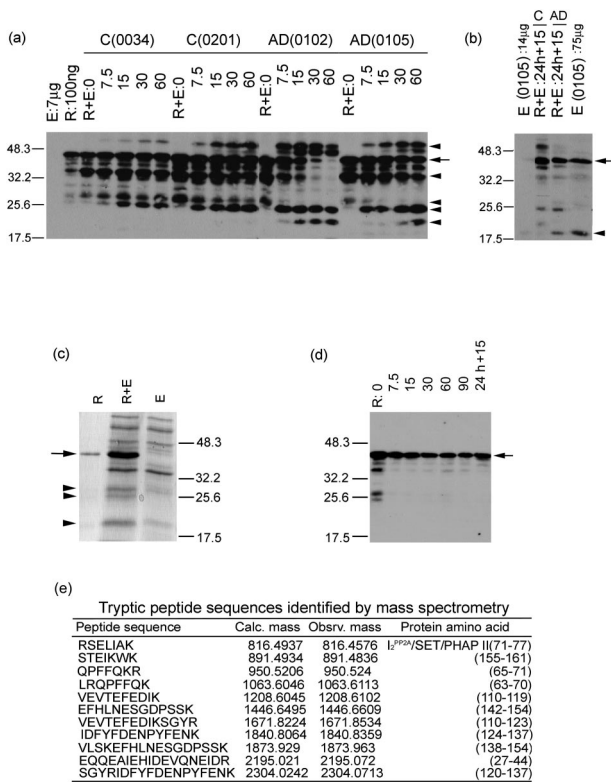


Figure 4. Digestion of recombinant human brain I₂^{PP2A} with AD and control brain extracts and identification of the cleavage products by Western blot and by mass spectrometry. **a:** Ten μg of recombinant I₂^{PP2A} was digested with 700 μg of temporal cortex extract from AD brains no. 0102 and no. 0105 and control (C) brain no. 0034 and no. 0201 for 0, 7.5, 15, 30, and 60 minutes, and 24 hours at 37°C. The digest containing 100 ng of recombinant I₂^{PP2A} and 7 μg of brain extract/gel lane was subjected to SDS-PAGE, followed by Western blots developed with antibody R1482 to I₂^{PP2A}. The **left** of **a** indicates the molecular weight markers, and on the **right**, the I₂^{PP2A} full-length 39 kd is indicated by an **arrow**, and the cleavage products generated by **arrowheads** (from **top** to **bottom**, the ~56-, ~34-, ~28-, ~25-, and ~22-kd polypeptides). The cleavage products were generated significantly earlier on digestion with AD than control brain extract. The I₂^{PP2A} signal was undetectable in 7 μg of brain extract (E) used as a control. R, recombinant human brain I₂^{PP2A}. **b:** After 24 hours of digestion, a second dose of the brain extract was added to the digest and incubated for another 15 minutes. The digestion with AD but not control brain extract generated a 20-kd I₂^{PP2A} polypeptide that co-migrated with the I₂^{PP2A} cleavage product seen in 75-μg AD brain extract (**right lane, arrowhead**); the 20-kd signal was undetectable in 14 μg of the AD brain extract (**left lane**), **arrow** indicates the 39-kd I₂^{PP2A}. **c:** Coomassie Blue-stained gel of 700 ng of recombinant I₂^{PP2A} (R), 7 μg AD brain extract (E), and digestion of 7 μg of recombinant I₂^{PP2A} with 7 μg of AD brain extract (R + E) for 90 minutes at 37°C. The polypeptide bands corresponding to ~28, ~25, and ~22 kd (**arrowheads**) were cut out and subjected to tryptic digest and mass spectrometry. **Arrow** shows the 39-kd I₂^{PP2A}. **d:** Recombinant of I₂^{PP2A} without brain extract was incubated at 37°C for 7.5, 15, 30, 60, and 90 minutes, and 24 hours 15 minutes. One hundred ng of recombinant I₂^{PP2A}/lane was subjected to SDS-PAGE, followed by Western blots developed with antibody R1482 to I₂^{PP2A}. The degradative products that were seen at the time 0 point disappeared as early as during 7.5 minutes of digestion. **Arrow** shows the 39-kd I₂^{PP2A}. **e:** The mass spectrometric analysis of ~25-kd polypeptide cut out from (R + E) showed it to be the amino terminal fragment of I₂^{PP2A}. Not shown in this figure, the ~28-kd polypeptide also was identified an amino terminal fragment of I₂^{PP2A}; the ~22-kd protein band, because of other proteins with the similar molecular weight from brain extract, could not be identified.

I₂^{PP2A}-immunopositive ~20-kd polypeptide that we had found increased in AD brain extract in Figure 3a. We found that the addition of a second dose of the brain extract after 24 hours of incubation as the source of the proteolytic activity generated a ~20-kd I₂^{PP2A}-immu-

nopositive polypeptide during 15, 30, and 60 minutes of incubation. However, digestion with the AD brain extract generated this ~20-kd polypeptide markedly more than the control brain extract (Figure 4b). Similar results were obtained by 30 or 60 minutes of further digestion (data not shown). AD or control brain extracts alone when treated identically showed only a background signal at ~20-kd position (Figure 4b). Similarly, incubation of recombinant I₂^{PP2A} alone did not result in the generation of the ~20-kd polypeptide (Figure 4d).

To further confirm that the cleavage products were from I₂^{PP2A}, digested products were subjected to SDS-PAGE, stained with Coomassie blue (Figure 4c), and the ~28-kd, ~25-kd, and ~22-kd polypeptide bands generated were sliced out and used for analysis by mass spectrometry. The mass spectrometric analysis of the tryptic digest of the ~28-kd and ~25-kd polypeptides revealed a complete match to that of I₂^{PP2A}/SET/PHAPII/TAF1 and showed that these polypeptides were from the amino terminal half or greater half of I₂^{PP2A} (Figure 4e). These tryptic peptide sequences lacked the C-terminal acidic region, indicating that these peptides were from the amino terminal region. The ~22-kd polypeptide, which was a mixture of the inhibitor and some unrelated brain proteins, could not be positively identified by mass spectrometry. All cleavage products were, however, positively identified with several antibodies to I₂^{PP2A} (data not shown). Amino terminal sequencing of the digest of the recombinant I₂^{PP2A} by AD brain extract revealed the presence of an ~20-kd amino terminal fragment (Tanimukai et al, in preparation).

Co-Localization of PP2A Inhibitors with PP2A Catalytic Subunit and with Hyperphosphorylated Tau in Neuronal Cytoplasm

Increase in the levels of mRNAs of I₁^{PP2A} and I₂^{PP2A}, and cleavage and translocation of I₂^{PP2A} from the nuclear to the cytoplasmic compartment in neurons in AD prompted us to investigate whether the PP2A inhibitors were involved in the hyperphosphorylation of tau in AD. We performed double-labeled immunohistochemical studies using specific antibodies against I₁^{PP2A}, I₂^{PP2A}, PP2A catalytic subunit, and to tau abnormally hyperphosphorylated at serine 396/404 (PHF1), Thr231/Ser 235 (M4), and Ser262/356 (12E8). Both PP2A inhibitors co-localized with PP2A catalytic subunit in neuronal cytoplasm in AD brains (Figure 5a). In addition, PP2A inhibitors were also co-localized with what appeared to be early- to middle-stage neurofibrillary tangles of the abnormally hyperphosphorylated tau in neuronal cytoplasm (Figure 5b, data not shown for mAb PHF1 and mAb M4). The neurons that expressed I₁^{PP2A} and I₂^{PP2A} mainly in the nucleus did not co-localize with phosphorylated tau immunoreactivity (Figure 5b), indicating that PP2A inhibitors in cytoplasm were probably responsible for tau hyperphosphorylation.

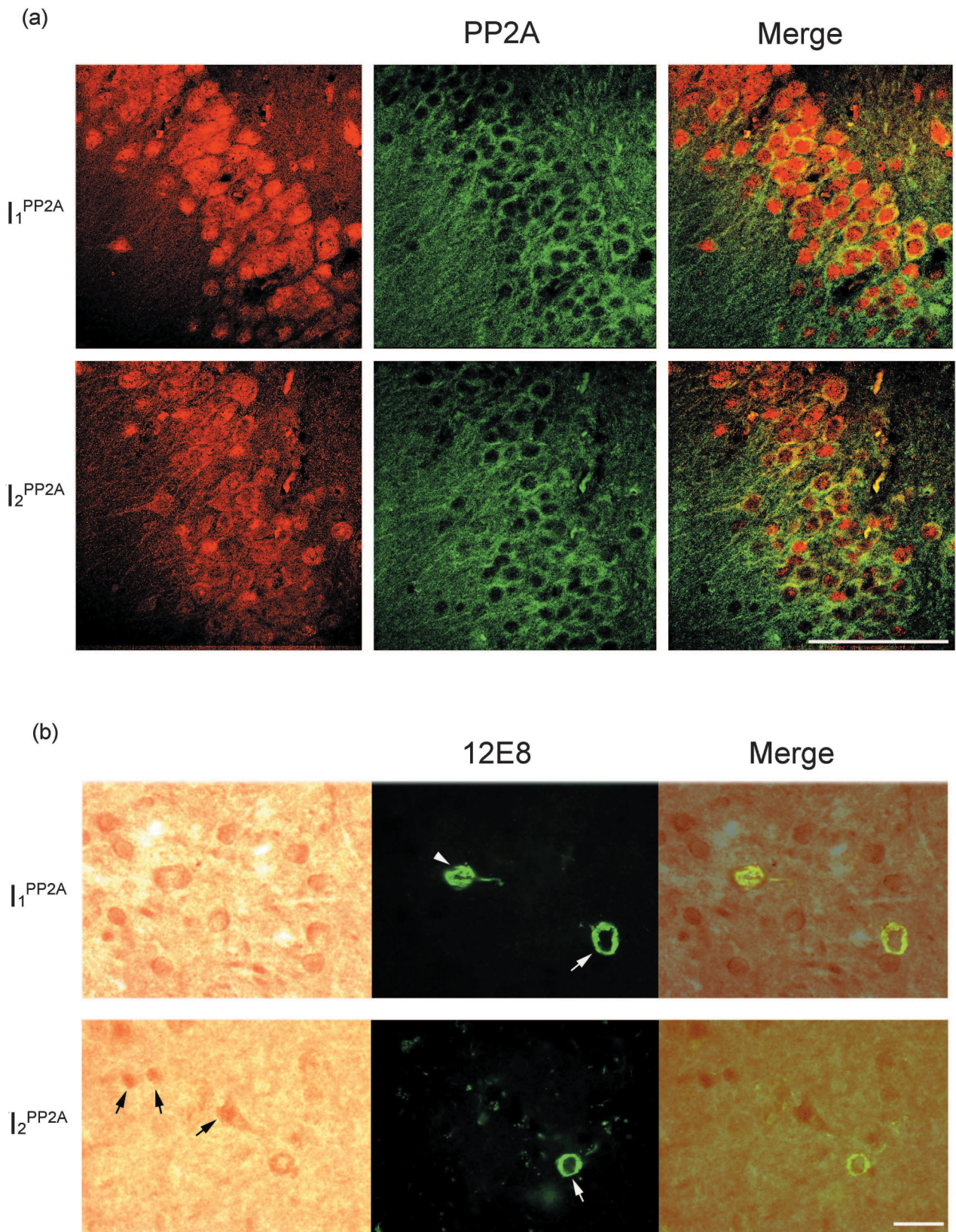


Figure 5. Co-localization of I₁^{PP2A} and I₂^{PP2A} with PP-2A and with phosphorylated tau in AD brain. **a:** Subcellular localizations of I₁^{PP2A}, I₂^{PP2A}, and PP-2A in AD hippocampus. Both I₁^{PP2A} and I₂^{PP2A} co-localized with PP-2A in neuronal cytoplasm. **b:** Both I₁^{PP2A} and I₂^{PP2A} were co-localized with mostly early-stage (**white arrowheads**) to middle-stage (**white arrows**) neurofibrillary changes as seen with phospho-dependent antibodies (12E8) to abnormally hyperphosphorylated tau in AD temporal cortex. The neurons that expressed I₂^{PP2A} mainly in the nucleus did not co-localize with phosphorylated tau (**black arrows**). Scale bars: 100 μ m (**a**); 25 μ m (**b**).

Discussion

Neurofibrillary degeneration of abnormally hyperphosphorylated tau is a primary and a pivotal lesion of AD and several tauopathies.³⁷ The present study demonstrates an intraneuronal increase in the AD neocortex in the mRNA expressions of I_1^{PP2A} and I_2^{PP2A} , the two major regulators of the intracellular PP2A activity, and the colocalization of these two inhibitors with PP2A and with the abnormally hyperphosphorylated tau and the early- to middle-stage neurofibrillary tangles. Selective cleavage and translocation of I_2^{PP2A} from its primary nuclear location to the cytoplasm in neurons of the neocortex in AD that undergoes neurofibrillary degeneration, shows a novel mechanism by which this PP2A inhibitor might contribute to the abnormal hyperphosphorylation of tau. These findings reveal for the first time the possible involvement of I_1^{PP2A} and I_2^{PP2A} in Alzheimer neurofibrillary pathology through the inhibition of PP2A activity and the resulting abnormal hyperphosphorylation of tau.

Neurofibrillary pathology in AD brain is known to start from entorhinal cortex in stages I/II of Braak, then spreads to the hippocampal formation, amygdala, and thalamus in stages III/IV. The pathology further develops in temporal proneocortex, and gradually invades the extended neocortical association areas in stage V, until finally the pathological process even progresses through the border fields into the primary areas in stage VI.³⁸ In this study, we used the tissue from the late stage of AD ie, stage V to VI. At this late stage, the majority of neurons in the hippocampus, especially the entorhinal cortex, are at advanced stages of neurofibrillary degeneration. The neuronal loss in the medial temporal cortex and other areas of the neocortex is relatively small at Braak stage V to VI of AD. As expected, we found that the temporal cortex had relatively mild tau pathology compared to entorhinal cortex and hippocampal formation (data not shown). In the present study, a statistically significant increase in the mRNA expressions of I_1^{PP2A} and I_2^{PP2A} was found both in the temporal cortex and the entorhinal cortex in AD. A relatively less marked increase in the expression of mRNAs of I_1^{PP2A} and I_2^{PP2A} in the entorhinal cortex as compared to temporal cortex in AD found in the present study is probably related to advanced stage of neurofibrillary degeneration in the former. In the AD cases at Braak stages V and VI, which were used for the present study, a very large number of neurons are at advanced stages of neurofibrillary degeneration as compared to a relatively much smaller number in the temporal cortex of the same brains. We believe a smaller increase in the expression of I_1^{PP2A} and I_2^{PP2A} mRNAs seen in the entorhinal cortex than the temporal cortex in the AD cases found in the present study reflect a larger number of neurons with advanced stages of neurofibrillary degeneration in the AD cases studied. Consistent with the present study, microarray analyses have also shown an up-regulation of the expression of acidic nuclear p32 and SET genes in AD hippocampus.¹⁴

AD is a slow but progressive neurodegenerative disorder with an average progression of 7 to 10 years. Thus,

the 15 to 25% increase in the PP-2A inhibitors found in the AD neocortex could have a considerable accumulative effect with time on the hyperphosphorylation of tau and the consequent neurofibrillary degeneration. In the present study the cerebellum, which is unaffected by neurofibrillary pathology in AD and was used as an internal control, did not show any significant changes in the mRNA expressions of I_1^{PP2A} or I_2^{PP2A} .

The immunohistochemical data in control brains showed that I_1^{PP2A} was localized in the nucleus and/or cytoplasm. On the other hand, I_2^{PP2A} was localized mainly in the nucleus, with a weak signal in the neuronal cytoplasm. These results are consistent with previous studies that showed that I_1^{PP2A} works as a shuttle protein between nucleus and cytoplasm³⁹ and that I_2^{PP2A} localized mainly in the neuronal nuclei in the mammalian brain.²⁸

The subcellular localization of I_1^{PP2A} was similar to that in control brain, whereas that of I_2^{PP2A} was changed with increased frequency from nucleus to the cytoplasmic dominant localization in neurons in AD temporal cortex. Similar translocation of I_2^{PP2A} was seen in AD hippocampus but not in AD cerebellum, indicating that this translocation was region-specific and was seen in the areas of the brain affected by neurofibrillary pathology. The corresponding biochemical data obtained from Western blots showed that I_2^{PP2A} immunoreactivity in nuclear fraction from AD temporal cortex was significantly decreased compared to that from the corresponding control tissue. In addition, the level of the 39-kd full-length I_2^{PP2A} in cytosol was reduced but at the same time, ~20-kd fragment of I_2^{PP2A} appeared and was significantly increased in AD compared to control cases. Consistent with these findings, previously it has been reported that the 20 kd of I_2^{PP2A} isolated from bovine kidney, which has the inhibitory activity, is derived from the cleavage of the 39-kd I_2^{PP2A} /SET/TAF1.¹⁹ Furthermore, in HeLa cells transfected with the amino terminal half of TAF1, the inhibitor is localized in the cytoplasm.³⁶ We postulated that the cleavage of the 39-kd I_2^{PP2A} and its translocation from the neuronal nucleus to the cytoplasm in the affected brain areas in AD might involve an increase in the activity of a specific hydrolase(s) in the diseased tissue. We found that the recombinant human brain I_2^{PP2A} was digested faster with AD than control brain extract, generating similar proteolytic fragments of the inhibitor.

The generation of the 20-kd I_2^{PP2A} from the full-length protein appears to take place in several steps. The 20-kd I_2^{PP2A} appeared to be the final product. It might have been cleaved from 22-kd I_2^{PP2A} because this polypeptide disappeared when the 20-kd polypeptide appeared during the digestion (Figure 4, a and b). In AD and control brain cytosols, the 22-kd I_2^{PP2A} was undetectable (Figure 3a). Although at present we do not know the identity of the protease(s) involved in the cleavage of I_2^{PP2A} in AD brain, PHAP-II (I_2^{PP2A} /SET/TAF1) has been shown to be cleaved in K562 cells by granzyme A.⁴⁰ Interestingly, Beresford and colleagues⁴¹ have also shown that recombinant SET protein is cleaved by granzyme A *in vitro* and

produces 25-kd, 22-kd, and 20-kd polypeptides. Granzyme A, a serine protease, is abundant in cytotoxic granules. Although there is no evidence of granzyme A in neurons, we speculate that some serine protease(s) in human brain might have similar role for this cleavage.

The cleavage and the translocation of I_2^{PP2A} might play a significant role in tau hyperphosphorylation because the 20-kd N-terminal fragment also has the PP2A inhibitory activity⁴² and PP2A is mainly localized in the neuronal cytoplasm, as shown in the present study and previously.⁴³ It has been reported that PP2A inhibitors inhibit PP2A activity by binding directly to the PP2A catalytic subunit.^{44,45} It remains to be investigated whether the cleaved I_2^{PP2A} binds easier than full-length I_2^{PP2A} to PP2A. In this study, we have observed the co-localization of PP2A inhibitors and PP2A catalytic subunit in the neuronal cytoplasm, suggesting that both PP2A inhibitors could bind and inhibit PP2A in the neuronal cytoplasm.

The present study showed the co-localization of PP2A inhibitors and tau abnormally hyperphosphorylated at Ser396/404, Thr231/Ser 235, and Ser262/356 in the neuronal cytoplasm. Most neurons that showed the co-localization between PP2A inhibitors and phosphorylated tau appeared to be in the early to middle stage of neurofibrillary pathology. These results and increase in the levels of mRNAs of PP2A inhibitors and redistribution of I_2^{PP2A} from neuronal nucleus to cytoplasm suggest that PP2A inhibitors might inhibit PP2A activity at the relatively early stage of neurofibrillary changes in AD brain. This speculation of the possible involvement of PP2A inhibitors in the hyperphosphorylation of tau is supported by a previous report in which I_1^{PP2A} and I_2^{PP2A} inhibited 50 to 80% of PP2A activity toward tau at the PHF1, 12E8, and M4 sites *in vitro*.²⁹

In conclusion, I_1^{PP2A} and I_2^{PP2A} , the two major regulators of intracellular PP2A activity, are selectively up-regulated in the areas of the AD brain affected with neurofibrillary pathology. In addition, I_2^{PP2A} , which is mainly localized in the nucleus as the full-length protein, is cleaved and its amino terminal half and/or greater halves, which are known to inhibit PP2A, are translocated to the cytoplasm in neurons in the affected areas of the brain in AD. I_1^{PP2A} and translocated I_2^{PP2A} co-localize both with PP2A and the early- to middle-stage neurofibrillary changes of abnormally hyperphosphorylated tau. These findings lead us to speculate a scenario in which the increase in the messages of I_1^{PP2A} and I_2^{PP2A} , plus the translocation of I_2^{PP2A} from the neuronal nucleus to the cytoplasm, results in an inhibition of PP2A activity in AD brain. The inhibition of PP2A activity probably leads to the abnormal hyperphosphorylation of tau not only by a decrease in the dephosphorylation but also by an increase in the phosphorylation of tau by tau protein kinases that are regulated by PP2A. Inhibition of PP2A has been shown to increase the activities of calcium calmodulin-dependent protein kinase II,⁴⁶ cyclic AMP-dependent protein kinase A,⁴⁷ and various members of the mitogen-activated protein kinase family.^{48–50} Thus, I_1^{PP2A} and I_2^{PP2A} offer previously unknown therapeutic targets for AD and tauopathies.

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References

1. Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI: Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci USA* 1986, 83:4913–4917
2. Iqbal K, Grundke-Iqbal I, Smith AJ, George L, Tung YC, Zaidi T: Identification and localization of tau peptide to paired helical filaments of Alzheimer disease. *Proc Natl Acad Sci USA* 1989, 86:5646–5650
3. Lee VM, Balin BJ, Otvos Jr L, Trojanowski JQ: A68: a major subunit of paired helical filaments and derivatized forms of normal tau. *Science* 1991, 251:675–678
4. Weingarten MD, Lockwood AH, Hwo SY, Kirschner MW: A protein factor essential for microtubule assembly. *Proc Natl Acad Sci USA* 1975, 72:1858–1862
5. Alonso A del C, Zaidi T, Grundke-Iqbal I, Iqbal K: Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proc Natl Acad Sci USA* 1994, 91:5562–5566
6. Alonso A del C, Grundke-Iqbal I, Iqbal K: Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. *Nat Med* 1996, 2:783–787
7. Alonso A del C, Grundke-Iqbal I, Barra HS, Iqbal K: Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of MAP1 and MAP2 and the disassembly of microtubules by the abnormal tau. *Proc Natl Acad Sci USA* 1997, 94:298–303
8. Gong CX, Singh TJ, Grundke-Iqbal I, Iqbal K: Phosphoprotein phosphatase activities in Alzheimer disease brain. *J Neurochem* 1993, 61:921–927
9. Gong CX, Shaikh S, Wang JZ, Zaidi T, Grundke-Iqbal I, Iqbal K: Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. *J Neurochem* 1995, 65:732–738
10. Gong CX, Lidsky T, Wegiel J, Zuck L, Grundke-Iqbal I, Iqbal K: Phosphorylation of microtubule-associated protein tau is regulated by protein phosphatase 2A in mammalian brain. Implications for neurofibrillary degeneration in Alzheimer's disease. *J Biol Chem* 2000, 275:5534–5544
11. Sun L, Liu SY, Zhou XW, Wang XC, Liu R, Wang Q, Wang JZ: Inhibition of protein phosphatases 2A and protein phosphatases 1 induced tau phosphorylation and impairment of spatial memory retention in rats. *Neuroscience* 2003, 118:1175–1182
12. Vogelsberg-Ragaglia V, Schuck T, Trojanowski JQ, Lee VM: PP2A mRNA expression is quantitatively decreased in Alzheimer's disease hippocampus. *Exp Neurol* 2001, 168:402–412
13. Loring JF, Wen X, Lee JM, Seilhamer J, Somogyi R: A gene expression profile of Alzheimer's disease. *DNA Cell Biol* 2001, 20:683–695
14. Blalock EM, Geddes JW, Chen KC, Porter NM, Marquesbery WR, Landfield PW: Incipient Alzheimer's disease: microarray correlation analyses reveal major transcriptional and tumor suppressor responses. *Proc Natl Acad Sci USA* 2004, 101:2173–2178
15. Sontag E, Luangpirom A, Hladik C, Mudrak I, Ogris E, Speciale S, White III CL: Altered expression levels of the protein phosphatase 2A A β Alphac enzyme are associated with Alzheimer disease pathology. *J Neuropathol Exp Neurol* 2004, 63:287–301

16. Sontag E, Hladik C, Montgomery L, Luangpirom A, Mudrak I, Ogris E, White III CL: Downregulation of protein phosphatase 2A carboxyl methylation and methyltransferase may contribute to Alzheimer disease pathogenesis. *J Neuropathol Exp Neurol* 2004, 63:1080–1091
17. Li M, Guo H, Damuni Z: Purification and characterization of two potent heat-stable protein inhibitors of protein phosphatase 2A from bovine kidney. *Biochemistry* 1995, 34:1988–1996
18. Li M, Makkinje A, Damuni Z: Molecular identification of I₁^{PP2A}, a novel potent heat-stable inhibitor protein of protein phosphatase 2A. *Biochemistry* 1996, 35:6998–7002
19. Li M, Makkinje A, Damuni Z: The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem* 1996, 271:11059–11062
20. Vaesen M, Barnikol-Watanabe S, Gotz H, Awani LA, Cole T, Zimmermann B, Kratzin HD, Hilschmann N: Purification and characterization of two putative HLA class II associated proteins: PHAPI and PHAPII. *Biol Chem Hoppe Seyler* 1994, 357:113–126
21. Matsuoka K, Taoka M, Satozawa N, Nakayama H, Ichimura T, Takahashi N, Yamakuni T, Song SY, Isobe T: A nuclear factor containing the leucine-rich repeats expressed in murine cerebellar neurons. *Proc Natl Acad Sci USA* 1994, 91:9670–9674
22. Chen TH, Brody JR, Romantsev FE, Yu JG, Kayler AE, Voneiff E, Kuhajda FP, Pasternack GR: Structure of pp32, an acidic nuclear protein which inhibits oncogene-induced formation of transformed foci. *Mol Biol Cell* 1996, 7:2045–2056
23. Ulitzur N, Rancano C, Pfeffer SR: Biochemical characterization of mapmodulin, a protein that binds microtubule-associated proteins. *J Biol Chem* 1997, 272:30577–30582
24. von Linden M, van Baal S, Wiegant J, Raap A, Hagemijer A, Grosveld G: Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene. *Mol Cell Biol* 1992, 12:3346–3355
25. Nagata K, Kawase H, Handa H, Yano K, Yamasaki M, Ishimi Y, Okuda A, Kikuchi A, Matsumoto K: Replication factor encoded by a putative oncogene, set, associated with myeloid leukemogenesis. *Proc Natl Acad Sci USA* 1995, 92:4279–4283
26. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlar EM: Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 1984, 34:939–944
27. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L: The Consortium to Establish A Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 1991, 41:479–486
28. Tanimukai H, Grundke-Iqbal I, Iqbal K: Inhibitors of protein phosphatases-2A: topology and subcellular localization. *Mol Brain Res* 2004, 126:146–156
29. Tsujio I, Zaidi T, Xu J, Kotula L, Grundke-Iqbal I, Iqbal K: Inhibitors of protein phosphatase-2A from human brain: structures, immunocytochemical localization and activities towards dephosphorylation of the Alzheimer type hyperphosphorylated tau. *FEBS Lett* (in press)
30. Greenberg SG, Davies P, Schein JD, Binder LI: Hydrofluoric acid-treated tau PHF proteins display the same biochemical properties as normal tau. *J Biol Chem* 1992, 267:564–569
31. Otvos Jr L, Feiner L, Lang E, Szendrei GI, Goedert M, Lee VM: Monoclonal antibody PHF-1 recognizes tau protein phosphorylated at serine residues 396 and 404. *J Neurosci Res* 1994, 39:669–673
32. Hasegawa M, Watanabe A, Takio K, Suzuki M, Arai T, Titani K, Ihara Y: Characterization of two distinct monoclonal antibodies to paired helical filaments: further evidence for fetal-type phosphorylation of the tau in paired helical filaments. *J Neurochem* 1993, 60:2068–2077
33. Seubert P, Mawal-Dewan M, Barbour R, Jakes R, Goedert M, Johnson GV, Litsky JM, Schenk D, Leiberburg I, Trojanowski JQ, Lee VM: Detection of phosphorylated Ser²⁶² in fetal tau, adult tau and paired helical filament tau. *J Biol Chem* 1995, 270:18917–18922
34. Malek SN, Katumuluwa AI, Pasternack GR: Identification and preliminary characterization of two related proliferation-associated nuclear phosphoproteins. *J Biol Chem* 1990, 265:13400–13409
35. Adachi Y, Pavlakis GN, Copeland TD: Identification and characterization of SET, a nuclear phosphoprotein encoded by the translocation break point in acute undifferentiated leukemia. *J Biol Chem* 1994, 269:2258–2262
36. Nagata K, Saito S, Okuwaki M, Kawase H, Furuya A, Kusano A, Hanai N, Okuda A, Kikuchi A: Cellular localization and expression of template-activating factor I in different cell types. *Exp Cell Res* 1998, 240:274–281
37. Iqbal K, Alonso A del C, El-Akkad E, Gong CX, Haque N, Khatoon S, Tanimukai H, Tsujio I, Grundke-Iqbal I: Pivotal role of neurofibrillary degeneration in Alzheimer disease and therapeutic targets. *Molecular Neurobiology of Alzheimer Disease and Related Disorders*. Edited by Takeda M, Tanaka T, Cacabelos R. Basel, Karger, 2004, pp 42–51
38. Braak H, Griffin K, Braak E: Neuroanatomy of Alzheimer's disease. *Alzheimer's Res* 1997, 3:235–247
39. Brennan CM, Gallouzi IE, Steitz JA: Protein ligands to HuR modulate its interaction with target mRNAs in vivo. *J Cell Biol* 2000, 151:1–14
40. Beresford PJ, Kam CM, Powers JC, Lieberman J: Recombinant human granzyme A binds to two putative HLA-associated proteins and cleaves one of them. *Proc Natl Acad Sci USA* 1997, 94:9285–9290
41. Beresford PJ, Zhang D, Oh DY, Fan Z, Greer EL, Russo ML, Jaju M, Lieberman J: Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J Biol Chem* 2001, 276:43285–43293
42. Saito S, Miyaji-Yamaguchi M, Shimoyama T, Nagata K: Functional domains of template-activating factor-I as a protein phosphatases 2A inhibitor. *Biochem Biophys Res Commun* 1999, 259:471–475
43. Pei JJ, Sersen E, Iqbal K, Grundke-Iqbal I: Expression of protein phosphatases (PP-1, PP2A, PP-2B and PTP-1B) and protein kinases (MAP kinase and P34cdc2) in the hippocampus of patients with Alzheimer disease and normal aged individuals. *Brain Res* 1994, 655:70–76
44. Janssens V, Goris J: Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signaling. *Biochem J* 2001, 353:417–439
45. Yu LG, Packman LC, Weldon M, Hamlett J, Rhodes JM: Protein phosphatase 2A, a negative regulator of the ERK signaling pathway, is activated by tyrosine phosphorylation of putative HLA class II-associated protein I (PHAPI)/pp32 in response to the antiproliferative lectin, jacalin. *J Biol Chem* 2004, 279:41377–41383
46. Bennecib M, Gong CX, Grundke-Iqbal I, Iqbal K: Inhibition of PP-2A upregulates CaMKII in rat forebrain and induces hyperphosphorylation of tau at Ser 262/356. *FEBS Lett* 2001, 490:15–22
47. Li L, Sengupta A, Haque N, Grundke-Iqbal I, Iqbal K: Memantine inhibits and reverses the Alzheimer type abnormal hyperphosphorylation of tau and associated neurodegeneration. *FEBS Lett* 2004, 566:261–269
48. An WL, Cowburn RF, Li L, Braak H, Alafuzoff I, Iqbal K, Grundke-Iqbal I, Winblad B, Pei JJ: Up-regulation of phosphorylated/activated p70 S6 kinase and its relationship to neurofibrillary pathology in Alzheimer's disease. *Am J Pathol* 2003, 163:591–607
49. Kins S, Kurosinski P, Nitsch RM, Gotz J: Activation of the ERK and JNK signaling pathways caused by neuron-specific inhibition of PP-2A in transgenic mice. *Am J Pathol* 2003, 163:833–843
50. Pei JJ, Gong CX, An WL, Winblad B, Cowburn RF, Grundke-Iqbal I, Iqbal K: Okadaic-acid-induced inhibition of protein phosphatase 2A produces activation of mitogen-activated protein kinases ERK1/2, MEK1/2, and p70 S6, similar to that in Alzheimer's disease. *Am J Pathol* 2003, 163:845–858