Involvement of I_2^{PP2A} in the abnormal hyperphosphorylation of tau and its reversal by Memantine

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Abstract **The activity of protein phosphatase (PP)-2A, which regulates tau phosphorylation, is compromised in Alzheimer disease brain. Here we show that the transient transfection of PC12 cells with inhibitor-2 (I_2^{PP2A}) of PP2A causes abnormal hyperphosphorylation of tau at Ser396/Ser404 and Ser262/Ser356. This hyperphosphorylation of tau is observed only when a subcellular shift of I_2^{PP2A} takes place from the nucleus to the cytoplasm and is accompanied by cleavage of I_2^{PP2A} into a 20 kDa fragment. Memantine, an un-competitive inhibitor of N-methyl-D-aspartate receptors, inhibits this abnormal phosphorylation of tau and cell death and prevents the I_2^{PP2A}-induced inhibition of PP2A activity in vitro. These findings demonstrate novel mechanisms by which I_2^{PP2A} regulates the intracellular activity of PP2A and phosphorylation of tau, and by which Memantine modulates PP2A signaling and inhibits neurofibrillary degeneration.**
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1. Introduction

Neurofibrillary degeneration of abnormally hyperphosphorylated tau is a hallmark of Alzheimer disease (AD) and related tauopathies [1]. The activity of phosphoseryl/phosphothreonyl protein phosphatase (PP)-2A, which regulates the phosphorylation of tau [2], is compromised in AD brain [3]. Bilateral injection of calyculin A, a potent inhibitor of both PP2A and PP1, into rat hippocampi produces tau hyperphosphorylation and behavioral impairment [4].

The intracellular activity of PP2A is regulated by two endogenous proteins called inhibitor-1 (I_1^{PP2A}) and I_2^{PP2A} [5,6]. They non-competitively inhibit PP2A activity with K_i values in the nanomolar range [5,6]. Several proteins homologous to I_2^{PP2A} have been described and include human SETα [7,8], PHAP-II [9] and TAF-1 [10]. TAF-1 in its complete form, like I_2^{PP2A}, is mainly a nuclear protein and contains a highly acidic C-terminal region that is involved in chromatin remodeling [10]. The N-terminal region of TAF-1 is responsible for its inhibi-

tory activity towards PP2A [11]. Full length TAF-1 is a nuclear protein, but TAF-1β, which lacks the C-terminal region, is localized in the cytoplasm and inhibits PP2A activity [12]. In AD brain, the transcription and expression of I_2^{PP2A} is increased and the inhibitor is translocated from its primary nuclear location to the cytoplasm accompanied by a cleavage of the full-length ∼39 kDa protein into a ∼20 kDa N-terminal fragment in the neurons [13]. Moreover, the I_2^{PP2A} cleavage activity is increased in the AD brain and it co-localizes both with PP2A and abnormally hyperphosphorylated tau in the neuronal cytoplasm [13].

Treatment of AD patients with Memantine (1-amino-3,5-dimethyladamantane hydrochloride), a low to moderate affinity, un-competitive inhibitor of glutamate gated NMDA receptor channels, results in functional improvement and reduction of care dependence [14]. The regulation of PP2A activity in neurons might, in part, be responsible for the neuron-healthy effects of Memantine. NMDA receptor has been reported to be in complex with PP2A, and stimulation of this receptor leads to dissociation of PP2A with subsequent reduction in PP2A activity [15]. We have recently reported that Memantine inhibits and reverses abnormal hyperphosphorylation and accumulation of tau in organotypic culture of rat hippocampal slices by reversing okadaic acid-induced inhibition of PP2A activity [16]. The exact mechanism by which Memantine modulates PP2A signaling has, however, been elusive.

Rat adrenal pheochromocytoma (PC12) cells provide a simple and reproducible model system to study various aspects of neuron biology and have been used as a reliable system to study N-methyl-D-aspartate receptor (NMDAR) properties [17]. Functional properties of NMDARs in PC12 cells are very similar to those described for recombinantly expressed NMDAR1A–NMDAR2C heteromers [18]. Employing PC12 cells as a model, the present study demonstrates novel mechanisms by which I_2^{PP2A} modulates PP2A activity and produces abnormal hyperphosphorylation of tau, and by which Memantine inhibits this pathology by directly affecting I_2^{PP2A}-induced inhibition of PP2A in the cell.

2. Materials and methods

2.1. Subcloning and generation of human I_2^{PP2A}

I_2^{PP2A} was originally cloned from human brain cDNA using oligonucleotide primers designed to the sequence of human kidney I_2^{PP2A} cDNA as described [19]. The I_2^{PP2A} sequence was amplified and subcloned into pCI-neo Mammalian Expression Vector (Promega, Madison, WI).

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2.2. Cell culture, transfection and Memantine treatment

We used PC12 cells stably transfected with tau_{441} [20]. Cell culture and transfection was done as described previously [19]. Memantine (Sigma, St. Louis, MO), where applicable, was added to the culture medium at 6 h post-transfection.

2.3. Western blots and immunocytochemistry

Western blots, immunocytochemical staining and confocal microscopy were carried out as described previously [19].

2.4. Inhibition of I_2^{PP2A} activity by Memantine

The in vitro inhibition of PP2A by I_2^{PP2A} and its reversal by Memantine were carried out by ELISA in which dephosphorylation of a tau phosphopeptide (h tau 194–207-KKK in which Ser 199 is phosphorylated) by bovine brain PP2A and its inhibition by recombinant human brain I_2^{PP2A} in the presence or absence of 100 nM Memantine was determined.

2.5. Cell toxicity assay

The effect of overexpression of I_2^{PP2A} on the viability of PC12 cells was determined by assaying the LDH release using CytoTox-ONE Assay Kit (Promega).

2.6. Statistical analysis

Mean values and S.D.s were computed for each group and analyzed by Student's t test. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Sub-cellular localization of I_2^{PP2A}

Tau_{441} stably transfected PC12 cells were transiently transfected with human I_2^{PP2A} and immunocytological localization was studied at 6, 24, 48, 72 and 96 h post-transfection and using I_2^{PP2A} polyclonal antibody R-42187. Analysis with laser confocal microscopy revealed detectable endogenous levels of I_2^{PP2A} in non-transfected cells, and a robust expression of the inhibitor above endogenous levels by 48 h post-transfection (Fig. 1a). In most of the cells, the inhibitor was located in the nucleus at this time point. By 72 h, majority of the transfected cells showed cytoplasmic localization whereas a few cells with nuclear staining of the inhibitor could still be seen (arrowheads in Fig. 1a). By 96 h, most of the cells showed cytoplasmic staining of the inhibitor. The expression of I_2^{PP2A} was the highest by 48 h but even by 96 h it was ~2-fold more than the mock expression level as quantitated by Western blot analysis of cell lysates at various time points post-transient transfection (Fig. 1b).

3.2. I_2^{PP2A} induced hyperphosphorylation of tau

We next investigated the effect of I_2^{PP2A} transfection on the biological correlates of PP2A activity. PP2A is a key enzyme in regulating the phosphorylation of tau at various phosphorylation sites including pS262 and pS396 [2]. We used hyperphosphorylation of tau as an indicator of depressed PP2A activity in the I_2^{PP2A} transfected cells. Since expression of an exogenous gene generally peaks by 48 h post-transient transfection, we prepared cell lysates at 48-h time point to analyze the cells for tau hyperphosphorylation by Western blots. We found no detectable differences in the phosphorylation levels of tau at either PHF-1 or 12E8 sites at 48 h post-transfection (data not shown). This lead us to speculate that since I_2^{PP2A} was mainly localized in the nucleus at 48 h (Fig. 1a), while PP2A is a cytosolic protein, I_2^{PP2A} was probably unable to inhibit PP2A activ-
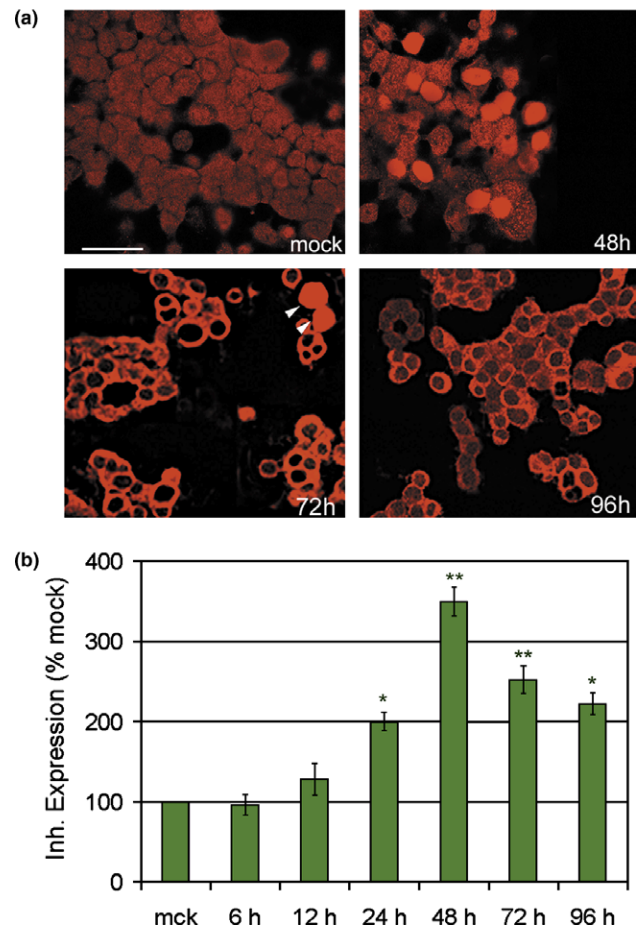

Fig. 1. Sub-cellular localization of I_2^{PP2A} in PC12 cells stably expressing human tau_{441} after transient transfection with human I_2^{PP2A}. (a) Confocal images of cells immunostained with anti-I_2^{PP2A} (R-42187) at different time intervals following transfection with I_2^{PP2A}. At 48 h post-transfection, majority of I_2^{PP2A} was seen in the nucleus. By 72–96 h, most of the inhibitor was observed in the cytoplasm with a few cells showing nuclear staining (arrow heads). Scale bar: 50 μm. (b) Time course of I_2^{PP2A} expression in PC12 cells. The expression of I_2^{PP2A} was determined by quantitative chemiluminescent (ECL) Western blots and data normalized to the values obtained from mock transfected cells. Data expressed as means ± S.D. Comparison was done between mock (pcDNA) transfected and I_2^{PP2A} transfected cells. * $P < 0.5$, ** $P < 0.01$.

ity due to spatial constraints. We therefore, carried out immunocytochemical analysis of the cells at both 48 and 96-h post-transfection. Consistent with the above scenario, confocal microscopic images of cultured cells at 48 h showed minimal, if any, hyperphosphorylation of tau at PHF-1 or 12E8 sites. However, at 96 h, a time when majority of the inhibitor was localized in the cytoplasm, we could observe a sharp increase in PHF-1 and 12E8 immunostaining (Fig. 2a and b).

3.3. Inhibition of hyperphosphorylation of tau by Memantine

We have previously shown that Memantine inhibits and reverses PP2A-inhibition-induced abnormal hyperphosphorylation of tau in organotypic rat hippocampal slice cultures [16]. However, the exact mechanism by which Memantine restored PP2A activity was not known. Since I_2^{PP2A} is one of the two naturally occurring modulators of PP2A signaling in cells, we postulated that Memantine might regulate I_2^{PP2A}
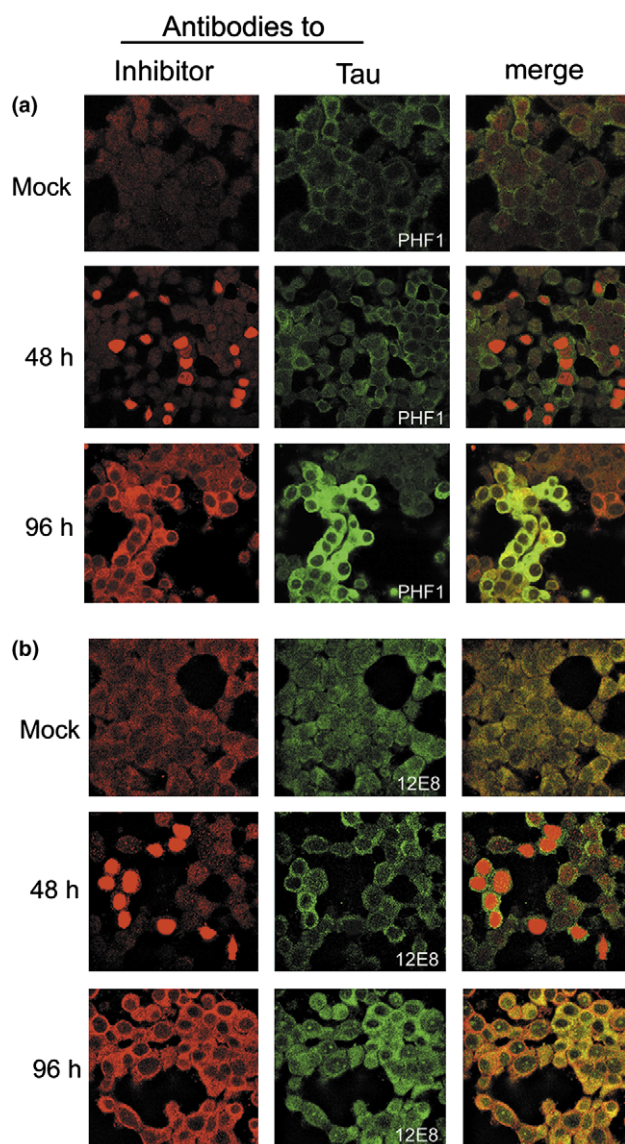
Fig. 2. Hyperphosphorylation of tau in PC12 cells stably expressing human tau$_{441}$ after transient transfection with human I$_2^{PP2A}$. Tau$_{441}$-PC12 cells were transiently transfected with human I$_2^{PP2A}$, fixed at 48 or 96 h post-transfection and double labeled with anti-I$_2^{PP2A}$, R-42187 (red) and either anti-phospho-tau (green) PHF-1 [against pS396/pS404 in (a)] or 12E8 [against pS262/pS356 in (b)] for analysis by confocal microscopy. Expression of I$_2^{PP2A}$ caused robust phosphorylation of tau at both PHF1 (a) and 12E8 (b) sites when the inhibitor was located in the cytoplasm (96 h), whereas minimal effect was observed when the inhibitor was localized in the nucleus (48 h).

induced inhibition of PP2A. We, therefore, sought out to test the effect of Memantine on the I$_2^{PP2A}$-induced tau hyperphosphorylation. PC12 cells were treated with 10 μM Memantine at 6 h post-transfection with I$_2^{PP2A}$ for a total of 90 h. Cells were fixed for immunocytochemistry at 96 h post-transfection. Immunocytochemically, Memantine clearly inhibited the abnormal phosphorylation of tau at PHF1 and 12E8 sites in treated PC12 cells (Fig. 3a). Western blot analysis of cell lysates was performed next using polyclonal antibody against I$_2^{PP2A}$ (R1482). Cells were separated according to their attachment to the culture plates at the time of harvest. Floating cells, plus cells that came off the culture plate during PBS wash, comprised a population in which the percentage of I$_2^{PP2A}$ transfected cells or cells expressing higher amounts of the inhibitor was greater than that in the attached population (Fig. 3b and d). While as much as ~30–40% of the I$_2^{PP2A}$ transfected cells detached, there was no significant cell detachment in the various control experiments to warrant separate analysis. For biochemical studies, the detached cells were analyzed separately from the attached cells. Western blots showed an increase in phosphorylation of tau at PHF1 and 12E8 sites in detached cells. Memantine restored the phosphorylation levels of both PHF1 and 12E8 sites to control levels (Fig. 3b and c). Immunocytochemical staining was not carried out on detached cells, but images were selected from the attached cells to show proof of the principle. Tau hyperphosphorylation, though detectable immunocytochemically, could not be observed biochemically in the attached cells.

TAF-1, which is homologous to I$_2^{PP2A}$, has been implicated in chromatin remodeling [10,21] and was identified as a stimulatory factor for replication and transcription when adenovirus DNA complexed with basic viral core proteins was used as a template for cell-free systems [22,23]. In order to confirm that the reversal of I$_2^{PP2A}$ induced tau hyperphosphorylation by Memantine was not due to the regulation of I$_2^{PP2A}$ expression in transfected cells, we compared the inhibitor levels in cell lysates obtained from both Memantine untreated and treated cells. Quantitative analysis failed to reveal any effect of Memantine on I$_2^{PP2A}$ protein levels (Fig. 3d). Thus, the effect of Memantine in restoring phosphorylation status of tau was not apparently due to the modulation of I$_2^{PP2A}$ expression.

Since transfection with I$_2^{PP2A}$ caused an increase in cell detachment, and detached cells comprised of a population of cells with a higher percentage of I$_2^{PP2A}$ immunoreactivity (Fig. 3d), we next sought out to see the level of cytotoxicity in these cells by Cytotox-1 assay. As expected, transfection with I$_2^{PP2A}$ caused a ~36% increase in cytotoxicity at 48 h post-transfection. This was reduced to ~13% after Memantine (10 μM) was added to the culture medium at 6 h post-transfection and maintained till analysis (Fig. 3e). Treatment of mock transfected cells with Memantine as a control also caused an unexpected small increase in cytotoxicity. The molecular events linking I$_2^{PP2A}$ overexpression and cell death in these cells remain to be investigated.

We had previously speculated that a selective cleavage and translocation of I$_2^{PP2A}$ from its primary nuclear localization to the cytoplasm in cortical neurons undergoing neurofibrillary degeneration in AD might represent a novel mechanism by which I$_2^{PP2A}$ inhibits PP2A activity, and leads to abnormal hyperphosphorylation of tau [13]. In the present study, immunocytochemical data showed an increase in phosphorylation of tau at PHF1 and 12E8 sites at 96 h post-transfection, and since this also represented a time point when I$_2^{PP2A}$ was mainly localized in the cytoplasm, we investigated whether the hyperphosphorylation of tau observed in the 96 h transfected cells was associated, at least in part, with a selective cleavage of I$_2^{PP2A}$ in the these cells. Western blot analysis indeed, showed the presence of a 20-kDa cleavage product of I$_2^{PP2A}$ in the detached cell population harvested at 96 h post-transfection (Fig. 4). We could also observe the presence of two other I$_2^{PP2A}$ cleavage fragments of ~25 kDa and ~28 kDa. These additional cleavage fragments were previously reported [13]. The detachment of transfected cells from the culture wells was associated both with increased level of expression of
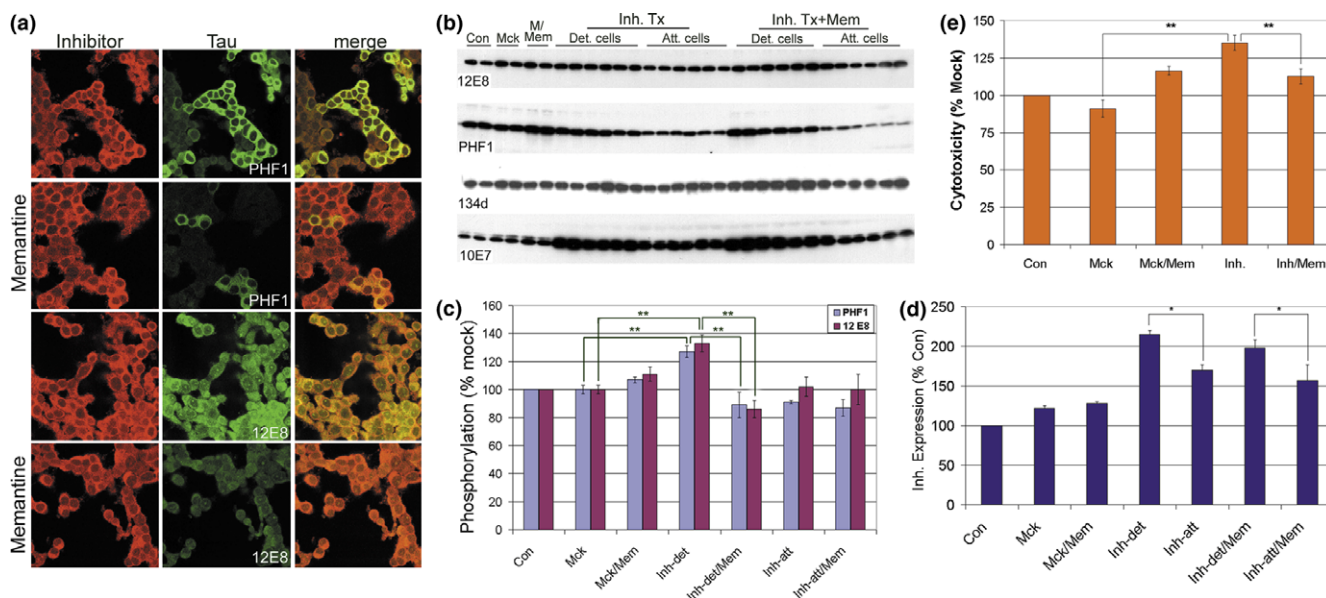
Fig. 3. Effect of Memantine on I_2^{PP2A} induced hyperphosphorylation of Tau at PHF1 and 12E8 sites in PC12 cells stably expressing human tau$_{441}$. Tau$_{441}$-PC12 cells were treated in the presence (+) or absence (−) of 10 μM Memantine at 6 h post-transfection with human I_2^{PP2A}. (a) At 96 h post-transfection, cells were fixed and double stained with anti-I_2^{PP2A} (R-42187, red) and anti phospho-tau (green) PHF1 (upper 2 panels) or 12E8 (bottom 2 panels). Images were obtained by laser confocal microscopy. Memantine reduced phosphorylation of tau at both sites. A similar experiment was run side by side and treated identically as in (a) except that cells that were detached from the culture plate at the time of harvest were separated from the attached cells for Western blot analysis (b) and quantitation (c). Transfection with human I_2^{PP2A} caused hyperphosphorylation of tau at PHF1 and 12E8 sites (27% and 36%, respectively), which was reversed by 10 μM Memantine treatment in detached cells. (d) The level of I_2^{PP2A} in detached cells was greater than in attached cells. Memantine had no effect on the levels of I_2^{PP2A}. Results represent up to six independent experiments. Antibodies used were mAb PHF1 (against pS396/pS404), mAb 12E8 (against pS262/pS356), 134d (total tau) and mAb 10E7 (full length I_2^{PP2A}). (e) I_2^{PP2A} induced cytotoxicity in PC12 cells stably expressing human tau$_{441}$. Cytotox-1 assay was used to quantify cytotoxic effects of I_2^{PP2A} transfection on PC12 cells. Transfection with human I_2^{PP2A} caused 36% increase in cytotoxicity which was decreased to ∼13% after 10 μM Memantine treatment. Results are presented as means ± S.D. $^*P < 0.05$, $^{**}P < 0.01$.
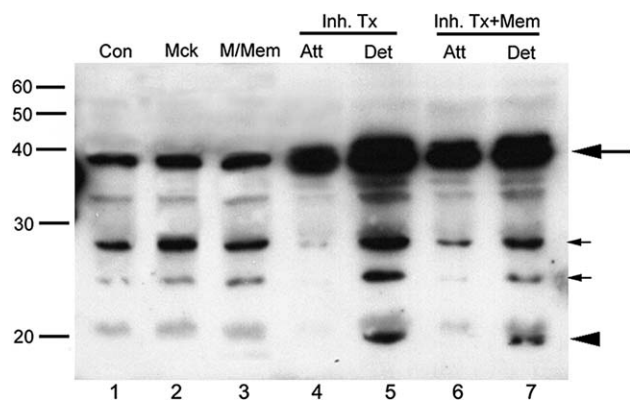

Fig. 4. Effect of Memantine on the cleavage of I_2^{PP2A} at 96 h post-transfection. PC12 cell lysates were prepared after 96 h of transfection with human I_2^{PP2A}. Cell lysates were separated based on their attachment to the culture plates at the time of harvest and analyzed by Western blots developed with polyclonal antibody R1482 against I_2^{PP2A}. Lanes 4–7 show expression of full length I_2^{PP2A} (arrow). Lanes 5 and 7 show a 20 kDa fragment of I_2^{PP2A} (arrowhead) which was observed in cell lysates prepared from detached cells in the respective treatment groups. Small arrows indicate ∼25 kDa and ∼28 kDa amino terminal fragments of I_2^{PP2A} also reported previously (see Section 3). Con (control), Mck (mock treated), M/Mem (mock with 10 μM Memantine), Inh.Tx (cells transfected with I_2^{PP2A} alone), Inh.Tx/Mem (cells transfected with I_2^{PP2A} and treated with 10 μM Memantine), Det (cells detached from the culture plate) and Att (cells attached to the culture plate at the time of harvest). Memantine, where indicated, was added to the culture medium at 6 h post-transfection for a total treatment time of 90 h.

I_2^{PP2A} and its amino terminal cleaved fragments; the increased cleavage probably contributed to increase in hyperphosphorylation of tau seen selectively in the detached cells. We could not detect any effect of Memantine on the cleavage of I_2^{PP2A}. Taken together, Memantine restored the I_2^{PP2A}-induced abnormal phosphorylation of tau to normal levels, but seemed to have no effect on the expression, sub-cellular localization or cleavage of I_2^{PP2A}. We next entertained the possibility of a direct interaction of Memantine with I_2^{PP2A}.

3.4. In vitro inhibition of PP2A activity by I_2^{PP2A} and its reversal by Memantine

Previously, we had demonstrated that Memantine does not have direct interaction with PP2A [16]. To investigate whether Memantine directly interacted with I_2^{PP2A}, we examined its effect on inhibition of PP2A activity by I_2^{PP2A}. I_2^{PP2A} inhibited the PP2A (purified from bovine brain) catalyzed dephosphorylation of the tau peptide at Tau-1 site. At 5 and 25 nM concentration, I_2^{PP2A} inhibited 11% and 33% of PP2A activity, respectively. This effect of I_2^{PP2A} on PP2A activity was inhibited by 100 nM Memantine (Fig. 5a). Memantine as such had no effect on PP2A activity (Fig. 5b).

4. Discussion

Independent of the etiology, neurofibrillary degeneration of abnormally hyperphosphorylated tau appears to be required
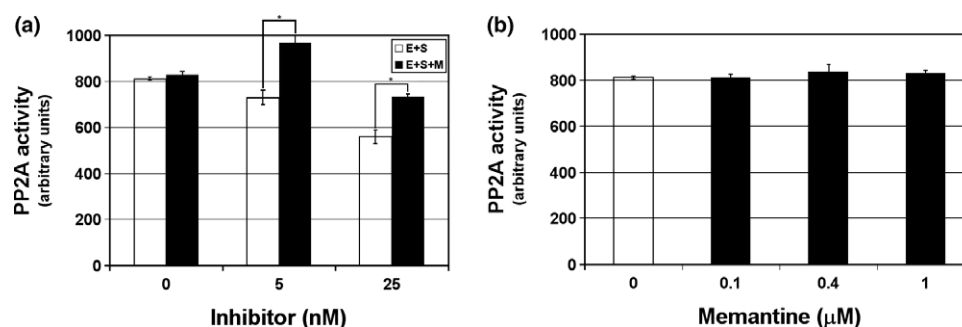
Fig. 5. In vitro inhibition of PP2A by I_2^{PP2A} and its reversal by Memantine. (a) Recombinant human brain I_2^{PP2A} inhibited the PP2A (bovine brain holoenzyme) catalyzed dephosphorylation of abnormally hyperphosphorylated tau peptide at the Tau-1 site in a dose dependant manner (open bars). Memantine (100 nM) reversed the I_2^{PP2A}-induced inhibition of PP2A activity (filled bars). (b) In the absence of I_2^{PP2A}, Memantine did not have any significant effect on PP2A activity. Results are presented as means ± S.D. *$P < 0.05$; E + S, enzyme + substrate; E + S + M, enzyme + substrate + memantine.

for the clinical expression of AD and related tauopathies. Previously, we have shown that I_2^{PP2A} is both overexpressed and selectively translocated from neuronal nucleus to cytoplasm in AD brain [13] and that the activity of PP2A is compromised by ∼20% in AD neocortex [3]. The present study demonstrates that probably the translocation of I_2^{PP2A} from the nucleus to the cytoplasm and its cleavage into the amino and carboxyl terminal halves are required for the abnormal hyperphosphorylation of tau. PP2A regulates phosphorylation of tau not only directly by dephosphorylation of tau but also by regulating the activities of several tau kinases [16,24–26]. Thus, inhibition of I_2^{PP2A} offers a promising target for the treatment of AD and related tauopathies.

We had previously shown that in AD temporal cortex and hippocampus, but not cerebellum, the transcription and expression of I_2^{PP2A} are significantly increased [13]. Furthermore, I_2^{PP2A} is selectively translocated in neurons from its primary nuclear localization to the cytoplasm accompanied by the appearance of a ∼20 kDa amino terminal cleavage fragment of the full length protein in the cytosol from cortical and hippocampal brain tissue in AD cases [13]. This 20 kDa fragment of I_2^{PP2A}, which has been reported by others as well [8,12], is derived from the cleavage of 39 kDa I_2^{PP2A}/SET/TAF-1 and has inhibitory activity towards PP2A [12]. Moreover, in HeLa cells transfected with amino terminal half of TAF1, the inhibitor localized to the cytoplasm [12]. The present study shows an increase in hyperphosphorylation of tau at pSer396/pSer404 and pSer262/pSer356 sites in PC12 cells transfected with full length I_2^{PP2A}, only at the time when I_2^{PP2A} is immunocytochemically observed in the cytoplasm. This increase in the phosphorylation of tau was observed in a population of cells with the highest expression of I_2^{PP2A} and the same cells also showed an increase in ∼25 kDa and ∼28 kDa amino terminal fragments, and the appearance of 20 kDa cleavage product of I_2^{PP2A}. Exactly what causes the translocation of I_2^{PP2A} from the nucleus to the cytoplasm and the mechanisms of the generation of its N-terminal cleavage fragments remain to be studied. It is currently unknown whether, along with the 20 kDa fragment, full length I_2^{PP2A} also translocates to the cytoplasm. I_2^{PP2A} is known to have several nuclear [12] and cytoplasmic functions [27]. The presence of a highly acidic C-terminal region brings about its nuclear retention [12] and I_2^{PP2A} lacking the C-terminal acidic domain is localized mainly in the cytoplasm. Protein transport represents a very efficient

way to rapidly modify the activity of such proteins by changing their localization, and involves a group of carrier proteins called Transportins (Exportins or Importins) [28]. It is very likely that I_2^{PP2A} has both nuclear localization signal (NLS) and nuclear export signal (NES) in its sequence. However, the exact nature of the processes that regulate the intracellular shift of I_2^{PP2A} remains to be understood. Regarding the generation of the 20 kDa cleavage fragment of I_2^{PP2A}, we have previously reported that recombinant human I_2^{PP2A} was digested faster with AD than control brain extract, generating similar proteolytic fragments of the inhibitor [13], but the specific protease(s) involved in the cleavage is not yet known.

PP2A activity is well known to be critical for cell survival and is thus, tightly regulated. We found 11% and 33% inhibition of PP2A activity by 5 nM and 25 nM I_2^{PP2A} in vitro, and a similar corresponding increase in phosphorylation of tau at PHF-1 and 12E8 sites. This increase in abnormal hyperphosphorylation of tau over a long period of time in AD, which on average has a progression period of 7–10 years, is apparently detrimental to the affected neurons.

We have previously reported that Memantine, a moderate affinity un-competitive inhibitor of NMDA receptors, can reverse okadaic acid-induced inhibition of PP2A in organotypic explant cultures of rat brain hippocampal slices, but the exact nature of such a rescue of PP2A activity by Memantine was not known. The present study also, for the first time, demonstrates a possible role of Memantine in modulating PP2A signaling by reversing I_2^{PP2A} induced inhibition of PP2A activity. We show that Memantine in cultured PC12 cells stably expressing tau_{441} was able to inhibit the abnormal hyperphosphorylation of tau caused by transient transfection of the cells with I_2^{PP2A}. Memantine had no effect on expression of the transgene suggesting that it probably alters the biological activity of I_2^{PP2A} directly. Furthermore, we show that Memantine could disinhibit the I_2^{PP2A} induced-inhibition of PP2A activity in vitro at therapeutic concentrations [30]. Taken together, the present study shows that Memantine restores normal phosphorylation state of tau, probably by downregulating I_2^{PP2A}'s ability to inhibit PP2A.

Memantine is a positively charged molecule and in order for it to influence PP2A activity it must gain entry into the cell. It was recently reported that the binding site of Memantine is an intracellular Mg^+ site on the NR-1 subunit of NMDAR [29]. Therefore, it is reasonable to assume that some of the drug

probably finds its way into the cytoplasm where it may interact with I_2^{PP2A}. Memantine acts as an un-competitive inhibitor of mostly open-gated NMDAR-associated Ca^{2+} channels. Under physiological conditions, the NMDA channel is activated for only brief periods of time due to relief of Mg^{2+} blockade. This contrasts with the situation in chronic neurodegenerative conditions like AD, where there is an overactivation of NMDAR [30]. In AD, for example, the brain metabolism is decreased [31] and therefore, the energetically deprived neurons become depolarized easily due to a failure in maintaining ionic homeostasis. This, in turn, may lead to the unbinding of the positively charged Mg^+ ion from its binding site, leading to the opening of NMDAR channels (despite the absence of increased glutamate) and influx of Ca^+. As more neurons die, the concentration of glutamate in the extracellular milieu is likely to increase, leading in turn to a persistent overactivation of NMDAR. Such an increase in NMDAR activity leads to the channels being excessively open (for relatively longer periods of time). It is this excessive opening of the NMDAR channels which allows Memantine to gain access to the internal Mg^{2+} binding site. Since AD is a slowly progressive disorder and the persistent NMDAR hyperactivity sets in probably several years after the clinical onset of the disease, it is quite logical to expect any effect of Memantine to occur only in the later stages of the disease. Although hyperphosphorylation of tau is an early event in the pathogenesis of AD, in the absence of NMDAR hyperactivity in early stages of the disease (a state when the channels are open only briefly and Memantine has limited chance of getting inside the cell), we would not expect Memantine to interact with I_2^{PP2A} and inhibit abnormal phosphorylation of tau. It is only when continued insults coupled with "metabolic-paresis" leads to persistent NMDAR hyperactivity; a common event in the late stages of many chronic neurodegenerative conditions, that Memantine is able to influence both the NMDAR as well as I_2^{PP2A} induced inhibition of PP2A activity, and henceforth, tau hyperphosphorylation. Decreased levels of hyperphosphorylated tau in the CSF of patients with moderate to severe AD receiving Memantine therapy could provide a very useful proof of principle of restoration of PP2A activity as a therapeutic target.

Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.06.021.

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