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Inhibitors of protein phosphatase-2A from human brain structures, immunocytological localization and activities towards dephosphorylation of the Alzheimer type hyperphosphorylated tau

Ichiro Tsujio, Tanweer Zaidi, Jiliu Xu, Leszek Kotula, Inge Grundke-Iqbal, Khalid Iqbal*

Department of Neurochemistry, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314-6399, USA

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Abstract Protein phosphatase (PP)-2A, which regulates the phosphorylation of tau, is regulated by two endogenous inhibitor proteins, I_1^{PP2A} and I_2^{PP2A}, in mammalian tissues. Here, we report the cloning of I_1^{PP2A} and I_2^{PP2A} from human brain, and show that in PC12 cells and in I_1^{PP2A} – GFP or I_2^{PP2A} – GFP transfected NIH3T3 and human neural progenitor cells, I_1^{PP2A} is localized mostly in the cell cytoplasm and I_2^{PP2A} mostly in the nucleus. The recombinant I_1^{PP-2A} and I_2^{PP-2A} inhibit PP-2A activity towards hyperphosphorylated tau in vitro; the dephosphorylation of the hyperphosphorylated tau at specific sites is selectively inhibited. Overexpression of I_1^{PP2A} as well as I_2^{PP2A} results in tau hyperphosphorylation and degeneration of PC 12 cells.

Keywords: Alzheimer disease; Abnormally hyperphosphory- lated tau; Protein phosphatase-2A inhibitor; Template activating factor; PHAP; LANP

1. Introduction

The presence of numerous neurons with neurofibrillary tangles of paired helical filaments (PHF) in the neocortex, especially the hippocampus, is a neuropathological hallmark of Alzheimer disease (AD). The microtubule associated protein tau is abnormally hyperphosphorylated in AD brain and is the major protein subunit of PHF [1–5]. The abnormally hyperphosphorylated tau is also present in the affected neurons as amorphous aggregates and as a cytosolic protein [2,6–10]. However, the mechanisms of hyperphosphorylation of tau are not yet established. Generally, the level of phosphorylation of a protein is regulated by the equilibrium between the activities of its protein kinases and phosphatases.

The activities of phosphoseryl/phosphothreonyl protein phosphatases (PP)-2A and PP-1 are compromised in AD brain [11,12], and the inhibition of activity of PP-2A by okadaic acid and not PP-2B activity by cyclosporin-A produces in metabolically active brain slices from adult rats the abnormal hyperphosphorylation of tau that inhibits its binding to microtubules and the promotion of their assembly in vitro [13]. Furthermore, the activities of several protein kinases which phosphorylate tau are regulated by PP-2A [14–17]. Thus, it is strongly suspected that PP-2A, which also regulates the activity of PP-1 through dephosphorylation of PP-1 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 PP-2A activity is regulated by two endogenous inhibitor proteins, I_1^{PP2A} and I_2^{PP2A}, in mammalian tissue (for review see [18]). These two proteins do not inhibit the activities of PP-1, PP-2B and PP-2C, the other three major serine/threonine protein phosphatases or affect the activities of a number of protein kinases studied in vitro [19]. Both I_1^{PP2A} and I_2^{PP2A} have been cloned from human kidney [20,21]. Both proteins inhibit holoenzyme forms of PP-2A, probably by binding directly to the catalytic subunit. The purified preparations of I_1^{PP2A} and I_2^{PP2A} inhibit PP-2A in vitro in a manner non-competitive with the substrate [19]. In intact cells, overexpression of I_2^{PP-2A} results in increased expression, DNA-binding and Ser-63 phosphorylation of c-Jun, and in higher transcriptional activity of activator protein-1 (AP-1) [22]. These effects are reversed by overexpression of hemagglutinin-tagged PP-2Ac, consistent with I_2^{PP-2A} acting as a PP-2A inhibitor in vivo. I_1^{PP2A} and I_2^{PP2A} associate with and modify the substrate specificity of PP-1 in the presence of physiological concentration of Mn^{2+}, suggesting a novel role for I_1^{PP2A} and I_2^{PP2A} in the coordination of PP-1 and PP-2A activities within cells [23].

I_1^{PP-2A} has been found to be the same protein as the putative histocompatibility leukocyte antigen class II-associated protein (PHAP)-I [21]. Proteins homologous to I_1^{PP-2A} have been isolated and described as mapmodulin [24], pp32 [25], and leucine-rich acidic nuclear protein (LANP) [26]. Proteins homologous to I_2^{PP-2A} have also been isolated and described as human SETα [21,27], PHAP-II, and template activating factor-1β (TAF-1β) [28]. SET, in its complete form, is a nuclear protein and contains a highly acidic C-terminal region that is involved in chromatin remodeling. SET itself is also a potent and specific inhibitor of PP-2A [21], and exerts its inhibitory activity via its N-terminal part [29]. TAF-1β protein which lacks the C-terminal region and also inhibits PP-2A activity

*Corresponding author. Fax: +1 718 494 1080.
E-mail address: iqbalk@worldnet.att.net (K. Iqbal).

Abbreviations: AP-1, activator protein-1; AD, Alzheimer disease; GFP, green fluorescent protein; GST, glutathione-S-transferase; LANP, leucine-rich acidic nuclear protein; MAP, microtubule associated protein; PHF, paired helical filaments; PCR, polymerase chain reaction; PHAP, putative histocompatibility leukocyte antigen class II-associated protein; TAF-1β, template activating factor-1β

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is localized mainly in the cytoplasm, while the full-sized TAF-1 is a nuclear protein [30]. However, these inhibitors have not yet been isolated and characterized from brain.

The present study describes the cloning and the characterization of I_1^{PP2A} and I_2^{PP2A} from human brain, the immunocytological localization of these inhibitors in cultured cells, and the inhibition of PP-2A catalyzed dephosphorylation of abnormally hyperphosphorylated tau at specific sites by these two inhibitors.

2. Materials and methods

2.1. Cloning and generation of brain I_1^{PP2A} and I_2^{PP2A}

Polymerase chain reaction (PCR) was used to generate probes specific for I_1^{PP2A} and I_2^{PP2A}. The 31-mer oligonucleotide 5′-CGA AGA TCT ATG GAG ATG GGC AGA CGG ATT C-3′ as the 5′ primer and the 33-mer oligonucleotide 5′-GCA GTC GAC GTC ATC ATC TTC TCC CTC ATC TTC as the 3′ primer to the sequence of human kidney I_1^{PP-2A} cDNA were designed. The 33-mer oligonucleotide 5′-CGT AGA TCT ATG TCG GCG CCG GCG GCC AAA GTC and the 33-mer oligonucleotide 5′-GCT GTC GAC GTC ATC TTC TCC TTC ATC CTC CTC were designed as the 5′ primer and the 3′ primer, respectively, to the sequence of human kidney I_2^{PP-2A} cDNA. The reaction mixture for PCR contained cDNA derived from human brain Marathon-Ready cDNA library (Clontech, Palo Alto, CA) as a template. PCR amplification was performed with 30 cycles, using a cycle of denaturation for 30 s at 92 °C, annealing for 40 s at 68 °C, and an extension for 2 min at 68 °C. The PCR product was digested with *Bgl*II and *Sal*I (Promega, Madison, WI), cloned into a pEGFP-N3 Vector (Clontech), and sequenced. To remove the green fluorescent protein (GFP) region of these two plasmids, they were retransformed with INV100 competent cells (Invitrogen, Carlsbad, CA), digested with *Sal*I and *Xba*I (Promega), and then treated with Klenow fragment (New England Biolabs, Beverly, MA) and religated.

2.2. Cell culture and transfections

NIH 3T3 cells (obtained from ATCC, Rockville, MD) were grown in 25 cm^2 flasks at 37 °C, containing 5% CO_2 in DMEM supplemented with 10% bovine calf serum. Cells were plated on coverslips and transfected with expression plasmids of I_1^{PP2A} – GFP or I_2^{PP2A} – GFP using Lipofectamine Plus Reagent according to the manufacturer's instructions (Life Technologies, Inc., Rockville, MD). At 24 h post-transfection, these cells were processed for indirect immunofluorescence.

Human neural progenitor cells were grown on dishes coated with 0.01% poly-D-lysine (Sigma, St. Louis, MO) in Neurobasal medium. Cells were transfected with expression plasmids as above using Lipofectamine Plus Reagent according to the manufacturer's instructions. At 72 h post-transfection, the human progenitor cells were processed for indirect immunofluorescence.

PC12 cells or tau 441 stably transfected PC12 cells were grown in 25 cm^2 flasks at 37 °C, maintained at 5% CO2 in RPMI medium 1640 (Gibco™) supplemented with 10% bovine calf serum, 5% horse serum, and 1% penicillin. Cells were plated in 8 well Lab-Tek II Chamber Slides (Nalge Nunc International, Naperville, IL) and then transfected with expression plasmid using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). At 24, 48 and 72 h post-transfection, cells were processed for indirect immunofluorescence and LDH assay.

2.3. Immunocytochemical staining

Human neural progenitor cells and NIH 3T3 cells transiently transfected with I_1^{PP2A} – GFP or I_2^{PP2A} – GFP as well as non-transfected PC12 cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) and immunostained with 1 μg/ml rabbit affinity purified antibody R-42089 to a synthetic peptide corresponding to amino acid residues 10–23 of human I_1^{PP-2A} [31], or with 2 μg/ml rabbit affinity purified antibody R-42187 to a synthetic peptide corresponding to amino acid residues 18–29 of human SET/I_2^{PP-2A} [31], followed by incubation with anti-rabbit Alexa 594 conjugate, 1:2000 dilution. The antibody R-42089 recognizes I_1^{PP2A} but not I_2^{PP2A}. Similarly, antibody R-42187 recognizes I_2^{PP2A} and not I_1^{PP2A} [31]. Representative images from several cells were generated using a Nikon PCM 2000 Confocal Imaging System.

In the case of PC12 cells transfected with I_1^{PP2A} or I_2^{PP2A}, at 72 h post-transfection, cells were fixed with 4% paraformaldehyde in 0.1 M PBS and were treated with 0.1% Triton-X in TBS for 5 min and were blocked with 5% defatted milk in TBS for 30 min and with primary antibodies in the same buffer overnight at 4 °C. The primary antibodies used were mAb PHF-1 (to phosphorylated Ser 396/404, 1:100) [32,33], mAb Tau-1 (to unphosphorylated Ser 195, 198, 199 or 202; 1:50 000) [1,34,35], mAb M4 (to phosphorylated Thr 231/Ser 235; 1:2000) [36], mAb 43D (to total tau; 1:2000), mAb DM1A (to alpha-tubulin; 1:2000) (Sigma), pAb 92e (to total tau; 1:5000) [37], pAb 134d (to total tau; 1:5000) [38], rabbit affinity purified antibody R-42089 to a synthetic peptide corresponding to amino acid residues 10–23 of kidney I_1^{PP2A}, and rabbit affinity purified antibody R-42187 to a synthetic peptide corresponding to amino acid residues 18–29 of kidney I_2^{PP2A}. Fluorescent secondary antibodies used were cy3 anti-rabbit and anti-mouse $(Fab_1)^2$ antibodies (1:1000; Molecular Probes, Eugene, OR). Representative images from several cells were generated using a Nikon PCM 2000 Confocal Imaging System.

2.4. Western blots

NIH3T3 cells grown in 175-cm^2 flasks were washed once and scraped in ice-cold PBS. Cells were then lysed in lysis buffer (50 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EGTA, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM PMSF, and 8 mM diisopropylfluorophosphate). The cell lysates were subjected to SDS–PAGE and transferred to PVDF membranes. The levels of GFP, I_1^{PP2A} and I_2^{PP2A} were visualized using anti-mouse GFP antibody (Quantum Biotechnology, Inc., Montreal, Canada), 1:1000 dilution, rabbit antibodies R-42089 (1 μg/ml) to I_1^{PP2A} and R-42187 (1 μg/ml) to I_2^{PP2A} [31]. As secondary antibodies, alkaline phosphatase-labeled goat anti-mouse, 1:2000 dilution, or anti-rabbit IgG, 1:2000, were used and membranes were developed by 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride. Western blots of GST – I_1^{PP2A} and GST – I_2^{PP2A} were developed as above using either monoclonal antibody B5E7 to GST [39] or antibodies to I_1^{PP2A} or I_2^{PP2A}.

2.5. Northern blots

Multiple Tissue Northern (MTN) blots (Clontech), which are hybridization-ready Northern blots prepared using Clontech's high-quality premium RNA, were used. The probe used was the ^{32}P-labeled PCR products, which was made with Random printed DNA labeling kit (Roche, Indianapolis, IN).

2.6. Generation of recombinant GST – I_1^{PP-2A}, GST – I_2^{PP2A}, I_1^{PP2A} and I_2^{PP2A}

The PCR products for I_1^{PP2A} and I_2^{PP2A} were separately digested with *Bgl*II and *Sal*I, cloned into a glutathione-S-transferase (GST) gene fusion vector, pGEX-4T-2 Vector (Amersham Pharmacia Biotech, Piscataway, NJ), and sequenced. Purification and detection of GST fusion protein produced in *E. coli* were carried out using the GST Gene Fusion system (Amersham Pharmacia Biotech). A single colony of E.coli cells containing recombinant PGEX 4T-2 with I_1^{PP2A} or I_2^{PP2A} plasmid was picked and used to inoculate 3 ml of 2X YTA medium at 37 °C with shaking overnight. Then, 1 ml of this liquid culture was employed to inoculate 1L 2X YTA medium at 37 °C with shaking until the AS600 nm reached 0.6–0.8. At this stage, IPTG was added to a final concentration of 0.2 mM and the cells were grown for an additional 3 h. The cultured cells were centrifuged at $7700 \times g$ for 20 min at 4 °C, and the pellets were drained and saved for the purification of the inhibitors. The pellets were suspended in lysis buffer (50 mM TBS, pH 7.6, 1 mM PMSF, 0.1 mM benzamidine and protease inhibitor cocktail [Sigma]) and sonicated on ice (pulsed 60%, 9 mm sonication tip) for 1.5 min for 5 times (interval time between sonications was 5 min) and centrifuged at $12\,000 \times g$ for 10 min, followed by $100\,000 \times g$ for 20 min. These pellets were discarded and the supernatant was passed through Glutathione Sepharose 4B column. The column was washed with TBS and then eluted with 10 mM glutathione, 50 mM Tris–HCl, pH 8.0. To purify the recombinant I_1^{PP2A} and I_2^{PP2A}, recombinant GST – I_1^{PP-2A} or GST – I_2^{PP2A} protein was treated with thrombin (Amersham Pharmacia Biotech) for 24 h (GST – I_1^{PP-2A}) or for 3 h

(GST – I_2^{PP2A}) in the Glutathione Sepharose 4B column and the flow-through and wash with TBS and 0.5 M NaCl in TBS were collected, combined, passed through HiTrap Benzamidine FF column (Amersham Pharmacia Biotech) to remove the thrombin from the inhibitors in the flow-through. The inhibitors were concentrated by ultrafiltration (Millipore, Bedford, MA) in 50 mM imidazole, pH 7.2, 0.2 mM EGTA, 0.02% BME and 0.1 mg/ml BSA and stored at −75 °C till used. The purity of the recombinant proteins was confirmed by SDS–PAGE and Western blots. The recombinant proteins were heated in sample buffer (in 20 mM Tris, pH 6.8, 1% SDS, 1% BME, and 10% glycerol/bromophenol blue), and then subjected to SDS–PAGE (10% polyacrylamide) and Coomassie blue staining and to Western blots developed with mouse anti-GST antibody, dilution of 1:1000, I_1^{PP2A} antibody (2.5 μg/ml) or I_2^{PP2A} antibody (5 μg/ml). The recombinant proteins were used for the inhibition of PP-2A activity in the phosphatase assay and for radioimmuno-dot-blot assay.

2.7. Preparation of [^{32}P] hyperphosphorylated tau and phosphatase assay

Phosphatase assays were carried out using ^{32}P-labeled hyperphosphorylated tau as a substrate. Hyperphosphorylation of recombinant human brain tau_{352} was performed by using 100 000 × *g* brain extract from a 20 day-old rat as the source of protein kinases as described previously [5,40]. The reaction was carried out at 32 °C using 0.25 mg/ml tau protein and 1 μl of brain extract (16 000 × *g* extract of 33% homogenate) per 20 μl of phosphorylation buffer (60 mM HEPES, pH 7.4, 8 mM $MgCl_2$, 5 mM EGTA, 2 mM ATP, 2 mM DTT, 20 nM calyculin A, and 1 mM 4-[2-aminoethylamino]-benzenesulfonyl fluoride [AEBSF, a serine protease inhibitor]). After 2 h and 8 h of incubation, NaF (17 mM) and ATP (cold plus ^{32}P, 2 mM), respectively, were added [5]. At the end of reaction, 5 mM BME and 100 mM NaCl were added and the reaction mixture was heated in a boiling water bath for 5 min. After heat treatment, the mixture was centrifuged at 16 000 × *g* for 15 min and tau which is heat stable was collected as supernatant. Tau from the supernatant was then precipitated and washed with 15% TCA/2 mM ATP three times and then with 10% TCA three times. The pellet was solubilized at 4 °C in 0.1 mM NaOH and then neutralized with 0.1 mM HCl to pH 7.5.

The inhibition of PP-2A activity by I_1^{PP2A} and I_2^{PP2A} was determined in 50 mM Tris, pH 7.0, 0.1 mM EDTA, 7.5 mM caffeine, and 10 mM BME using $PP\text{-}2A_1$ purified from bovine brain as a holoenzyme [41], ^{32}P-labeled hyperphosphorylated tau as a substrate, and recombinant inhibitors as such or as GST fusion proteins. Reactions were initiated with ^{32}P-labeled hyperphosphorylated tau, and to stop the reaction after 20 min at 30 °C samples were chilled on ice for 10 min. ^{32}P-labeled tau was then separated from free ^{32}P using paper chromatography [42]. The radioactivity was measured by Cerenkov Method in a scintillation counter. These samples were also used for radioimmuno-dot-blot assays, after storage at −75 °C for more than 10 half-life periods of ^{32}P till the radioactive signal was negligible.

2.8. LDH assay for cell viability

The effect of the overexpression of I_1^{PP2A} or I_2^{PP2A} on the degeneration of PC12 cells was determined by assaying the LDH release using the Cyto Tox-ONE™ Assay kit (Promega). PC12 cells were transfected with I_1^{PP2A}, I_2^{PP2A}, N3 (mock) or pcDNA (mock) in 96 well microtiter plates. AT 24, 48 and 72 h post-transfection, the LDH release was assayed, and degeneration calculated as: % cell death = (experimental − culture medium background) × 100/(maximum LDH release × culture medium background).

2.9. Radioimmunodot blotting assay

Levels of phosphorylated and unphosphorylated taus were determined by radioimmunodot blot assays [8,43]. Samples were diluted (2 ng/μl) in dilution buffer (50 mM Tris, pH 7.6, 0.1% SDS, 200 mM NaCl, 1 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin) and were applied onto nitrocellulose membranes at 3 μl/cm^2. Membranes were blocked for 1 h with 5% defatted milk, then blots were overlaid with phospho-independent or phospho-dependent tau antibodies (see Table 1) at room temperature overnight with shaking. After washing three times with TBS, the blots were overlaid with ^{125}I-labeled anti mouse or rabbit IgG for 2 h at room temperature. The blots were washed with TBS containing 0.1% Tween 20 overnight, then washed three times for 30 min, followed by drying the blots and counting the radioactivity in a Fuji BAS 1500 phosphorimager.

Table 1
Tau antibodies employed

Antibody	Type	Epitope
134d[a]	Poly	Total tau
P Thr-231	Poly	pThr-231
M4	Mono	pThr-231/pSer-235
P Ser-262	Poly	pSer-262
12E8	Mono	pSer-262/356
P Ser-396	Poly	pSer-396
PHF-1	Mono	pSer-396/404

[a]Phospho-independent and total tau.
P: phospho-dependent and phosphorylated epitope.

3. Results

Cloning of I_1^{PP2A} and I_2^{PP2A} from human brain and mRNA expression in various tissues and brain regions. To determine the structures of brain I_1^{PP2A} and I_2^{PP2A}, we cloned these cDNAs of proteins from human brain using human Marathon-Ready cDNA library and primers generated for the human kidney I_1^{PP2A} and I_2^{PP2A} cDNAs. The nucleotide sequences of the brain cDNAs of I_1^{PP2A} and I_2^{PP2A} were 7.56 kb and 8.40 kb, respectively (Fig. 1).

The mRNAs of I_1^{PP2A} and I_2^{PP2A} were investigated by Northern blot analysis. The $I_1^{PP\text{-}2A}$ probe hybridized in Northern blots to three transcripts of 4.2, 3.7 and 2.2 kb in all tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas) examined (Fig. 2(a)). Strong signals in kidney and skeletal muscle, moderate signals in brain, placenta and pancreas and weak signals in lung were observed (Fig. 2(a-i) to (a-iv)). In brain, the 3.7 kb transcript was the most prominent and the 2.2 kb signal was the weakest of all the three transcripts. $I_1^{PP\text{-}2A}$ probe hybridized in all brain regions (amygdala, caudate nucleus, corpus callosum, hippocampus, brain [whole] and thalamus) examined (Fig. 2(b)). The strongest signal was detected in amygdala (Fig. 2(b-i) to (b-iv)).

The size of SET gene mRNA, which is homologous to $I_2^{PP\text{-}2A}$, was previously reported to be 2.7 and 2.0 kb on Northern blots in mouse tissues with human SET cDNA probe [30]. In the present study, Northern blots analysis showed that $I_2^{PP\text{-}2A}$ probe hybridized prominently to two transcripts of 2.7 kb and 1.9 kb and weakly to a transcript of 4.0 kb in all tissues examined (Fig. 2(c)). The strong signals were detected in placenta and brain (Fig. 2(c-i) to (c-iv)). $I_2^{PP\text{-}2A}$ probe hybridized in all brain regions examined (Fig. 2(d)). Strong signals were detected in amygdala, hippocampus and thalamus (Fig. 2(d-i) to (d-iv)). These results suggested that I_1^{PP2A} and I_2^{PP2A} mRNAs were localized in all human tissues, including the areas of the brain, i.e., amygdala and hippocampus that are most affected by neurofibrillary degeneration in AD.

Isolation of recombinant human brain I_1^{PP2A} and I_2^{PP2A} and Inhibition of PP-2A activity towards hyperphosphorylated tau by these inhibitors. To determine the activities of I_1^{PP2A} and I_2^{PP2A}, we generated these inhibitors as recombinant GST fusion proteins and then cleaved them from GST with thrombin. SDS–PAGE and Western blots of the isolated I_1^{PP2A} and I_2^{PP2A} had molecular size of ∼30 and ∼40 kDa, respectively (Fig. 3). The GST – $I_1^{PP\text{-}2A}$ and GST – $I_2^{PP\text{-}2A}$ had apparent molecular size of ∼60 and ∼70 kDa, respectively.

Human brain $I_1^{PP\text{-}2A}$

agatct
Bgl II

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1   atggagatgg gcagacggat tcatttagag ctgcggaaca ggacgccctc tgatgtgaaa
61  gaacttgtcc tggacaacag tcggtcgaat gaaggcaaac tcgaaggcct cacagatgaa
121 tttgaagaac tggaattctt aagtacaatc aacgtaggcc tcacctcaat cgcaaacttа
181 ccaaagttaa acaaacttaa gaagcttgaa ctaagcgata acagagtctc aggggcctg
241 gaagtattgg cagaaaagtg tccgaacctc acgcatctaa atttaagtgg caacaaaatt
301 aaagacctca gcacaataga gccactgaaa aagttagaaa acctcaagag cttagacctt
361 ttcaattgcg aggtaaccaa cctgaacgac taccgagaaa atgtgttcaa gctcctcccg
421 caactcacat atctcgacgg ctatgaccgg gacgacaagg aggcccctga ctcggatgct
481 gagggctacg tggagggcct ggatgatgag gaggaggatg aggatgagga ggagtatgat
541 gaagatgctc aggtagtgga agacgaggag gacgaggatg aggaggagga aggtgaagag
601 gaggacgtga gtggagagga ggaggaggat gaagaaggtt ataacgatgg agaggtagat
661 gacgaggaag atgaagaaga gcttggtgaa gaagaaaggg gtcagaagcg aaaacgagaa
721 cctgaagatg agggagaaga tgatgactaa gtcgac
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Sal I

Human brain $I_2^{PP\text{-}2A}$

agatct
Bgl II

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1   atgtcggcgc cggcggccaa agtcagtaaa aaggagctca actccaacca cgacggggcc
61  gacgagacct cagaaaaaga acagcaagaa gcgattgaac acattgatga agtacaaaat
121 gaaatagaca gacttaatga acaagccagt gaggagattt tgaaagtaga acagaaatat
181 aacaaactcc gccaaccatt ttttcagaag aggtcagaat tgatcgccaa aatcccaaat
241 ttttgggtaa caacatttgt caaccatcca caagtgtctg cactgcttgg ggaggaagat
301 gaagaggcac tgcattattt gaccagagtt gaagtgacag aatttgaaga tattaaatca
361 ggttacagaa tagattttta ttttgatgaa aatccttact ttgaaaataa agttctctcc
421 aaagaatttc atctgaatga gagtggtgat ccatcttcga agtccaccga aatcaaatgg
481 aaatctggaa aggatttgac gaaacgttcg agtcaaacgc agaataaagc cagcaggaag
541 aggcagcatg aggaaccaga gagcttcttt acctggttta ctgaccattc tgatgcaggt
601 gctgatgagt taggagaggt catcaaagat gatatttggc caaacccatt acagtactac
661 ttggttcccg atatggatga tgaagaagga gaaggagaag aagatgatga tgatgatgaa
721 gaggaggaag gattagaaga tattgacgaa gaaggggatg aggatgaagg tgaagaagat
781 gaagatgatg atgaagggga ggaaggagag gaggatgaag gagaagatga ctaggtcgac
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Sal I

Fig. 1. Nucleotide sequence of human brain $I_1^{PP\text{-}2A}$ and $I_2^{PP\text{-}2A}$ cDNAs. Underlines indicate the oligonucleotides used to generate the cDNAs and location of the introduced *Bgl*II and *Sal*I sites. Genbank™ accession numbers of human brain I_1^{PP2A} and I_2^{PP2A} are AY349171 and AY349172, respectively.

To study the activities of the recombinant inhibitors towards PP-2A, we carried out PP-2A assays with ^{32}P-labeled hyperphosphorylated tau_{352} as a substrate. Previously, it was reported that the purified preparations of $I_1^{PP\text{-}2A}$ and $I_2^{PP\text{-}2A}$ inhibited PP-2A-catalyzed dephosphorylation of ^{32}P-labeled myelin basic protein, ^{32}P-labeled histone H1, ^{32}P-labeled pyruvate dehydrogenase complex, and ^{32}P-labeled phosphorylase [19]. Because of interest in the regulation of PP-2A activity towards hyperphosphorylated tau, we carried out PP-2A assay with ^{32}P-labeled hyperphosphorylated tau_{352} as a substrate. The recombinant $I_1^{PP\text{-}2A}$, $I_2^{PP\text{-}2A}$ and GST – $I_1^{PP\text{-}2A}$ inhibited but neither GST nor GST – I_2^{PP2A} inhibited PP-2A activity in vitro with hyperphosphorylated tau as a substrate (Fig. 3). Half-maximal inhibition of PP2A with $I_1^{PP\text{-}2A}$ and GST – $I_1^{PP\text{-}2A}$occurred at about 600 nM, and with $I_2^{PP\text{-}2A}$ at about 400 nM. The apparent Ki for $I_1^{PP\text{-}2A}$ and GST – $I_1^{PP\text{-}2A}$ was about 480 nM, and for $I_2^{PP\text{-}2A}$ was about 300 nM. The Kis for $I_1^{PP\text{-}2A}$, $I_2^{PP\text{-}2A}$ with ^{32}P-labeled hyperphosphorylated tau_{352} as a substrate were ~10–20 times higher than those with other ^{32}P-labeled substrates reported previously [19].

Differential inhibition of PP-2A activity towards AD specific phosphorylated sites of tau by I_1^{PP2A} *and* I_2^{PP2A}. Tau is abnormally hyperphosphorylated at specific sites in AD and different sites have different effects on the biological activity of tau [44,45]. We therefore investigated the inhibition of PP-2A activity towards different abnormally phosphorylated sites of tau by I_1^{PP2A} and I_2^{PP2A}. For these studies, we first established the time kinetics of the dephosphorylation of the hyperphosphorylated tau by PP-2A at each specific site using phospho-dependent antibodies and a polyclonal antibody to total tau (see Table 1). The dephosphorylation reaction was found to be linear up to 15 min incubation at 32 °C and during this incubation less than 15% of the substrate was dephosphorylated (data not shown). Employing 10 min incubation at 32 °C, we determined both the dephosphorylation of tau by PP-2A at each specific site (Table 2) and the inhibition of this activity by $I_1^{PP\text{-}2A}$, $I_2^{PP\text{-}2A}$ or GST – $I_1^{PP\text{-}2A}$ employing their IC_{50} concentrations towards hyperphosphorylated tau in the above studies. PP-2A was found to dephosphorylate tau with different efficiency at different sites (Table 2). The dephosphorylation of tau at 12E8 (Ser-262/356) and at Thr-231 was found to be most and least efficient by PP-2A. $I_1^{PP\text{-}2A}$ inhibited the dephosphorylation of tau most efficiently at 12E8 (Ser-262/356) and PHF-1 (Ser-396/404) sites, while $I_2^{PP\text{-}2A}$ had the corre-

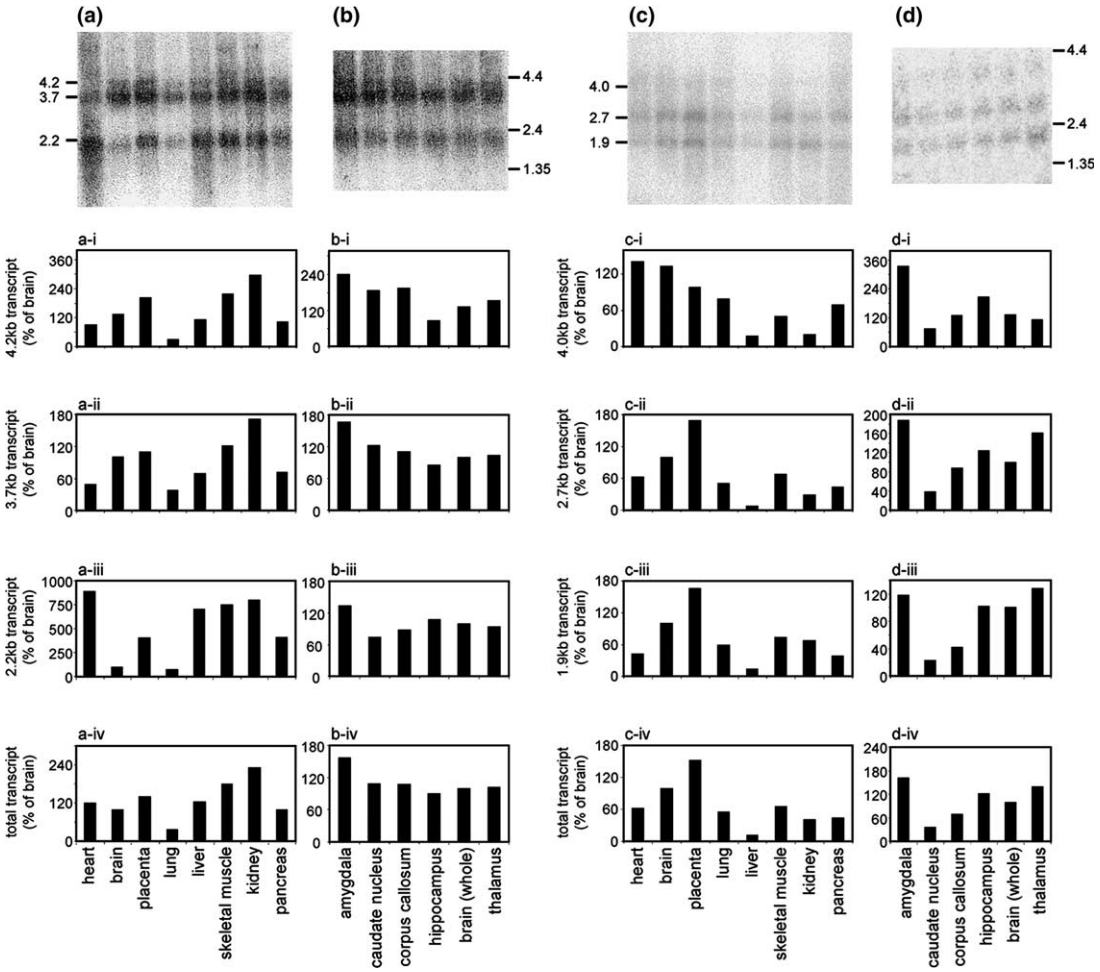

Fig. 2. Northern blots showing the e xpression of I_1^{PP2A} and I_2^{PP2A} mRNAs in different human tissues and brain regions. The cDNA probes used were PCR products. I_1^{PP2A} probe (a,b) or I_2^{PP2A} probe (c,d) was hybridized to RNAs of various human tissues (a,c) or brain regions (b,d). Northern blots in a, b, c and d were scanned and values obtained were normalized to brain as 100% (i–iv). The size (kb) of the transcripts detected are indicated on the left and the standards on the right of the panels.

sponding higher activity towards M4 (Thr-231/Ser-235) site (Table 3). Like I_1^{PP2A}, the GST – I_1^{PP2A} fusion protein inhibited the PP-2A activity towards the hyperphosphorylated tau but the relative inhibitory activities of the two proteins were variable at each phosphorylation site studied, especially at 12E8 site.

Immunocytological localization of I_1^{PP2A} *and* I_2^{PP2A} *in cultured cells*. To determine the cytological localization of I_1^{PP2A} and I_2^{PP2A}, NIH3T3 cells and human neural progenitor cells transfected with I_1^{PP2A} – GFP or I_2^{PP2A} – GFP, and untransfected PC12 cells were immunostained with antibodies to I_1^{PP2A} or I_2^{PP2A}. Endogenous I_1^{PP2A} was localized both in the cytoplasm and nuclei, whereas endogenous I_2^{PP2A} was seen mostly in the nuclei of PC12 cells (Fig. 4(a)). Overexpression of $I_1^{PP\text{-}2A}$ – GFP was found both in the cytoplasm and the nucleus but mostly in the former in NIH3T3 cells and in human neural progenitor cells. Overexpression of $I_2^{PP\text{-}2A}$ – GFP was observed mostly in the nucleus both in NIH3T3 cells and human progenitor cells. The overexpression of the GFP fusion proteins was confirmed biochemically in the cell lysates. Western blots developed with antibodies to GFP revealed a 60 kDa ($I_1^{PP\text{-}2A}$ – GFP) band and a 70 kDa ($I_2^{PP\text{-}2A}$ – GFP) band in $I_1^{PP\text{-}2A}$ transfected and $I_2^{PP\text{-}2A}$ transfected NIH3T3 cells, respectively (Fig. 4(b)).

Regulation of phosphorylation of tau and induction of cell death by I_1^{PP2A} *and* I_2^{PP2A}. To study whether I_1^{PP2A} and/or I_2^{PP2A} regulate the phosphorylation of tau and cause cell death, PC12 cells stably transfected with human brain tau_{441} or the untransfected cells were transiently transfected with I_1^{PP2A} or I_2^{PP2A} and its effect on abnormal phosphorylation of tau at various sites in the cells was studied immunocytochemically.

Overexpression of I_1^{PP2A} resulted in a marked increase in the phosphorylation of tau at 12E8 and PHF-1 sites and a moderate increase at M4 and Tau-1 sites (Fig. 5(a)). Mock transfection, i.e., vector alone of PC12 cells, did not reveal any significant changes in the phosphorylation of tau at any of the above sites studied (data not shown). Immunocytochemical staining with two different phospho-independent polyclonal antibodies revealed that the tau_{441} stably transfected PC12 cells employed for the studies had a similar expression of total tau (data not shown). Overexpression of I_2^{PP2A} resulted in an increase in the phosphorylation of tau at 12E8, PHF-1, M4 and Tau-1 sites (Fig. 5(b)); the increase was most noticeable at the 12E8 site. Mock transfected cells revealed no detectable change

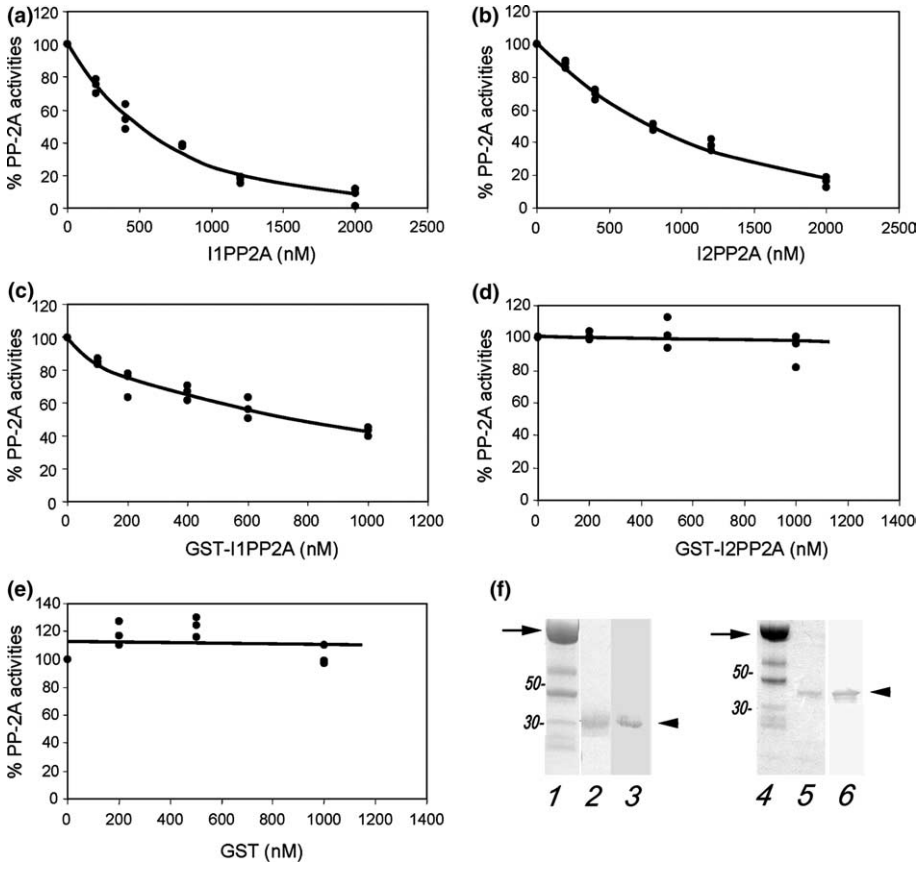

Fig. 3. Effect of recombinant human $I_1^{PP\text{-}2A}$, $I_2^{PP\text{-}2A}$, GST – $I_1^{PP\text{-}2A}$, GST – $I_2^{PP\text{-}2A}$ and GST on PP-2A. (a–e) The activity of PP-2A was measured as described in Section 2 with ^{32}P-labeled hyperphosphorylated tau_{352} as substrate in the presence of the indicated concentrations of purified recombinant $I_1^{PP\text{-}2A}$ (a), and $I_2^{PP\text{-}2A}$ (b), GST-$I_1^{PP\text{-}2A}$(c), GST-$I_2^{PP\text{-}2A}$(d) and GST (e). (f) SDS–PAGE of recombinant GST – I_1^{PP2A} (lane 1, arrow), GST – I_2^{PP2A} (lane 4, arrow), purified I_1^{PP2A} (lane 2), I_2^{PP2A} (lane 5) and Western blots of purified I_1^{PP2A} (lane 3) and I_2^{PP2A} (lane 6) developed with corresponding antibodies.

in the phosphorylation of tau at any one of the above sites studied (data not shown). These findings suggested that both I_1^{PP2A} and I_2^{PP2A} regulated the phosphorylation of tau at some of the same sites known to be abnormally hyperphosphorylated in AD brain.

The abnormally hyperphosphorylated tau, which sequesters normal tau, MAP1 and MAP2 and depolymerizes microtubules [8–10], is suspected to lead to neurodegeneration in AD and other tauopathies. We, therefore, investigated the effect of the overexpression of I_1^{PP2A} and I_2^{PP2A} on the viability of the PC12 cells by the Cyto Tox-ONE™ assay. We found that in a post-transfection time-dependent manner, both I_1^{PP2A} and I_2^{PP2A} as such and as well as GFP-fusion proteins increased the cell death by ~twofold during 72 h when compared with the corresponding mock transfected cells (Fig. 5(c)). These findings suggested that inhibition of PP-2A activity by I_1^{PP2A} or I_2^{PP2A} alone is sufficient both to produce the abnormal hyperphosphorylation of tau as well as result in an increase in cell death.

Table 2
Dephosphorylation of hyperphosphorylated tau at various sites by PP-2A

	Phosphorylated (%)[a]
Control	100
Thr-231	68.5 ± 6.1
M4	59.2 ± 8.3
Ser-262	41.3 ± 6.1
12E8	19.3 ± 3.6
Ser-396	42.1 ± 3.9
PHF-1	38.1 ± 4.8

[a]Phosphorylation before treatment with PP-2A as 100%.

4. Discussion

The activity of PP-2A, a major phosphoseryl/phosphothreonyl protein phosphatase, is believed to be regulated by I_1^{PP2A} and I_2^{PP2A}. However, neither the structures nor the activities of these inhibitors from brain had been reported previ-

Table 3
Inhibition of PP-2A catalyzed dephosphorylation of tau at various sites by I_1^{PP2A}, I_2^{PP2A} or GST – I_1^{PP2A}

	Dephosphorylation (%)[a]		
	I_1^{PP2A}	I_2^{PP2A}	GST – I_1^{PP2A}
Thr 231	73.6 ± 12.6	58.7 ± 11.4	96.2 ± 10.4
M4	51.6 ± 7.8	24.6 ± 6.2	41.3 ± 5.0
Ser 262	38.2 ± 11.0	36.6 ± 6.1	39.8 ± 10.2
12E8	26.8 ± 5.1	37.1 ± 3.4	58.9 ± 10.1
Ser 396	48.6 ± 6.2	52.0 ± 3.8	68.1 ± 6.8
PHF-1	31.8 ± 4.6	43.0 ± 6.2	39.0 ± 4.8

[a]Dephosphorylation of tau by PP-2A in the absence of inhibitors as 100%.

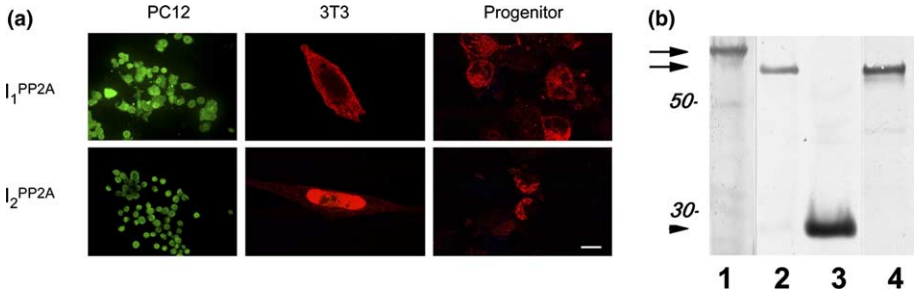

Fig. 4. Immunocytochemical staining of untransfected PC12 cells, and NIH 3T3 cells and human neural progenitor cells transiently transfected with $I_1^{PP\text{-}2A}$ – GFP or $I_2^{PP\text{-}2A}$ – GFP. (a) PC12 cells and at 24 h post-transfection, NIH3T3 cells and at 72 h post-transfection, progenitor cells were processed for indirect immunofluoresence. Scale bar: PC12 cells, 5 mm bar = 17.5 μm; NIH3T3 and neural progenitor cells, 5 mm bar = 6 μm. (b) Western blots of NIH 3T3 cells transiently transfected with mock plasmid I-GFP (lane 3) or $I_1^{PP\text{-}2A}$ – GFP *(lane 2 4) or* $I_2^{PP\text{-}2A}$ – GFP*(lane 1)*. The levels of GFP, $I_1^{PP\text{-}2A}$ and $I_2^{PP\text{-}2A}$ were visualized using monoclonal antibody to GFP (lanes 2 and 3), polyclonal antibodies, R-42089 to $I_1^{PP\text{-}2A}$ (lane 4) and R-42187 to $I_2^{PP\text{-}2A}$ (lane 1). $I_1^{PP\text{-}2A}$ – GFP or $I_2^{PP\text{-}2A}$ – GFP fusion proteins were detected in cells transfected with $I_1^{PP\text{-}2A}$ and $I_2^{PP\text{-}2A}$, respectively. Arrow shows GFP fusion $I_1^{PP\text{-}2A}$ or $I_2^{PP\text{-}2A}$ and arrowhead shows GFP.

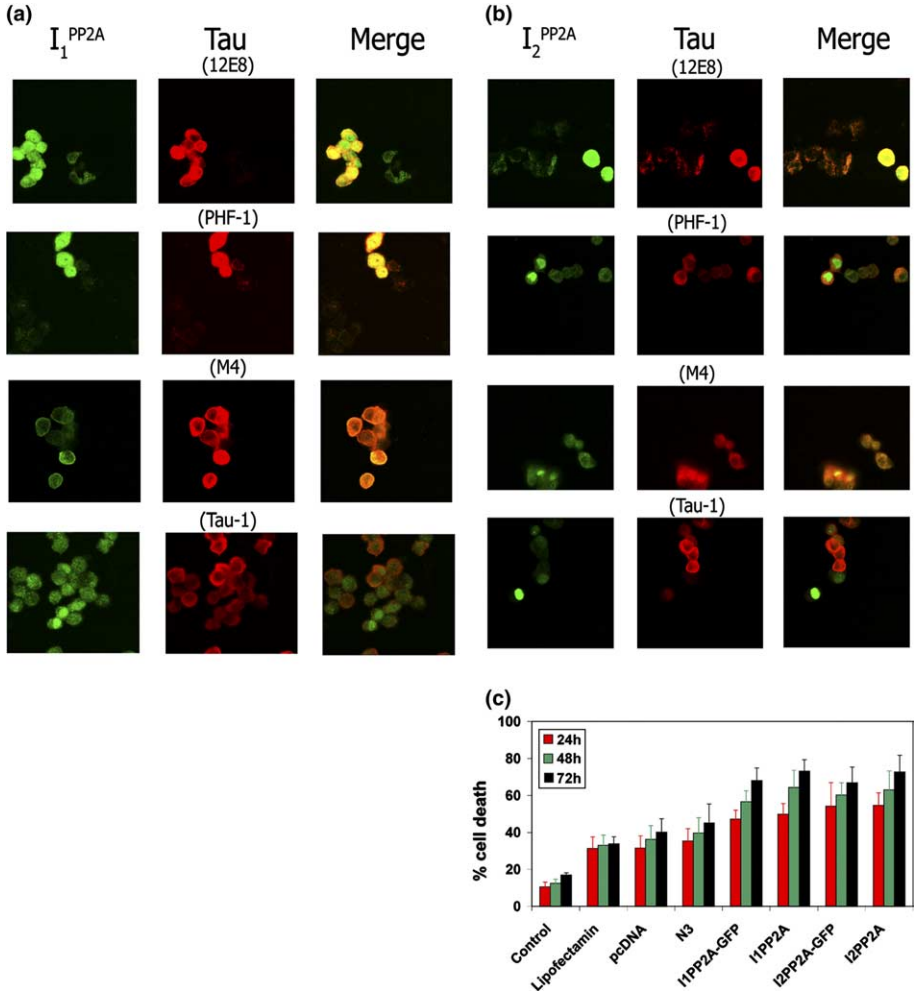

Fig. 5. Immunocytochemical staining and cell survival of tau_{441} stably transfected PC12 cells transiently transfected with I_1^{PP2A} *or* I_2^{PP2A}. (a,b) At 72 h post-transfection, cells were processed for double immunofluorescence using rabbit polyclonal antibody to I_1^{PP2A} or to I_2^{PP2A} and mouse monoclonal phosphodependent antibodies to tau as primary antibodies, followed by Oregon Green-labeled goat anti-rabbit (green; left panels), and cy3-labeled anti-mouse $(Fab^1)_2$ (red; middle panels) as secondary antibodies (1:1000; Jackson Immuno Research). Mock transfected cells not shown in this figure did not show any significant changes in the immunochemical staining with any of the above antibodies. (c) PC12 cells were transfected with I_1^{PP2A}, I_2^{PP2A}, N3 (mock) or pcDNA (mock) plasmids. At 24 (red bar), 48 (green bar) and 72 (black bar) hours post-transfection, the viability of cells was measured with the Cyto Tox-ONE™ Assay Kit (Promega). % cell death = (experimental – culture medium background)/(maximum LDH release – culture medium background). Differences between I_1^{PP2A} or I_2^{PP2A} and mock transfected cells (pcDNA or N3), $P < 0.05$.

ously. The present study shows for the first time the presence of I_1^{PP2A} and I_2^{PP2A} in human brain and demonstrates in a site specific manner the differential inhibition of PP-2A activity towards Alzheimer-like hyperphosphorylated tau by these two inhibitors. The data presented in this study also show that both I_1^{PP2A} and I_2^{PP2A} regulate the phosphorylation of tau and that overexpression of either one of these inhibitors is sufficient to cause call death. All these findings taken together raise an intriguing possibility that the abnormal hyperphosphorylation of tau produced by inhibition of PP-2A activity might be sufficient to produce neurodegeneration of the Alzheimer type.

The nucleotide sequences of cDNAs of I_1^{PP2A} and I_2^{PP2A} cloned from human brain were found to be identical to those reported previously for PHAP-I and human kidney I_1^{PP2A} [21,28] and SET/I_2^{PP2A} [21,27]. Northern blot analysis revealed the presence of three transcripts of 4.2, 3.7 and 2.2 kb for I_1^{PP-2A} in human brain and other tissues studied. In the case of I_2^{PP-2A}, the Northern blots showed two prominent transcripts of 2.7 and 1.9 kb and a third weak signal of 4.0 kb in human brain and other tissues examined. Employing the human brain cRNA probes of I_1^{PP2A} and I_2^{PP2A} from the present study, we found 3.7 and 2.1 kb I_1^{PP2A} and 2.9 and 2.0 kb I_2^{PP2A} transcripts in rat brain [31]. A previous study reported that I_2^{PP-2A} homologous gene, Set α, encoded two transcripts of 2.0 and 2.7 kb in adult mouse tissue [27]. It appears that in addition to the two transcripts corresponding to the rodent tissues, the human tissues contain novel larger transcripts of 4.2 kb I_1^{PP2A} and 4.0 kb I_2^{PP2A}. Whether more than one transcript observed in Northern blots code for a single protein and are due to different lengths of poly A tails or might represent alternatively spliced mRNAs coding for proteins of different sizes remains to be investigated both in the case of I_1^{PP2A} and I_2^{PP2A}.

PP-2A activity is compromised in AD brain [11,12] and has been speculated to be a cause of the hyperphosphorylation of tau and neurofibrillary degeneration. Amygdala and hippocampus are most affected by neurofibrillary changes in AD brain. In the present study, the hybridization of I_1^{PP2A} and I_2^{PP2A} probes in Northern blots of the various areas of the human brain was especially strong in amygdala and hippocampus among various brain regions. This finding raises an intriguing possibility of an involvement of these PP-2A inhibitors in Alzheimer neurofibrillary degeneration. Consistent with this scenario, in situ hybridization and immunohistochemical studies have shown a wide distribution and a co-localization of these inhibitors with PP-2A in mammalian brain, especially in pyramidal cell layers of CA1-3 regions and in the amygdaloid nucleus [31].

In non-transfected PC12 cells, endogenous I_1^{PP2A} was localized to cytoplasm and nucleus, whereas the endogenous I_2^{PP2A} was observed mostly in the nucleus. A similar subcellular localization of these inhibitors was observed by us in rat brain [31]. Both NIH3T3 and human neural progenitor cells could be successfully transfected with $I_1^{PP2A}-GFP$ and $I_2^{PP2A}-GFP$ fusion proteins. The overexpression of $I_1^{PP2A}-GFP$ was observed mostly in the cytoplasm, whereas that of $I_2^{PP2A}-GFP$ was seen mainly in the nucleus in both NIH3T3 and human neural progenitor cells. These immunocytological localizations of I_1^{PP2A} and I_2^{PP2A} are in agreement with the previous reports [28,30]. Studies on template activating factor-1 which is the same as I_2^{PP2A} have shown that on cleavage the amino-terminal half of the protein, which like the whole protein, also inhibits PP-2A activity, is both in the nuclear and cytoplasmic locations [30]. Thus, not only I_1^{PP2A} but also I_2^{PP2A} can modulate the PP-2A activity of the neuronal cytoplasmic pool where tau is localized.

Previously, it was reported that both I_1^{PP2A} and I_2^{PP2A} purified from bovine kidney inhibited the PP-2A-catalyzed dephosphorylation of [^{32}P]MBP, [^{32}P]histone H1, [^{32}P]pyruvate dehydrogenase complex and [^{32}P]phosphorylase A [19]. In the present study, recombinant I_1^{PP2A}, I_2^{PP2A}, and $GST-I_1^{PP2A}$ but not $GST-I_2^{PP2A}$ inhibited the PP-2A activity in vitro towards [^{32}P] hyperphosphorylated human brain tau_{352}. These findings showed for the first time that the PP-2A inhibitors can affect phosphorylation of tau, which is known to be regulated by PP-2A. The loss of PP-2A inhibitor activity in $GST-I_2^{PP2A}$ fusion protein was probably due to the unavailability of the inhibitor domain due to some conformational change induced in this protein. The Kis of the recombinant human brain I_1^{PP2A} and I_2^{PP2A} towards [^{32}P] hyperphosphorylated tau were 480 and 300 nM, respectively. These Ki values are about 10–20 fold higher than those reported previously towards other [^{32}P] substrates by inhibitors purified from bovine kidney. These differences might be both due to differences in the substrates and the sources of the inhibitors.

Tau in AD brain is abnormally hyperphosphorylated at multiple sites (see [44]). However, the phosphorylation of only some of these sites has been shown to affect the biological activity of tau; the phosphorylation of tau at Thr-231, Ser-262 and Ser-396 inhibits its binding to microtubules and their assembly. The present study revealed that I_1^{PP2A} and I_2^{PP2A} inhibited the PP-2A catalyzed dephosphorylation of tau differentially and with site-specific preference at these functionally important sites. While I_1^{PP2A} was more effective than I_2^{PP2A} in inhibiting the dephosphorylation of the PHF-1 site, the reverse appeared to be the case at the M4 site. In all the cases, the phospho-dependent monoclonal antibodies to tau distinguished better than the corresponding polyclonal antibodies the differences between the inhibition of the dephosphorylation by I_1^{PP2A} and I_2^{PP2A}.

The overexpression of I_1^{PP2A} or I_2^{PP2A} in PC12 cells revealed that these inhibitors regulated the phosphorylation of tau at several of the same sites, which are known to be abnormally hyperphosphorylated in AD and other tauopathies, and caused cell death. Like in vitro studies above in PC12 cells, the phosphorylation of tau at the PHF-1 site was more markedly increased in the I_1^{PP2A}-transfected cells than in the I_2^{PP2A}-transfected cells, suggesting a differential effect of the two inhibitors on the dephosphorylation of tau by PP-2A. A marked hyperphosphorylation of tau at the 12E8 site in both I_1^{PP2A}- and I_2^{PP2A}-transfected PC12 cells is probably both due to an inhibition of PP-2A activity as well as an increase in the activity of CaMKinase II, the activity of which is regulated by PP-2A [15]. CaMKinase II has been previously shown by us to be a major 12E8 site tau kinase in the brain [46]. Similarly, the increase in the phosphorylation of tau at the PHF-1, M4 and Tau-1 sites could be both due to inhibition of dephosphorylation by PP-2A as well as increase in the activities of one or more tau kinases, the activities of which are regulated by PP-2A [17,47].

In conclusion, I_1^{PP2A} and I_2^{PP2A}, the regulators of PP-2A activity, are present in mammalian brain. In human brain, the mRNAs of these two PP-2A inhibitors are especially

prominent in amygdala and hippocampus, the two areas of the brain most affected by neurofibrillary pathology. I_1^{PP2A} is localized in both cytoplasm and the nucleus, whereas I_2^{PP2A} is mostly seen in the nucleus. The recombinant human brain I_1^{PP2A} and I_2^{PP2A} are biologically active and can inhibit the PP-2A catalyzed dephosphorylation of the AD type abnormally hyperphosphorylated tau in vitro. I_1^{PP2A} and I_2^{PP2A} inhibit the dephosphorylation of tau in a site specific preference and differentially at some of the major abnormally phosphorylated sites seen in AD. Both I_1^{PP2A} and I_2^{PP2A} regulate the phosphorylation of tau at several of the sites abnormally hyperphosphorylated in AD and cause cell death. All these findings taken together suggest that the downregulation of I_1^{PP2A} and/or I_2^{PP2A} might inhibit abnormal hyperphosphorylation of tau and consequent neurofibrillary degeneration in AD and other tauopathies. We have previously shown that the restoration of the okadaic acid-inhibited PP-2A activity to the normal levels by memantine in rat hippocampal slices in culture inhibits the abnormal hyperphosphorylation of tau and neurodegeneration [47].

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References

[1] Grundke-Iqbal, I., Iqbal, K., Tung, Y.-C., Quinlan, M., Wisniewski, H.M. and Binder, L.I. (1986) Abnormal phosphorylation of the microtubule associated protein tau in Alzheimer cytoskeletal pathology. Proc. Natl. Acad. Sci. USA 83, 4913–4917.

[2] Iqbal, K., Grundke-Iqbal, I., Zaidi, T., Merz, P.A., Wen, G.Y., Shaikh, S.S., Wisniewski, H.M., Alafuzoff, I. and Winblad, B. (1986) Defective brain microtubule assembly in Alzheimer's disease. Lancet 2, 421–426.

[3] Iqbal, K., Grundke-Iqbal, I., Smith, A.J., George, L., Tung, Y.-C. and Zaidi, T. (1989) Identification and localization of a tau peptide to paired helical filaments of Alzheimer's disease. Proc. Natl. Acad. Sci. USA 86, 5646–5650.

[4] Lee, V.M., Balin, B.J., Otvos Jr., L. and Trojanowski, J.Q. (1991) A68: A major subunit of paired helical filaments and derivitized forms of normal tau. Science 251, 675–678.

[5] Alonso, A. del C., Zaidi, T., Novak, M., Grundke-Iqbal, I. and Iqbal, K. (2001) Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. Proc. Natl. Acad. Sci. USA 98, 6923–6928.

[6] Bancher, C., Brunner, C., Lassmann, H., Budka, H., Jellinger, K., Wiche, G., Seitelberger, F., Grundke-Iqbal, I., Iqbal, K. and Wisniewski, H.M. (1989) Accumulation of abnormally phosphorylated tau precedes the formation of neurofibrillary pathology in Alzheimer disease. Brain Res. 477, 90–99.

[7] Köpke, E., Tung, Y.-C., Shaikh, S., Alonso, A., Iqbal, K. and Grundke-Iqbal, I. (1993) Microtubule associated protein tau: abnormal phosphorylation of non-paired helical filament pool in Alzheimer disease. J. Biol. Chem. 268, 24374–24384.

[8] Alonso, A. del C., Zaidi, T., Grundke-Iqbal, I. and Iqbal, K. (1994) Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. Proc. Natl. Acad. Sci. USA 91, 5562–5566.

[9] Alonso, A. del C., Grundke-Iqbal, I. and Iqbal, K. (1996) Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. Nature Med. 2, 783–787.

[10] Alonso, A. del C., Grundke-Iqbal, I., Barra, H.S. and Iqbal, K. (1997) Abnormal phosphorylation of tau and mechanism of Alzheimer neurofibrillary degeneration: sequestration of MAP1 and MAP2 and the disassembly of microtubules by the abnormal tau. Proc. Natl. Acad. Sci. USA 94, 298–303.

[11] Gong, C.-X., Singh, T.J., Grundke-Iqbal, I. and Iqbal, K. (1993) Phosphoprotein phosphatase activities in Alzheimer disease brain. J. Neurochem. 61, 921–927.

[12] Gong, C.-X., Shaikh, S., Wang, J.-Z., Zaidi, T., Grundke-Iqbal, I. and Iqbal, K. (1995) Phosphatase activity towards abnormally phosphorylated: decrease in Alzheimer disease brain. J. Neurochem. 65, 732–738.

[13] Gong, C.-X., Lidsky, T., Wegiel, J., Zuck, L., Grundke-Iqbal, I. and Iqbal, K. (2000) The phosphorylation state of microtubule-associated protein tau is regulated by protein phosphatase 2A in mammalian brain. J. Biol. Chem. 275, 5534–5544.

[14] Tanaka, T., Zhong, J., Iqbal, K., Trenkner, E. and Grundke-Iqbal, I. (1998) The regulation of phosphorylation of tau in SY5Y neuroblastoma cells: the role of protein phosphatases. FEBS Lett. 426, 248–254.

[15] Bennecib, M., Gong, C.-X., Grundke-Iqbal, I. and Iqbal, K. (2000) Role of protein phosphatase-2A and -1 in the regulation of GSK-3, cdk5 and cdc2 and the phosphorylation of tau in rat forebrain. FEBS Lett. 485, 87–93.

[16] Bennecib, M., Gong, C.-X., Grundke-Iqbal, I. and Iqbal, K. (2001) Inhibition of PP-2A upregulates CaMKII in rat forebrain and induces hyperphosphorylation of tau at Ser 262/356. FEBS Lett. 490, 15–22.

[17] Pei, J.-J., Gong, C.-X., An, W.-L., Winblad, B., Cowburn, R.F., Grundke-Iqbal, I. and Iqbal, K. (2003) 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. 163, 591–607.

[18] Oliver, C.J. and Shenolikar, S. (1998) Physiologic importance of protein phosphatase inhibitors. Front. Biosci. 3, 961–972.

[19] Li, M., Guo, H. and Damuni, Z. (1995) Purification and characterization of two potent heat-stable inhibitor protein phosphatase 2A from bovine kidney. Biochemistry 34, 1988–1996.

[20] Li, M., Makkinje, A. and Damuni, Z. (1996) Molecular identification of I_1^{PP-2A}, a novel potent inhibitor of protein phosphatase 2A. Biochemistry 35, 6998–7002.

[21] Li, M., Makkinje, A. and Damuni, Z. (1996) The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. J. Biol. Chem. 271, 11059–11062.

[22] Al-Murrani, S.W.K., Woodgett, J.R. and Damuni, Z. (1999) Expression of I_2^{PP-2A}, an inhibitor of protein phosphatase 2A, induces c-Jun and AP-1 activity. Biochem. J. 341, 293–298.

[23] Katayose, Y., Li, M., Al-Murrani, S.W.K., Shenolikar, S. and Damuni, Z. (2000) Protein phosphatase 2A inhibitors, I_1^{PP-2A} and I_2^{PP-2A}, associate with and modify the substrate specificity of protein phosphatase 1. J. Biol. Chem. 275, 9209–9214.

[24] Ulitzur, N., Rancano, C. and Pfeffer, S.R. (1997) Biochemical characterization of mapmodulin, a protein that binds microtubule-associated proteins. J. Biol. Chem. 272, 30577–30582.

[25] Chen, T.-H., Brody, J.R., Romantsev, F.E., Yu, J.G., Kayler, A.E., Voneiff, E., Kuhajda, F.P. and Pasternack, G.R. (1996) Structure of pp32, an acidic nuclear protein which inhibits oncogene-induced formation of transformed foci. Mol. Biol. Cell. 7, 2045–2056.

[26] Matsuoka, K., Taoka, M., Satosawa, N., Nakayama, H., Ichimura, T., Takahashi, N., Yamakuni, T., Song, S.-Y. and Isobe, T. (1994) A nuclear factor containing the leurine-rich repeats expressed in murine cerebellar neurons. Proc. Natl. Acad. Sci. USA 91, 9670–9674.

[27] von Lindern, M., van Baal, S., Wiegant, J., Raap, A., Hagemeijer, A. and Grosveld, G. (1992) 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. 12, 3346–3355.

[28] Vaesen, M., Barnikol-Watanabe, S., Gotz, H., Awni, L.A., Cole, T., Zimmermann, B., Kratzin, H.D. and Hilschmann, N. (1994) Purification and characterization of two putative HLF class II associated proteins: PHAPI and PHAPII. Biol. Chem. Hoppe-Seyler 375, 113–126.
[29] Saito, S., Miyaji-Yamaguchi, M., Shimoyama, T. and Nagata, K. (1999) Functional domains of template-activating factor-1 as a protein phosphatase 2A inhibitor. Biochem. Biophys. Res. Commun. 259, 471–475.
[30] Nagata, K., Saito, S., Okuwaki, M., Kawase, H., Furuya, A., Kusano, A., Hanai, N., Okuda, A. and Kikuchi, A. (1998) Cellular localization and expression of template-activating factor 1 in different cell types. Exp. Cell Res. 240, 271–281.
[31] Tanimukai, H., Grundke-Iqbal, I. and Iqbal, K. (2004) Inhibitors of protein phosphatase-2A: topography and subcellular localization. Mol. Brain. Res. 126, 146–156.
[32] Greenberg, S.G., Davies, P., Schein, J.D. and Binder, L.I. (1992) Hydrofluoric acid-treated tau PHF proteins display the same biochemical properties as normal tau. J. Biol. Chem. 26, 564–569.
[33] Otvos Jr., L., Feiner, L., Lang, E., Szendrei, G.I., Goedert, M. and Lee, V.M.Y. (1994) Monoclonal antibody PHF-1 recognizes tau protein phosphorylated at serine residues 396 and 404. J. Neurosci. Res. 39, 669–673.
[34] Binder, L.I., Frankfurter, A. and Rebhun, L.I. (1985) The distribution of tau in the mammalian central nervous system. J. Cell Biol. 101, 1371–1378.
[35] Szendrei, G.I., Lee, V.M.Y. and Otvos Jr., L. (1993) Recognition of the minimal epitope of monoclonal antibody Tau-1 depends upon the presence of a phosphate group but not its location. J. Neurosci. Res. 34, 243–249.
[36] Hasegawa, M., Watanabe, A., Takio, K., Suzuki, M., Arai, T., Titani, K. and Ihara, Y. (1993) 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. 60, 2068–2077.
[37] Grundke-Iqbal, I., Vorbrodt, A.W., Iqbal, K., Tung, Y.C., Wang, G.P. and Wisniewski, H.M. (1988) Microtubule-associated polypeptides tau are altered in Alzheimer paired helical filaments. Mol. Brain Res. 4, 43–52.
[38] Tatebayashi, Y., Iqbal, K. and Grundke-Iqbal, I. (1999) Dynamic regulation of expression and phosphorylation of tau by fibroblast growth factor 2 in neural progenitor cells from adult rat hippocampus. J. Neurosci. 19, 5245–5254.
[39] Ziemnicka-Kotula, D., Xu, J., Gu, H., Potempska, A., Kim, K.S., Jenkins, E.C., Trenkner, E. and Kotula, L. (1998) Identification of a candidate spectrin SH3 binding protein suggests a general mechanism of association of tyrosine kinases with the spectrin-based membrane skeleton. J. Biol. Chem. 273, 13681–13692.
[40] Zheng-Fischhofer, Q., Biernat, J., Mandelkow, E.M., Illenberger, S., Godemann, R.L. and Mandelkow, E. (1998) Sequential phosphorylation of Tau by glycogen synthase kinase-3beta and protein kinase A at Thr212 and Ser214 generates the Alzheimer-specific epitope of antibody AT100 and requires a paired-helical-filament-like conformation. Eur. J. Biochem. 252, 542–552.
[41] Cohen, P., Alemany, S., Hemmings, B.A., Resink, T.J., Stralfors, P. and Tung, H.Y. (1988) Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. Methods Enzymol. 159, 390–408.
[42] Huang, K.P. and Robison, J.C. (1976) A rapid and sensitive assay method for protein kinase. Anal. Biochem. 72, 593–599.
[43] Khatoon, S., Grundke-Iqbal, I. and Iqbal, K. (1992) Brain levels of microtubule associated protein tau are elevated in Alzheimer's disease brain: a radioimmunoslot-blot assay for nanograms of the protein. J. Neurochem. 59, 750–753.
[44] Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K. and Ihara, Y. (1995) Proline-directed and non-proline-directed phosphorylation of PHF-tau. J. Biol. Chem. 270, 823–829.
[45] Sengupta, A., Kabat, J., Novak, M., Wu, Q., Grundke-Iqbal, I. and Iqbal, K. (1998) Phosphorylation of tau at both Thr 231 and Ser 262 is required for maximal inhibition of its binding to microtubules. Arch. Biochem. Biophys. 357, 299–309.
[46] Sironi, J.J., Yen, S.H., Gondal, J.A., Wu, Q., Grundke-Iqbal, I. and Iqbal, K. (1998) Ser-262 in human recombinant tau protein is a markedly more favourable site for phosphorylation by CaMKII than PKA or PhK. FEBS Lett 436, 471–475.
[47] Li, L., Sengupta, A., Haque, N., Grundke-Iqbal, I. and Iqbal, K. (2004) Memantine inhibits and reverses the Alzheimner type abnormal hyperphosphorylation of tau and associated neurodegeneration. FEBS Lett 566, 261–269.