Cytoplasmic Retention of Protein Phosphatase 2A Inhibitor 2 (I$_2$$^{PP2A}$) Induces Alzheimer-like Abnormal Hyperphosphorylation of Tau*

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**Background:** In Alzheimer brain, I$_2$$^{PP2A}$ is translocated from the neuronal nucleus to the cytoplasm and promotes abnormal hyperphosphorylation of Tau.

**Results:** Inactivation of nuclear localization signal (NLS) causes retention of I$_2$$^{PP2A}$ in the cell cytoplasm, where it promotes Tau hyperphosphorylation by affecting PP2A signaling.

**Conclusion:** Retention of I$_2$$^{PP2A}$ in cell cytoplasm results in Tau hyperphosphorylation.

**Significance:** The study provides potential tools for investigating Tau-based therapeutics.

Abnormal hyperphosphorylation of Tau leads to the formation of neurofibrillary tangles, a hallmark of Alzheimer disease (AD), and related tauopathies. The phosphorylation of Tau is regulated by protein phosphatase 2A (PP2A), which in turn is modulated by endogenous inhibitor 2 (I$_2$$^{PP2A}$). In AD brain, I$_2$$^{PP2A}$ is translocated from neuronal nucleus to cytoplasm, where it inhibits PP2A activity and promotes abnormal phosphorylation of Tau. Here we describe the identification of a potential nuclear localization signal (NLS) in the C-terminal region of I$_2$$^{PP2A}$ containing a conserved basic motif, **179**RK**181**, which is sufficient for directing its nuclear localization. The current study further presents an inducible cell model (Tet-Off system) of AD-type abnormal hyperphosphorylation of Tau by expressing I$_2$$^{PP2A}$ in which the NLS was inactivated by **179**RK**181** → AAA along with **169**KR**169** → AA mutations. In this model, the mutant NLS (mNLS)-I$_2$$^{PP2A}$ (I$_2$$^{PP2A}$AA-AAA) was retained in the cell cytoplasm, where it physically interacted with PP2A and inhibited its activity. Inhibition of PP2A was associated with the abnormal hyperphosphorylation of Tau, which resulted in microtubule network instability and neurite outgrowth impairment. Expression of mNLS-I$_2$$^{PP2A}$ activated CAMKII and GSK-3β, which are Tau kinases regulated by PP2A. The immunoprecipitation experiments showed the direct interaction of I$_2$$^{PP2A}$ with PP2A and GSK-3β but not with CAMKII. Thus, the cell model provides insights into the nature of the potential NLS and the mechanistic relationship between I$_2$$^{PP2A}$-induced inhibition of PP2A and hyperphosphorylation of Tau that can be utilized to develop drugs preventing Tau pathology.

Abnormal hyperphosphorylation and aggregation of microtubule-associated protein Tau into paired helical filaments/neurofibrillary tangles is a hallmark of neurodegenerative tauopathies, including Alzheimer disease (AD),2 frontotemporal dementias, tangle-only dementia, Pick disease, argyrophilic grain disease, progressive supranuclear palsy, corticobasal degeneration, Guam parkinsonism dementia complex, dementia pugilistica, and traumatic brain injury/chronic traumatic encephalopathy (1, 2). Tau is a highly soluble and unfolded protein that stabilizes the assembly of microtubules. However, Tau abnormal hyperphosphorylation negatively regulates its microtubule binding, dissociation of Tau from microtubules, and sequestration of normal Tau and other microtubule-associated proteins, causing its aggregation, breakdown of the microtubule network, and eventually cell death (3–7). Studies have shown that Tau normally contains 2–3 mol of phosphate/mol of Tau, but it is 3–4-fold more phosphorylated in AD brain (8). The number of neurofibrillary tangles correlates with progressive neuronal dysfunction, synaptic loss, and functional decline in humans and transgenic mouse models (9–12). Although the triggering mechanism leading to Tau hyperphosphorylation is yet to be clarified, it is well recognized that an imbalanced regulation in Tau protein kinases and phosphatases can directly cause AD-like Tau hyperphosphorylation (1).

Protein phosphatase 2A (PP2A) is the major brain Tau phosphatase that regulates Tau phosphorylation, both directly and indirectly regulating the activities of several Tau kinases, which include glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase 5, Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII), MAPK (MEK1/2), ERK1/2, and protein kinase A (1). PP2A accounts for ∼70% of Tau phosphatase activity in the human brain, regulating nearly all Tau phosphorylation sites (13, 14), and its activity is compromised in the AD brain (15, 16). The activity of PP2A is regulated by two endogenous inhibitors, I$_1$$^{PP2A}$ and I$_2$$^{PP2A}$ (17, 18), along with post-translational...
modifications, including phosphorylation at Tyr\textsuperscript{307} (19), which inactivates PP2A, and methylation at Leu\textsuperscript{609} (20), which activates PP2A. I\textsubscript{2}\textsuperscript{PP2A}, also known as SET\textalpha{}, TAF-1\textbeta{}, and PHAPII, is a nuclear protein that regulates cell cycle (21), cell proliferation (22), and cell motility (23). Moreover, I\textsubscript{2}\textsuperscript{PP2A} controls gene transcription (24) by regulating histone acetylation (25) and is possibly involved in neuronal apoptotic pathways in AD brain (26). We have shown previously that both the mRNA and protein expressions of I\textsubscript{2}\textsuperscript{PP2A} are up-regulated, and I\textsubscript{2}\textsuperscript{PP2A} is selectively cleaved at Asn\textsuperscript{175} into two fragments, N-terminal and C-terminal fragments (I\textsubscript{2}\textsuperscript{NTF} and I\textsubscript{2}\textsuperscript{CTF}), by asparaginyl endopeptidase and is translocated from its primary localization in the nucleus to the cytoplasm (27–29). Because PP2A and Tau are localized in the cytoplasm, the increased neuronal cytoplasmic residing of I\textsubscript{2}\textsuperscript{PP2A} in the AD brain leads to the inhibition of PP2A, Tau hyperphosphorylation, and formation of neurofibrillary tangles (28, 30, 31). More recently, we have shown that I\textsubscript{2}\textsuperscript{CTF} alone sufficiently induces Tau pathology and cognitive impairment in a non-transgenic rat model of AD (32). As a nuclear protein, I\textsubscript{2}\textsuperscript{PP2A} requires a specific sequence called the nuclear localization signal (NLS) to be targeted to the nucleus. Indeed, the NLS of I\textsubscript{2}\textsuperscript{PP2A} has been reported at 168KRSSQTFQNASKRR\textsuperscript{181}, and targeted expression of I\textsubscript{2}\textsuperscript{PP2A} is found to be associated with neuronal death (33). Therefore, cytoplasmic withholding of I\textsubscript{2}\textsuperscript{PP2A} regulated by NLS and its association with PP2A appear to be the key upstream molecular mechanism controlling the abnormal phosphorylation of Tau, which is considered one of the earliest signs of neuronal degeneration that precede Tau aggregation/neurofibrillary tangles in AD and related tauopathies.

In this study, we first show that the functional NLS of I\textsubscript{2}\textsuperscript{PP2A} is localized at 179RKR\textsuperscript{181}, which controls the shuttle of I\textsubscript{2}\textsuperscript{PP2A} between the cellular nucleus and cytoplasm. Second, we report a PC12 stably expressing human Tau\textsubscript{441} cell line that allows the inducible expression of mNLS-I\textsubscript{2}\textsuperscript{PP2A} (168KR\textsuperscript{169} → AA/179RKR\textsuperscript{181} → AAA) based on the Tet-Off system. Employing this cell line, we found that cytoplasmic accumulation of I\textsubscript{2}\textsuperscript{PP2A} is associated with inhibition of PP2A activity and activation of Tau kinases regulated by PP2A, hyperphosphorylation of Tau, and promotion of microtubule instability. The mNLS-I\textsubscript{2}\textsuperscript{PP2A} cell model can be useful in screening of therapeutic drugs for AD and other tauopathies.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors**—To create a wild type I\textsubscript{2}\textsuperscript{PP2A} response vector in the Tet-Off system, PCR was performed using primers based on the WT human I\textsubscript{2}\textsuperscript{PP2A} sequence (GenBank\textsuperscript{TM} number AY349172): sense primer, 5′-ACATCGGATCCATGCGCGCCGGCCGCGCTGGGC-3′ (BamHI-I\textsubscript{2}\textsuperscript{PP2A}, N-terminal positions 1–24 of human I\textsubscript{2}\textsuperscript{PP2A} and a BamHI site); antisense primer, 5′-ATAAGAATTCGCGGCGCTAGTGGGTAGTCATCCTTCCTCCTTCCCTC-3′ (C-terminal 805–834 of human I\textsubscript{2}\textsuperscript{PP2A} and an HA tag plus NotI site). The PCR product was digested with BamHI and NotI and ligated into a pTRE2hyg vector previously digested with BamHI-N-I\textsubscript{2}\textsuperscript{PP2A} mutant; sense primer 5′-GAGATCCATGTCGGCGCCGGCGCCGCGCTGGGC-3′ (BamHI-I\textsubscript{2}\textsuperscript{PP2A}, N-terminal positions 1–24 of human I\textsubscript{2}\textsuperscript{PP2A} and a BamHI site) and antisense primer 5′-GGTAAAGAGCTCTCTTGATCTCGGTACTGGCGCCGCGCTGGGC-3′ (reverse sequence 510–573 of human I\textsubscript{2}\textsuperscript{PP2A} mutant); sense primer 5′-ATTCAAAAACCGAGATATAGAGGAGAAGAACATCGTATGGGTAGTCATCCTTCCTCCTATCTCTCCTC-3′ (C-terminal 805–834 of human I\textsubscript{2}\textsuperscript{PP2A} and an HA tag plus NotI site) were individually incubated with pGEX-6P-1-I\textsubscript{2}\textsuperscript{PP2A} as a template in the first PCR. The PCR products were gel-purified, combined, and incubated with BamHI-N-I\textsubscript{2}\textsuperscript{PP2A} and NotI-C-I\textsubscript{2}\textsuperscript{PP2A} primers to synthesize full-length I\textsubscript{2}\textsuperscript{PP2A} RKR\textsuperscript{179} → AAA cDNA in the second PCR. The resulting product was digested with NotI and BamHI and inserted into NotI and BamHI sites of pTRE2hyg to generate the pTRE2hyg I\textsubscript{2}\textsuperscript{PP2A} RKR\textsuperscript{179} → AAA mutation. pTRE2hyg I\textsubscript{2}\textsuperscript{PP2A} mutants were generated by the same procedure.

**Cell Culture, Transfection, and Differentiation**—COS-7 cells (obtained from ATCC (Manassas, VA) were grown in 25-cm\textsuperscript{2} flasks at 37 °C, containing 5% CO\textsubscript{2} in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum. To investigate the localization I\textsubscript{2}\textsuperscript{PP2A} and its mutants, cells were plated on 4-well Lab-Tek II Chamber Slides coated with poly-L-lysine (Naige Nunc International, Naperville, IL) and transfected with expression plasmids using FuGENE 6 (Roche Applied Science).

In order to generate a stable cell line of Tau\textsubscript{441}/I\textsubscript{2}\textsuperscript{PP2A} or its mutants, PC12 pheochromocytoma cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% heat-inactivated horse serum plus 5% fetal calf serum. Cells were co-transfected with pCI-neoTau\textsubscript{441}, pTet-Off, and pTRE2hyg (vector) or pTRE2hyg containing human I\textsubscript{2}\textsuperscript{PP2A}WT, I\textsubscript{2}\textsuperscript{PP2AA}, and I\textsubscript{2}\textsuperscript{PP2AA-AAA} tagged with HA using Lipofectamine 2000 (Invitrogen). The pTet-Off vector contains the neomycin resistance gene as a selectable marker. Drug selection was carried out for 2 weeks by seeding 1 × 10\textsuperscript{5} cells/well in 96-well tissue culture plates (Nunc, Roskilde, Denmark) containing the same media supplemented with 400 μg/ml G418 (Sigma) and 100 μg/ml hygromycin B (EMD Biosciences). Among the G418-hygromycin-resistant clones, 20 clones were selected randomly and transferred into 6-well tissue culture plates (Nunc) for cell expansion in the presence of 200 μg/ml G418 and 100 μg/ml hygromycin. Overexpression of I\textsubscript{2}\textsuperscript{PP2A} has been reported to be associated with cellular death (26, 30). We therefore maintained cells at a low dose of doxycycline (Dox; Sigma) (100 ng/ml) to keep a low level of I\textsubscript{2}\textsuperscript{PP2A} expression. For the present study, cells were grown without Dox for 72–96 h to allow the expression of I\textsubscript{2}\textsuperscript{PP2A}. The Tau\textsubscript{441}/I\textsubscript{2}\textsuperscript{PP2A} cell lines were screened by Western blot analysis of HA expression in the presence or absence of 1 μg/ml Dox. For studying the effect of mNLS-I2PP2A-induced hyperphosphorylation of tau on the number and the length of neurites, stably
transfected PC12 cells were differentiated into neurons by adding 100 ng/ml NGF to the culture medium for 5 days.

Subcellular Fractionation—Cytoplasmic and nuclear fractions were prepared from cells as described previously with minor modifications (29). Briefly, the cells were scraped and washed with cold PBS, and centrifuged at 200 × g for 7 min. The pellet was resuspended in lysis buffer comprising 50 mM Tris-HCl, pH 7.4, 0.32 mM sucrose, 150 mM NaCl, 10% glycerol, 1 mM sodium vanadate, 50 mM sodium fluoride, 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and homogenized for 1 min on ice using a Potter-Elvehjem homogenizer set to 600 rpm with 5–10 gentle stokes. The lysate was then inspected, and if intact cells were still evident, the homogenization was repeated. The cell lysate was centrifuged at 750 × g for 10 min in a swinging bucket rotor. The supernatant was kept, and the pelat was resuspended with half of the initial volume of the lysis buffer with 3–4 strokes and centrifuged as above. The second supernatant was combined with the previous one, and the pelat was resuspended with the same buffer and saved as the nuclear fraction. The pooled supernatant was centrifuged at 100,000 × g for 1 h, and the resulting supernatant was saved as the cytosolic fraction. After protein measurement, the samples were boiled in Laemmli’s buffer and used for Western blots.

PP2A Activity Assay—PP2A activity was assayed in cell lysates using the phosphatase ELISA described previously (29). Briefly, 96-well plates were precoated with a 17-aa phosphopeptide corresponding to Tau aa 194–207 in which Ser199 was replaced with phosphoserine and to which KKK were added at the C terminus (coating buffer, 35 mM NaHCO3). After overnight blocking with a protein-free blocking solution (Pierce), the enzymatic reaction was performed by adding 2 μg of cell lysate protein in 60 μl of reaction buffer (50 mM Tris-HCl, pH 7.0, 2 mM MnCl2, 0.1 mg/ml BSA, 20 mM β-mercaptoethanol) at 30 °C for 30 min (in the presence or absence of 15 mM of okadaic acid) in a moist chamber. The reaction was stopped by adding 150 μl of Pierce blocking solution containing 50 mM NaF, followed by an overnight incubation with monoclonal antibody Tau-1, which recognizes Tau unphosphorylated at Ser198, Ser199, or Ser202 (30). HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) were treated with antigen retrieval buffer containing 7 mM citric acid and 10 mM sodium citrate in microwave for 2 min followed by primary antibody treatment. The primary antibodies used in the indicated dilutions were as follows: anti-HA (1:3000, Sigma-Aldrich), anti-PP2Ac (R123d, 1:300), anti-PP2Ac (1483, 1:200) (29), anti-α-tubulin (DM1A, 1:2000; Sigma), and anti-GSK-3β (1:300; Cell Signaling) overnight at 4 °C. An Alexa-488/555-conjugated appropriate combination of anti-mouse/rabbit secondary antibody (1:50; Jackson Laboratory) was used for fluorescent labeling. The cells on slides were rinsed with PBS, mounted with Fluorogel (Electron Microscopy Sciences, Hatfield, PA), and examined under a laser confocal microscope (Nikon Eclipse 90i, Melville, NY). For quantitative analysis, the images were captured using the ×40 objective, and the antibody staining was quantified by measuring mean pixel intensity with the software Image Pro-Plus version 5.0 (Media Cybernetics, Silver Spring, MD), as described previously (39, 40). Each experimental condition was calculated using the average number of 5 fields/well for 3–4 wells. The numbers of cells bearing neurites and the length of

Cell Model of Tau Hyperphosphorylation

Immunofluorescent Staining—Histopathologically confirmed AD and age-matched control brain paraffin sections (Sun Health Research Institute, Brain Donation Program, Sun City, AZ) were treated with antigen retrieval buffer containing 7 mm citric acid and 10 mm sodium citrate in microwave for 2 min followed by primary antibody treatment. The primary antibodies used in the indicated dilutions were as follows: anti-α-Tau (1:3000, Sigma-Aldrich), anti-PP2Ac (R123d, 1:300), anti-PP2Ac (1483, 1:200) (29), anti-α-tubulin (DM1A, 1:2000; Sigma), and anti-GSK-3β (1:300; Cell Signaling) overnight at 4 °C. An Alexa-488/555-conjugated appropriate combination of anti-mouse/rabbit secondary antibody (1:50; Jackson Laboratory) was used for fluorescent labeling. The cells on slides were rinsed with PBS, mounted with Fluorogel (Electron Microscopy Sciences, Hatfield, PA), and examined under a laser confocal microscope (Nikon Eclipse 90i, Melville, NY). For quantitative analysis, the images were captured using the ×40 objective, and the antibody staining was quantified by measuring mean pixel intensity with the software Image Pro-Plus version 5.0 (Media Cybernetics, Silver Spring, MD), as described previously (39, 40). Each experimental condition was calculated using the average number of 5 fields/well for 3–4 wells. The numbers of cells bearing neurites and the length of
the neurites were analyzed by 25–30 randomly selected NGF-differentiated PC12 cells using ImageJ software, as described previously (41).

Statistical Analysis—The data were analyzed with Student’s t test when comparing means between two groups or with one-way analysis of variance plus Bonferroni post hoc multiple comparison test to compare mean values across multiple treatment groups. In all cases, a probability statistic \( p < 0.05 \) was taken to indicate significance. All data are expressed throughout as means ± S.E.

RESULTS

Mutant NLS-I\(_{2}\)PP2A Translocates to the Cell Cytoplasm from the Nucleus and Inhibits PP2A Activity—We previously reported that I\(_{2}\)PP2A is translocated from neuronal nucleus to the cytoplasm, co-localizes with PP2A, and promotes abnormal hyperphosphorylation of Tau by inhibiting PP2A in AD brain (27, 28), suggesting the critical role of I\(_{2}\)PP2A translocation and its association with PP2A in the pathogenesis of AD. Because the NLS of proteins plays an important role in facilitating the nuclear translocation, we focused on the 168KRSSQTQNKASRAR181 region of I\(_{2}\)PP2A, as described by Qu et al. (33). We further carried out multiple alignments of I\(_{2}\)PP2A sequence among different species and found that 178RKR181 emerged as the most conserved sequence, suggesting its important functional implication (Fig. 1A). To identify the critical region of I\(_{2}\)PP2A NLS, we generated various mutants of this protein (Fig. 1B) and overexpressed them in COS7 cells transiently. Confocal microscopy analysis of their subcellular localization revealed that the mutants I\(_{2}\)PP2A AAA and I\(_{2}\)PP2AA AA-AAA were

![Image](https://example.com/image.png)

**FIGURE 1. Identification of nuclear localization signal of I\(_{2}\)PP2A.** A, the structural similarities between human, chimpanzee, pig, bovine, rat, mouse, and Drosophila were analyzed to determine the conservation and homology of potential I\(_{2}\)PP2A NLS among various species. B, schematic diagram of the constructs of I\(_{2}\)PP2A and its NLS mutants employed to study their intracellular localization. C, photomicrographs of COS7 cells transiently transfected with vector (vec), HA-tagged human I\(_{2}\)PP2A (I\(_{2}\)PP2A WT), and its NLS mutants (I\(_{2}\)PP2AAA, I\(_{2}\)PP2AAAA, and I\(_{2}\)PP2AAA-AAA) for the identification of critical regions of I\(_{2}\)PP2A required for its translocation from the cell nucleus to the cytoplasm. Following 48 h of transfection with HA-tagged I\(_{2}\)PP2A (WT and NLS mutants), COS7 cells were subjected to immunocytochemical staining with anti-HA, and TOPRO-3 was used for nuclear staining. Mutations at amino acid residues 178–181 markedly increased the cytoplasmic retention of I\(_{2}\)PP2A. D, COS7 cells transiently transfected with R179A-I\(_{2}\)PP2A and K180A-I\(_{2}\)PP2A, followed by immunostaining with anti-HA (I\(_{2}\)PP2A). Immunofluorescence images indicate that lysine 180 is required for I\(_{2}\)PP2A nuclear localization (i.e. NLS). Scale bar, 50 μm.
present in a diffuse manner throughout the cells, whereas the wild type \(I_2^{PP2A}\) and \(I_2^{PP2A-AAA}\) were localized mainly in the nuclei (Fig. 1C), but low cytoplasmic staining can also be observed at higher microscopic gain (data not shown), suggesting that the \(178^{RKR}\) motif is required for the nuclear localization of \(I_2^{PP2A}\). We further observed that mutation of \(Lys^{180}\) to Ala alone confined the \(I_2^{PP2A}\) into the cytoplasm of COS7 cells (Fig. 1D).

Next we evaluated whether translocation of \(I_2^{PP2A}\) is associated with inhibition of PP2A activity. As expected, total cell lysates from all transfections of \(I_2^{PP2A}\) constructs inhibited PP2A activity significantly, independent of their cellular localization (Fig. 2A). We found similar expressions of endogenous and various constructs of HA-tagged exogenous \(I_2^{PP2A}\) (Fig. 2, B and C). In order to investigate the effect of the cytoplasmic localization of \(I_2^{PP2A}\) on PP2A activity, we fractionated the total cell lysate into cytosolic and nuclear fractions where histone-3 and \(\beta\)-actin were used as markers for nuclear and cytosolic fractions, respectively (Fig. 2, D–G). When we assayed PP2A activity with cytosolic fractions, only NLS mutants \(I_2^{PP2A-AAA}\) and \(I_2^{PP2A-AA-AAA}\) significantly inhibited the activity (Fig. 2H). These results suggest that the translocation of \(I_2^{PP2A}\) from neuronal nucleus to cytosol is the critical step in inhibition of PP2A activity.

**Direct Interaction of PP2Ac with Translocated mNLS-I\(_2^{PP2A}\) Leads to Inhibition of PP2A Activity—Abnormal hyperphosphorylation of Tau is considered a key event leading to neurofibrillary tangle formation, which correlates with dementia in AD (42, 43). Based on the present finding that translocated mutant NLS-I\(_2^{PP2A}\) inhibits PP2A activity, the major phosphatase in the brain, we developed a Tet-Off cellular model of AD-type abnormal hyperphosphorylation of Tau by stably overexpressing HA-tagged mNLS-I\(_2^{PP2A}\) (I\(_2^{PP2A-AA-AAA}\)) along with human Tau \(\Delta_{344}\) in PC12 cells. We also developed stably transfected cell lines for vector and \(I_2^{PP2A-WT}\) in an identical manner. To validate this model, we first determined the PP2A activity in the cytosolic fraction of the cell lines. PP2A activity was significantly decreased in mNLS-I\(_2^{PP2A}\) cells as compared with controls (vec) (Fig. 3A). PP2A activity was also decreased by \(I_2^{PP2A-WT}\) but did not reach statistical significance (Fig. 3A). We then characterized the methylation sensitivity of PP2Ac antibodies used in the present study by treating PC12 cell lysates with NaOH, which causes the demethylation of the PP2Ac pool (34). We found that R123d and BD PP2Ac antibodies recognize total PP2Ac, whereas 1D6 recognizes the demethylated PP2Ac pool (Fig. 3B). Next, we examined whether translocated mNLS-I\(_2^{PP2A}\) can bind to PP2Ac and thus inhibit its activity. To study the interaction between PP2Ac and mNLS-I\(_2^{PP2A}\), the cytosolic fraction of PC12 cell lysates was also used to immunoprecipitate endogenous PP2AC, using rabbit polyclonal antibody R123d to PP2Ac. Western blots developed with HA antibody clearly showed significantly higher interaction between \(I_2^{PP2AA-AA-AAA}\) and PP2Ac as compared with \(I_2^{PP2A-WT}\) (Fig. 3, C and D). It is also evident that \(I_2^{PP2A-WT}\) also co-immunoprecipitated with cytosolic PP2Ac, possibly caused by the overexpression of the transgene. To further confirm these findings, we carried out immunofluorescence analysis with stably transfected cell lines using antibodies to PP2Ac (R123d) and \(I_2^{PP2A}\) (HA). A diffuse pattern of \(I_2^{PP2A}\) staining predominantly localized to the nucleus was observed in \(I_2^{PP2A-WT}\) cells (Fig. 3E). In contrast, PP2AC and \(I_2^{PP2A}\) were found to co-localize in the cytoplasmic compartment in mNLS-I\(_2^{PP2A}\) (I\(_2^{PP2A-AA-AAA}\)) cells (Fig. 3E). These findings suggest that the direct physical interaction of NLS mutant-I\(_2^{PP2A}\) with PP2A catalytic subunit could be involved in PP2A inhibition.

**Effect of mNLS-I\(_2^{PP2A}\) on Tau Phosphorylation and Neurite Outgrowth—**PP2A is the most implicated protein phosphatase in regulating Tau phosphorylation because it accounts for more than ~70% of Tau dephosphorylation activity (13). Inhibition of PP2A activity has been reported to be associated with abnormal hyperphosphorylation of Tau in cultured cells and in the brain (16, 29, 32, 40–46). In the current study, we therefore investigated the functional consequences of the inhibition of PP2A activity due to direct interaction of PP2Ac with mNLS-I\(_2^{PP2A}\) in the I\(_2^{PP2AA-AA-AAA}\) cells as compared with I\(_2^{PP2A-WT}\) cells. Because both \(I_2^{PP2AA-WT}\) and \(I_2^{PP2AA-AA-AAA}\) were Tet-Off cell lines, we determined abnormal hyperphosphorylation of Tau at 12E8 (Ser\(^{265}/\)Ser\(^{356}\)) and PHF1 (Ser\(^{390}/\)Ser\(^{404}\)) sites in the presence or absence of 1 \(\mu\)g/ml Dox for 72 h. The level of the abnormal hyperphosphorylation of Tau at 12E8 and PHF1 sites, which are considered major sites in AD-type neurofibrillary degeneration, was increased significantly both in \(I_2^{PP2AA-WT}\) (\(p < 0.05\)) and \(I_2^{PP2AA}\) (\(p < 0.01\)) as compared with the vector cells (Fig. 4). However, the extent of Tau hyperphosphorylation at 12E8 (\(p = 0.054\)) and PHF1 (\(p = 0.046\)) sites in mNLS-I\(_2^{PP2AA}\) was higher than in \(I_2^{PP2AA-WT}\) cells. Treatment with Dox normalized the level of \(I_2^{PP2A}\) transgene expression and the associated increase in abnormal hyperphosphorylation of Tau (Fig. 4). No significant change was detected in total Tau level as determined by human-specific anti-Tau. 43D, among the cell lines (Fig. 4A).

The functional aftermath of abnormal hyperphosphorylation of Tau is the disruption of microtubule stability, which could reduce the number and the length of neurite outgrowth (41). To investigate this possibility, we differentiated the stable cell lines of \(I_2^{PP2AA-WT}\) and \(I_2^{PP2AA-AA-AAA}\) with 100 ng/ml NGF for 5 days and then carried out immunofluorescence using rabbit polyclonal antibody to HA and mouse monoclonal antibody DM1A to tubulin. The morphology of cells expressing \(I_2^{PP2AA-AA-AAA}\) was significantly altered, with marked decreases in the number and length of neurites. The neurite outgrowth was also reduced in \(I_2^{PP2AA-WT}\) cells but was not statistically significant when compared with controls (vec) (Fig. 5, A–C). The number of neurite-bearing cells was reduced to ~22 and ~39%, whereas the mean neurite length was reduced to ~17 and ~49% in PC12 cells stably expressing \(I_2^{PP2AA-WT}\) and mNLS-I\(_2^{PP2AA}\), respectively (Fig. 5). These data imply that the abnormal hyperphosphorylation of Tau by mNLS-I\(_2^{PP2AA}\) suppresses its microtubule binding and assembly, which eventually impairs the neurite outgrowth and neuronal morphology.

**Effect of mNLS-I\(_2^{PP2A}\) on GSK-3\(_{\beta}\) and CAMKII Signaling Pathways—**The abnormal hyperphosphorylation of Tau that results from the inhibition of PP2A activity is probably due to not only a direct decrease in the dephosphorylation by PP2A but also an increase in phosphorylation of Tau by Tau kinases that are regulated by PP2A. Among the Tau kinases in the brain,
GSK-3β, CAMKII, ERK1/2, MEK1/2, and P70S6 kinase are regulated by PP2A (47). To explore the involvement of PP2A-regulated Tau kinases, we measured the levels of total and activated/inactivated forms of these kinases by Western blots. The levels of phosphorylated/activated CaMKII increased, whereas phosphorylated/inactivated GSK-3β at Ser9 decreased noticeably in mNLS-I2 PP2A cells (Fig. 6, A and B). However, we detected no significant change in the levels of phosphorylated Tau kinases. The NLS mutant I2 PP2A translocates from the cell nucleus to the cytoplasm and inhibits PP2A activity. A, following 48 h of transient transfection with human I2 PP2AWT and its NLS mutants (I2 PP2AA, I2 PP2AAA, and I2 PP2AA-AAA), COS7 cells were lysed, and PP2A activity was measured by ELISA using total cell lysate. I2 PP2AWT along with all of its NLS mutants inhibited PP2A activity. B, the expression of endogenous and exogenous I2 PP2A-HA was determined by Western blots. C, the quantitation of blots in B. The exogenous I2 PP2A expression did not show any significant difference among the groups. D and F, nuclear and cytoplasmic fractions were prepared from COS7 cells transiently transfected (48 h), and the expression of I2 PP2A in each fraction was analyzed by Western blots. Histone-3 and β-actin were used as markers of nuclear and cytoplasmic fractions, respectively. E and G, quantitative analysis of blots in D and F, respectively. The cytoplasmic retention of I2 PP2A is associated with inactivation of NLS. H, PP2A activity assayed in the cytosolic fraction showed a significant decrease in NLS-I2 PP2A mutants (I2 PP2AA and I2 PP2AA-AAA). Data are expressed as mean ± S.E. (error bars) for three separate experiments. *, p < 0.05; **, p < 0.01.
ERK1/2, MEK1/2, and p70 S6 kinase among the cells. CAMKII phosphorylates Tau at Ser 262/Ser356 (12E8 site), whereas GSK-3β/H9252 is the responsible kinase phosphorylating Tau at both Ser396 and Ser404 (PHF1 site) (48). Treatment with KN-93 (CAMKII inhibitor) but not with LiCl (GSK-3β/H9252 inhibitor) significantly \( p < 0.05 \) inhibited the mNLS-I2PP2A-induced Tau hyperphosphorylation at 12E8 site (Fig. 6, C and D). On the contrary, LiCl treatment significantly \( p < 0.05 \) prevented the Tau phosphorylation at PHF1 but not at the 12E8 site induced by mNLS-I2PP2A expression (Fig. 6, C and D). Combined treatment of both inhibitors (KN-93 and LiCl) showed a synergistic effect in reversing the hyperphosphorylation of Tau in mNLS-I2PP2A cells. These results suggested that mNLS-I2PP2A promotes abnormal hyperphosphorylation of Tau both by directly inhibiting PP2A and by up-regulating the activities of Tau kinases regulated by PP2A.

However, the question remains to be answered as to whether mNLS-I2PP2A has any direct interaction with CAMKII or GSK-3β. CAMKII phosphorylates Tau at Ser 262/Ser356 (12E8 site), whereas GSK-3β/H9252 is the responsible kinase phosphorylating Tau at both Ser396 and Ser404 (PHF1 site). Treatment with KN-93 (CAMKII inhibitor) but not with LiCl (GSK-3β/H9252 inhibitor) significantly \( p < 0.05 \) inhibited the mNLS-I2PP2A-induced Tau hyperphosphorylation at 12E8 site (Fig. 6, C and D). On the contrary, LiCl treatment significantly \( p < 0.05 \) prevented the Tau phosphorylation at PHF1 but not at the 12E8 site induced by mNLS-I2PP2A expression (Fig. 6, C and D). Combined treatment of both inhibitors (KN-93 and LiCl) showed a synergistic effect in reversing the hyperphosphorylation of Tau in mNLS-I2PP2A cells. These results suggested that mNLS-I2PP2A promotes abnormal hyperphosphorylation of Tau both by directly inhibiting PP2A and by up-regulating the activities of Tau kinases regulated by PP2A.

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However, the question remains to be answered as to whether mNLS-I2PP2A has any direct interaction with CAMKII or GSK-3β.
which might also contribute to the activation of these kinases. To address this issue, we performed co-immunoprecipitation studies with cell lysates from COS7 cells transiently transfected with a combination of I₂_pp2aa-AAA-HA/CAMKII-GFP or I₂_pp2aa-AAA-Myc/GSK-3β-HA. I₂_pp2aa co-immunoprecipitated with GSK-3β along with PP2Ac, whereas no in vivo association of CAMKII with I₂_pp2aa was detected (Fig. 7, A and B). Furthermore, protein phosphatase 1 (PP1) is reported to dephosphorylate GSK-3β at Ser9 and activate the kinase activity (49). Because mNLS-I₂_pp2aa is found to be associated with GSK-3β with undefined functional implications, it is reasonable to speculate that I₂_pp2aa might affect GSK-3β indirectly through PP1. Thus, we also evaluated the direct interaction between I₂_pp2aa and PP1 by co-immunoprecipitation experiments. We did not find any association of I₂_pp2aa with PP1 in our cell model (Fig. 7A). Furthermore, Western blot analysis of PP1 did not show any significant change between I₂_pp2aaWT and mNLS-I₂_pp2aa cells used in the study (Fig. 7, C and D). In addition, GSK-3β and I₂_pp2aa were found to co-localize in cytoplasmic compartments of COS7 cells double-labeled with anti-GSK-3β and anti-HA (I₂_pp2aa-AAA-AAA) (Fig. 7E). Next, we asked the question whether or not similar cross-talk between I₂_pp2aa and GSK-3β is also evident in AD brain. Consistent with previous reports (27, 50), a significantly enhanced I₂_pp2aa staining was detected in the cytoplasm of hippocampus of AD brains as compared with age-matched control brains, and the increased I₂_pp2aa was co-localized with GSK-3β (Fig. 7F).

FIGURE 4. mNLS-I₂_pp2aa promotes abnormal hyperphosphorylation of Tau in Tau441-Tet-Off-mNLS-I₂_pp2aa PC12 cells, an inducible cell model. A, cells were treated with or without Dox (1 μg/ml) for 72 h, and the cell lysates were used to measure Tau phosphorylation by Western blots developed with 12E8 (Ser(P)262/Ser(P)356) and PHF1 (Ser(P)396/Ser(P)404) Tau antibodies. B, quantitative analysis showing the suppression of the expressions of I₂_pp2aaWT and I₂_pp2aa-AAA-AAA with Dox. C, quantitative analysis showing Tau hyperphosphorylation at 12E8 and PHF1 sites expressed as mean ± S.E. (error bars) after normalization with total Tau (43D). The abnormal hyperphosphorylation of Tau at 12E8 and PHF1 sites seen in a Dox-dependent manner was higher in I₂_pp2aa-AAA-AAA than in I₂_pp2aaWT cells due to the cytoplasmic localization of the former. *, p < 0.05; **, p < 0.01.

DISCUSSION

Tauopathies are age-associated neurodegenerative diseases characterized by neurofibrillary pathology made up of abnormally hyperphosphorylated Tau, and the severity of these lesions directly correlates with dementia. Given the magnitude and the impact of tauopathies, including AD, on public health, there is increasing interest in the field to decipher the molecular mechanism underlying the Tau pathology as well as identifying the therapeutic interventions where disease-relevant cellular models are of crucial importance. We have previously shown that the activity of PP2A, the major Tau phosphatase regulating abnormally hyperphosphorylated Tau, is decreased in AD brains due to the cleavage and translocation of I₂_pp2aa from the neuronal nucleus to the cytoplasm by asparagine endopeptidase, possibly as a consequence of brain acidosis (27, 29). As observed in AD brain, we recently reported a selective increase in the cleavage of I₂_pp2aa and inhibition of PP2A activity in the lateral sclerosis cases (46), suggesting I₂_pp2aa-PP2A signaling as a common molecular target in different neurodegenerative diseases. To clarify whether cytoplasmic localization of I₂_pp2aa is vital for its association and inhibition of PP2A and thus neurofibrillary degeneration, we identified the potential NLS of I₂_pp2aa and, employing the mutated (inactivated) NLS, we generated a cell model of the hyperphosphorylated Tau that exhibits the following characteristic features. 1) The system is developed on PC12 cells based on the mutation of NLS of I₂_pp2aa,
which restricts it to cytoplasmic localization where both PP2A and expressed human Tau441 are present. 2) The expression of mNLS-I_2PP2A can be switched on and off in response to doxycycline (Tet-Off system) in order to control the inhibition of PP2A activity and resultant hyperphosphorylation of Tau. 3) The expression of mNLS-I_2PP2A is associated with the down-regulation of PP2A activity along with up-regulation of PP2A-regulated Tau kinases, possibly synergistically increasing the phosphorylation of Tau and impairment in microtubule network and neurite outgrowth.

I_2PP2A is predominantly a nuclear protein; hence, the NLS of I_2PP2A is supposed to be the central element controlling its nuclear localization. Previous studies revealed two NLSs for I_2PP2A; one is close to the N terminus, 6AKVSKK11 (51), and other one is in the area of the I_2PP2A cleavage site at Asn175, 168KRSSQTQNKASRKR181 (33). Based on the sequence comparison analysis among different species, we found 168KRSSQTQNKASRKR181 as an apparent conserved region of I_2PP2A and inferred that this region is a potential NLS. By using site-directed mutagenesis in combination with immunofluorescence analysis, we confirmed the NLS in the I_2CTF in the current study and further narrowed it down to 179RKR181 because mutations of these three amino acid residues cause the I_2PP2A to be diffusely distributed throughout the cytoplasm.

The present study clearly suggests that NLS at amino acids 179–181 is sufficient to determine its subcellular localization. The most likely reason is that NLS at amino acids 6–11 is neutralized by phosphorylation at Ser9 in contrast to the NLS at 179–181 because the phosphorylation of Ser9 is considered to be critical for its interaction and thus inhibition of PP2A (23). However, one cannot rule out the existence of double NLS in a protein similar to that found in 5-lipoxygenase (52). In the present study, cell fractionation and subsequent analysis with subcellular markers followed by PP2A activity further confirmed

![Cell Model of Tau Hyperphosphorylation](image)

**FIGURE 5. Effect of I_2PP2A on neuronal morphology and neurite outgrowth in PC12 cell.** PC12/Tau stably transfected cells expressing vector (Vec), I_2PP2AWT, or mNLS-I_2PP2A (I_2PP2AA-AAA) were differentiated with 100 ng/ml NGF for 5 days and then processed for double immunofluorescence using anti-tubulin (DM1A) and anti-HA (I_2PP2A). A, representative confocal images. B and C, quantitative analysis of cells bearing neurites and neurite length, respectively. The morphology of cells expressing mNLS-I_2PP2A was significantly altered with a marked decrease in the number and length of neurites as compared with vector and I_2PP2AWT cells. Scale bar, 50 μm. Error bars, S.E. *, p < 0.05; **, p < 0.01.
that cytoplasmic localization of $I_2^{PP2A}$ is associated with inhibition of PP2A activity, which is considered to be the major regulator of Tau hyperphosphorylation as seen in tauopathies. Thus, utilizing this observation, we developed an inducible cellular model of Tau hyperphosphorylation by overexpressing mNLS-$I_2^{PP2A}$ ($I_2^{PP2A}$AA-AAA) along with human Tau$_{441}$ in PC12 cells.

PP2A has been reported to interact physically with its endogenous inhibitors $I_1^{PP2A}$ and $I_2^{PP2A}$ and its cleavage fragments and thus inhibits its activity (31, 41). In the present study, we validated the newly developed cellular model in which cytoplasmic localization of $I_2^{PP2A}$ leads to increased physical association with PP2Ac and inhibits its activity. Co-localization studies with PP2Ac and $I_2^{PP2A}$ clearly demonstrated a significant cross-talk in the cytoplasmic compartment, which is consistent with the reduced PP2A activity. Co-immunoprecipitation experiments further confirmed that the increased physical interaction between PP2A and $I_2^{PP2A}$ facilitates the inhibition of PP2A activity in our cell model.

Next, we addressed the question of whether or not PP2A inhibition promotes the abnormal hyperphosphorylation of Tau. Using a small molecule inhibitor of GSK-3/β, we observed that cytoplasmic retention of $I_2^{PP2A}$ resulted in increased phosphorylation of Tau at the 12E8 and PHF1 sites. This effect was found to be dependent on the presence of LiCl, indicating that PP2A inhibition contributes to the hyperphosphorylation of Tau in our cellular model.

FIGURE 6. Activation of CAMKII and GSK-3/β in response to cytoplasmic retention of $I_2^{PP2A}$. Lysates from stably expressing vector (vec), $I_2^{PP2A}$WT, or mNLS-$I_2^{PP2A}$ ($I_2^{PP2A}$AA-AAA) were used to detect the activation/inactivation of Tau protein kinases. A, Western blot patterns of total and phosphorylated kinases; B, quantitation of phosphorylated kinases after normalization with the level of corresponding kinase; C and D, cell lines were incubated with LiCl (1 mM) and/or KN-93 (10 μM) for 24 h, and the cell lysates were used to analyze the levels of hyperphosphorylation of Tau at 12E8 and PHF1 sites. C, representative Western blots. D, quantitation of blots from C showed that $I_2^{PP2A}$-induced Tau hyperphosphorylation at the 12E8 site was inhibited by KN93 and not by GSK-3/β inhibitor LiCl, whereas the PHF1 site was inhibited by LiCl and not by KN93 alone. KN93 together with LiCl yielded the highest inhibition. Data are expressed as mean ± S.E. (error bars) from 3–4 separate experiments. *, $p<0.05$; **, $p<0.01$. 

Cell Model of Tau Hyperphosphorylation
Tau and microtubule instability as a functional consequence of cytoplasmic retention of $I_2^{PP2A}$ in this cell model. We found a marked increase in the abnormal hyperphosphorylation of Tau in cells at Ser262/356 (12E8) and Ser396/404 (PHF1) sites, two prominent abnormal hyperphosphorylated sites found in PHF-Tau in AD brain (53). Ser262 is located in the microtubule (MT) binding domain of Tau, and phosphorylation at this site has been shown to induce MT disassembly, whereby the new unbound pool of Tau is susceptible to self-assembly into PHFs (14, 43, 54, 55). Phosphorylation of Tau at Ser262 in combination with Thr212 and Thr231 results not only in loss of its normal function but also gain of a toxic activity that causes disruption of microtubule networks and cell death (3, 14). With respect to Ser262, there is evidence that Tau phosphorylation at this site plays a critical role in Aβ42-induced Tau toxicity because Tau-induced toxicity was prevented by using a transgenic fly expressing a nonphosphorylatable form of Tau at Ser262 (56). The phosphorylation of Tau at Ser262 and Ser356 has another functional consequence: priming the phosphorylation at other sites, which increases further its microtubule inhibitory activity and self-aggregation into filaments, because we previously showed that a prior phosphorylation of Tau at Ser262/356 containing the Ser/Thr-X motif by CAMKII, a non-proline-dependent protein kinase (PDPK) was found to stimulate a subse-

**FIGURE 7. In vivo association of $I_2^{PP2A}$ with GSK-3β.** A and B, COS7 cells were transiently transfected with a combination of $I_2^{PP2A}$AA-AAA-HA/CAMKII-GFP or $I_2^{PP2A}$AA-AAA-MYC/GSK-3β-HA. Cell lysates were subjected to immunoprecipitation (IP) with anti-HA, and the Western blots of immunoprecipitates were probed with anti-GFP for CAMKII, anti-HA/MYC for $I_2^{PP2A}$, anti-PP2Ac, and anti-PP1. $I_2^{PP2A}$ was found to be associated with GSK-3β and with PP2Ac but not with CAMKII and PP1. C, the total level of PP1 was detected by Western blots in vector, $I_2^{PP2A}$WT, and mNLS-$I_2^{PP2A}$ cell lines. D, quantitation of blots in C. Data are expressed as mean ± S.E. (error bars) from two separate experiments. E, COS7 cells were transiently co-transfected with GSK-3β-GFP and HA-tagged $I_2^{PP2A}$WT or mNLS-$I_2^{PP2A}$ (I2$^{PP2A}$AA-AAA), and cells were then stained with anti-HA ($I_2^{PP2A}$). The co-localization of the two is demonstrated by yellow color in the merged image, suggesting the cross-talk of translocated $I_2^{PP2A}$ with GSK-3β in the cytoplasm. Scale bar, 20 μm. F, co-localization of $I_2^{PP2A}$ and GSK-3β in the cytoplasm of hippocampal neurons in AD brain. Scale bar, 50 μm.
quent phosphorylation catalyzed by GSK-3β, a PDPK (57). In contrast to Ser262, Ser396 and Ser404 are present in the C-terminal domain and have only a moderate influence on Tau-MT interaction but are up-regulated in AD and other tauopathies (58, 59). Moreover, phosphorylation of Tau at the PHF1 (Ser396/404) site in combination with the AT8 (Ser199/Ser202/Ser205) site has been shown to induce a compaction of the paper clip folding of Tau that generates a pathological conformation, which aggregates somewhat more readily (60). Thus, it appears that the missorting of I2PP2A into the cytoplasm probably contributes to the deficit in PP2A activity, which in turn promotes the hyperphosphorylation and loss of biological function of Tau in our cellular model.

There is significant evidence that abnormal hyperphosphorylation of Tau results in Tau dysfunction due to the disturbances in microtubule dynamics, axonal transport, and neurite outgrowth, which synergistically contribute to the pathogenic processes. In the current study, we found that mNLS-I2PP2A decreased not only the mean neurite length but also the average number of NGF-induced differentiated cells bearing neurites. Our data are consistent with our previous observation that I1PP2A, another endogenous inhibitor of PP2A, impairs MT network and neurite outgrowth via hyperphosphorylation of Tau (41). Although our data do not imply a direct role of I2PP2A in microtubule instability, it is well recognized that once Tau is abnormally hyperphosphorylated, it can disrupt microtubules by sequestering normal microtubule-associated proteins (61). There is indirect evidence that Tau hyperphosphorylation might facilitate neurite retraction (62). Pharmacological inhibition, dominant negative down-regulation, and siRNA knockdown of PP2Ac lead to neurite retraction and inhibition of axiogenesis (63). Thus, I2PP2A could contribute to the destabilization of microtubules indirectly through PP2A-dependent phosphorylation states of Tau in our cell model. However, we cannot rule out the possibility that the effects of PP2A observed in the present study on neurite outgrowth and microtubule stability are partially Tau-independent because PP2A is not only the major Tau phosphatase but also the major brain phosphatase, which plays a crucial role in regulating most cellular functions, including neurite outgrowth (64, 65).

In addition to the direct action of PP2A on Tau, the activation of Tau protein kinases can also induce Tau hyperphosphorylation. For instance, the proline-independent 12E8 site is phosphorylated by CaMKII, PKA, and MARK (66, 67). Ser396 is almost exclusively phosphorylated by GSK-3β, whereas both GSK-3β and Cdk5 phosphorylate Tau at Ser404. We found a significant activation of CaMKII, which is consistent with the report that the treatment of metabolically active rat brain slices by a PP2A inhibitor, okadaic acid, activated CaMKII activity and promoted Tau hyperphosphorylation at the 12E8 site (68). However, the levels of phosphorylated ERK1/2, MEK1/2, and p70 S6 kinase did not change among the cells. Overexpression of I1PP2A has been shown to increase the levels of ERK1 and ERK2 in HeLa cells (69), whereas Fukukawa (70) reported the opposite; overexpression of I2PP2A results in suppression of EGF-stimulated ERK activation, and knocking down I2PP2A by siRNA resulted in enhancement of the MEK/ERK pathway in HeLa cells. Phosphorylated ERK1/2 was reported to be reduced in head and neck squamous cell carcinoma stably expressing shRNA against I2PP2A (71). The exact reason for this discordance is not clear, but it is apparent that the regulation of the MEK/ERK pathway by I2PP2A is possibly a cell type-specific phenomenon; however, it demands further investigation. Additionally, we noticed a significant activation of GSK-3β, as detected by a decrease in inhibitory phosphorylation at Ser9. Our current finding is consistent with previous reports that showed that accumulation of I2PP2A or overexpression of the C-terminal cleaved fragment of I2PP2A, I2CTFP, results in an increase in GSK-3β activity (32, 72). Co-treatment with LiCl (GSK-3β inhibitor) and KN-93 (CAMKII inhibitor) significantly reduced the I2PP2A-induced Tau hyperphosphorylation. The exact reason underlying the GSK-3β activation in our model is not clear. However, the direct physical interaction between I2PP2A and GSK-3β could possibly explain the observed phenomenon. Another possible reason could be that PP1 activation could lead to the dephosphorylation of Ser9-GSK-3β associated with its activation. Consistent with this possibility, it has been reported that I1PP2A and I2PP2A results in a marked increase in PP1c activity in the presence of a nearly physiological concentration of Mn2+ in a substrate-specific manner (73). Although we did not measure PP1 activity, we could not detect any change in total PP1 protein level. On the other hand, coimmunoprecipitation experiments clearly suggest a physical association of I1PP2A with GSK-3β in contrast with CAMKII. Consistent with our finding, Liu et al. (72) showed that overactivation of GSK-3β inhibits PP2A through up-regulation of I1PP2A, indicating a positive correlation between I1PP2A and GSK-3β activity. We further confirmed the in vivo association of GSK-3β with I1PP2A in AD brain, where both are co-localized in the neuronal cytoplasm. Collectively, these results (Fig. 8) suggest 1) that inhibition of PP2A caused by cytoplasmic translocation of I2PP2A mimics the abnormal hyperphosphorylation of Tau, as observed in AD brain; 2) that the effect of mNLS-I2PP2A is modulated both directly by PP2A and indirectly by the Tau kinases that are regulated by PP2A; and 3) that the in vivo
association of $I_2^{PP2A}$ with GSK-3β could imply a direct role of $I_2^{PP2A}$ in activation of GSK-3β.

In conclusion, our study demonstrates a potential NLS at amino acids 179–181, which regulates the translocation of $I_2^{PP2A}$ from neuronal cytoplasm to nucleus, and offers the development of a Tet-Off-inducible cell model of abnormal hyperphosphorylation of Tau in which the NLS of $I_2^{PP2A}$ is mutated, and it is translocated to the cell cytoplasm. Cyttoplasmic retention of $I_2^{PP2A}$ results in inhibition of PP2A directly and indirectly by PP2A-regulated Tau kinases, all of which eventually lead to the hyperphosphorylation of Tau. The restoration of PP2A activity through the inhibition of $I_2^{PP2A}$–PP2A interaction offers a promising therapeutic target for preventing the neurofibrillary degeneration of the abnormally phosphorylated Tau. Our cellular model of tauopathies is suitable for screening drugs/compounds that can attenuate the abnormal hyperphosphorylation of Tau.

Acknowledgments—We thank Dr. Zane Martin Jones for helpful comments on the manuscript, Dr. George Merz for assistance with confocal microscopy, and Janet Murphy for secretarial assistance.

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Hyperphosphorylation of tau protein is a key event in the pathogenesis of Alzheimer’s disease (AD) and tauopathies. While the hyperphosphorylation of tau is critical for the formation of neurofibrillary tangles (NFTs), the mechanisms underlying this process are still largely unknown. In this review, we discuss the role of protein phosphatase 2A (PP2A) in the regulation of tau phosphorylation.

PP2A is a heterotrimeric protein phosphatase that acts as a major regulator of phosphorylation in the brain. Its catalytic subunit is essential for the association of B regulatory subunit with tau, and its dysfunction has been linked to AD. In a recent study, the authors investigated the role of PP2A in tau phosphorylation. They found that PP2A regulates tau phosphorylation directly and also indirectly through the modulation of protein kinase (PK) activity.

In AD, hyperphosphorylation of tau induces the formation of NFTs, which are a hallmark of the disease. The phosphorylation of tau at multiple sites, including Ser262, Thr231, and Ser396, is thought to contribute to the formation of NFTs. The authors propose that the dysregulation of PP2A might contribute to the hyperphosphorylation of tau in AD.

Overall, the authors highlight the importance of PP2A in the regulation of tau phosphorylation and suggest that targeting PP2A might be a potential therapeutic strategy for the treatment of tauopathies.
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Cell Model of Tau Hyperphosphorylation