Memantine inhibits and reverses the Alzheimer type abnormal hyperphosphorylation of tau and associated neurodegeneration

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Abstract Memantine, an *N*-methyl-D-aspartate (NMDA) receptor antagonist, reduces the clinical deterioration in moderate-to-severe Alzheimer disease (AD) for which other treatments are not available. The activity of protein phosphatase (PP)-2A is compromised in AD brain and is believed to be a cause of the abnormal hyperphosphorylation of tau and the consequent neurofibrillary degeneration. Here we show that memantine inhibits and reverses the PP-2A inhibition-induced abnormal hyperphosphorylation and accumulation of tau in organotypic culture of rat hippocampal slices. Such restorative effects of memantine were not detected either with 5,7-dichlorokynurenic acid or with D(−)-2-amino-5-phosphopentanoic acid, NMDA receptor antagonists active at the glycine binding site and at the glutamate binding site, respectively. These findings show (1) that memantine inhibits and reverses the PP-2A inhibition-induced abnormal hyperphosphorylation of tau/neurofibrillary degeneration and (2) that this drug might be useful for the treatment of AD and related tauopathies.

Keywords: Alzheimer disease; Protein phosphatase-2A; NMDA receptor; Tau hyperphosphorylation; Memantine; Neurofibrillary degeneration

1. Introduction

Neurofibrillary degeneration, which is a hallmark of Alzheimer disease (AD) and related tauopathies such as frontotemporal dementia, correlates directly with dementia [1,2]. Microtubule associated protein tau is abnormally hyperphosphorylated in these neurodegenerative diseases and in this form is the major protein subunit of the neurofibrillary changes [3]. The biological activity of tau is regulated by its degree of phosphorylation [4]. While normal tau promotes the assembly and maintains the structure of microtubules, the abnormally hyperphosphorylated form of this protein in AD cytosolic abnormally hyperphosphorylated tau (AD P-tau) instead sequesters normal tau, microtubule-associated protein 1 (MAP1) and MAP2 and causes disassembly of microtubules [5,6]. This toxic property of the AD P-tau, which through the breakdown of the microtubule network can compromise axonal transport and lead to neurodegeneration, appears to be solely due to its abnormal hyperphosphorylation because dephosphorylation by a phosphatase restores it into a normal-like protein in vitro [6,7]. The activity of phosphoseryl/phosphothreonyl protein phosphatase (PP)-2A, which regulates the phosphorylation of tau [8,9] is compromised in AD brain [10,11] and is believed to be a cause of the abnormal hyperphosphorylation of tau. A recent study has shown that the *N*-methyl-D-aspartate (NMDA) receptor is in a complex with PP-2A and that stimulation of this receptor can lead to the dissociation of PP-2A from the complex and the reduction of PP-2A activity in cultured cells [12]. Memantine is a low to moderate affinity uncompetitive NMDA receptor antagonist which leads to functional improvement and reduces care dependence in moderate to severe AD patients [13,14].

We show the effect of memantine on the abnormal hyperphosphorylation of tau and the protein kinases (PKAs) and PPs involved in organotypic culture of rat hippocampal slices in which PP-2A activity was inhibited by okadaic acid (OA). Memantine reversed the OA-induced decrease in PP-2A activity, increase in calcium, calmodulin-dependent protein kinase II (CaMKII) and cyclin AMP-dependent PKA activities and abnormal hyperphosphorylation of tau to the control level, and inhibited the neurodegeneration associated with these changes. It reversed the decrease and aggregation of MAP2 and the phosphorylation and aggregation of neurofilament-heavy/medium (NF-H/M) subunits. Such effects of memantine were not detected either with 5,7-dichlorokynurenic acid (DK) or with D(−)-2-amino-5-phosphonopentanoic acid (AP), NMDA receptor antagonists active at the glycine binding and the glutamate binding sites. These findings suggest (1) that memantine can inhibit and reverse the Alzheimer type abnormal hyperphosphorylation of tau and neurofibrillary degeneration and (2) that this effect of memantine might involve modulation of PP-2A signaling in addition to its activity as an NMDA antagonist.

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Abbreviations: AD, Alzheimer disease; AD P-tau, AD cytosolic abnormally hyperphosphorylated tau; AP, D(−)-2-amino-5-phosphonopentanoic acid; cdk5, cyclin dependent protein kinase 5; CaMKII, calcium, calmodulin-dependent protein kinase II; DK, 5,7-dichlorokynurenic acid; LDH, lactate dehydrogenase; MAP, microtubule associated protein; MAPK, mitogen-activated protein kinase; NF-H/M, neurofilament-heavy/medium; NMDA, *N*-methyl-D-aspartate; OA, okadaic acid; PHF, paired helical filaments; PMSF, phenylmethyl sulfonyl fluoride; PP, protein phosphatase

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2. Materials and methods

2.1. Hippocampal organotypic cultures

Organotypic cultures of rat hippocampal slices were prepared from 20–30 day old Wistar rats and cultured with the interface method as described previously [15]. Slices were treated with OA (Ammonium salt from CalBiochem, La Jolla, CA) to inhibit PP-2A activity, glutamate (Sigma–Aldrich, St. Louis, MO) as an NMDA receptor agonist and memantine (a gift from Merz Pharmaceuticals, Frankfurt, Germany), DK (Sigma–Aldrich) and AP (Sigma–Aldrich) as NMDA receptor antagonists. When the slices were treated, the reagents were applied into the culture medium. At different time points, the slices collected with a brush were washed twice and then homogenized at 4 °C using a Teflon-glass homogenizer in 50 mM HEPES, pH 7.0, 10 mM β-merceptoethanol (BME), 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 2.0 mM benzamidine and 2.0 μg/ml each of aprotinin, leupeptin and pepstatin. The homogenate was then divided into two parts, one was centrifuged at $16\,000 \times g$ for 15 min and the supernatant was used to assay activities of PP-2A and PP-1. The rest of the homogenate was diluted 1:1 with a phosphatase inhibitor cocktail (20 mM β-glycerophosphate, 2 mM Na_3VO_4 and 100 mM NaF, pH 7.0) and either used for Western blots or centrifuged at $16\,000 \times g$ for 15 min and the resulting supernatant used to determine the kinase activities.

2.2. Protein phosphatase assays

Activities of PP-2A and PP-1 were assayed towards [^{32}P] phosphorylase-a as a substrate as described previously [16]. A PP-1 specific inhibitor, inhibitor-1 was included in the assays for PP-2A activity. PP-1 activity was calculated by subtracting the PP-2A activity from the total phosphorylase-a phosphatase activity (PP-1/PP-2A) assayed in the absence of inhibitor-1.

2.3. PKA assays

CaMKII activity was assayed as described [17] using a reaction mixture containing 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM BME, 10 μg/ml calmodulin (CaM), 20 μM syntide (Sigma, St. Louis MO, USA), 0.06 mg/ml slice extract and 200 μM [γ^{32}P] ATP. The activity of cyclic AMP-dependent PKA was determined as above except the reaction mixture contained 70 mM $NaHPO_4$, pH 6.8, 14 mM $MgCl_2$, 1.4 mM EDTA, 30 μM malantide (Sigma, St. Louis, MO, USA), 200 μM [γ^{32}P] ATP and 0.06 mg/ml slice extract. Mitogen-activated protein kinase (MAPK) activity was determined as described previously [18].

2.4. Radioimmuno-dot-blots and Western blots

Levels of phosphorylation of tau at different sites were assayed by the radioimmuno-dot-blots of the slice homogenates (4 μg protein/dot) as described previously [19]. The primary tau antibodies used were as follows: polyclonal antibodies (pAbs) pS-262 (1:1000) to P-Ser-262 (Bio-source), pS-212 (1:1000) to P-Ser 212 (Bio-source), pS-214 (1:1000) to P-Ser 214 (Bio-source), R145d (1:3000) to P-Ser 422 [18], R134d (1:5000) to total tau [20] or monoclonal antibodies (mAbs) PHF-1 (1:200) to P-Ser 396/404 [21,22] and 12E8 (1:500) to P-Ser-262/356 [23]. The phosphorylation of tau at Ser-262 and the levels of MAP2 and phosphorylated NF-H/M were assayed by [^{125}I] Western blots (30 μg homogenate/lane), using pAb pS-262 (1:1000, Biosource) or mAb SMI 31 to phosphoneurofilaments-H/M subunits (pNF-H/M), or mAb SMI 52 to MAP2 (both from Sternberger Monoclonals, Inc.) as primary antibodies and [^{125}I] radiolabeled secondary antibodies (Amersham).

2.5. Immunohistochemistry

After different treatments some of the hippocampal slices were fixed in periodate/lysine/paraformaldehyde solution [24] at 33 °C for 5 h and then kept in 1% Triton X-100 in PBS (pH 7.4) and 0.1% NaN_3 for 72 h at room temperature. The slices were then incubated in blocking solution containing PBS, 0.1% Triton X-100 and 10% normal horse serum for 3 h at room temperature. Thereafter, the slices were rinsed in PBS and incubated for 2 days in primary antibody at 4 °C. The primary antibodies used were as follows: mAb SMI 31 to pNF-H/M (1:10 000), mAb SMI 52 to MAP2 (1:20 000) and pAb pS-262 (1:1000) to tau phosphorylated at Ser-262. The immunoreactivity was visualized by incubation with peroxidase-conjugated goat antimouse/rabbit IgG (1:1000, Jackson) for 3 h at 37 °C, followed by 0.05% diaminobenzidine (DAB) and H_2O_2 (0.01%) for 10 min.

2.6. Lactate dehydrogenase (LDH) activity and protein

The LDH released into the culture medium from the slices was determined colorimetrically using Cytotox 96R Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) according to the manufacturer's protocol. Protein concentrations were assayed by the modified Lowry method [25].

3. Results

3.1. Okadaic acid (OA) inhibits PP-2A and stimulates CaMKII activity

OA is an extensively studied experimental irreversible non-competitive inhibitor of PP-2A and PP-1 [26]. We elected to employ for the present study as a model the organotypic culture of rat hippocampal slices in which the PP-2A activity was inhibited by OA. This ex vivo system provides a direct and practical access to mammalian brain for studying the effect of pharmacological compounds on the biology of specific proteins and the cascades involved.

OA inhibited PP-2A activity in the hippocampal slice cultures in a time- (3–48 h) and concentration- (10–1000 nM) dependent manner (Fig. 1(a)). We found that 10 nM OA inhibited ~20% of PP-2A activity during 24 h treatment. OA concentrations of 100 nM and 1 μM resulted in ~40% and ~65% inhibition of PP-2A activity, respectively, during 24 h treatment. Treatment up to 48 h at either concentration of OA produced an additional 20–30% inhibition of PP-2A activity. In agreement with previous studies in which metabolically active rat brain slices were treated with 0.1–5 μM OA up to 3 h [8,9,27] no inhibition of PP-1 activity was detected (Fig. not shown).

Several PKA activities are known to be regulated by reversible phosphorylation and some of these kinases are substrates for PP-2A. We found that the CaMKII activity increased with increase in the inhibition of PP-2A activity by OA treatment (Fig. 1(b)). An increase of ~20%, ~70% and ~140%, respectively, was observed in cultures treated with 10, 100 and 1000 nM OA for 3–48 h. An increase of ~20% was observed in PKA activity in the slice cultures treated with 100 nM OA for 24 h or 48 h but not for 3 h (Fig. 2(c)). However, no significant change in the activities of GSK-3 or cyclin dependent protein kinase 5 (cdk5) in the OA-treated cultures was detected (Fig. not shown). The cell death in the cultures as determined by assaying LDH activity released in the culture medium was markedly increased both with increase in the OA concentration up to 1 μM and duration of the treatment up to 48 hours studied (Fig. 1(c)). To keep cytotoxic effects of OA low and to have a model of a significant inhibition of PP-2A activity, we chose the treatment of the slice cultures with 100 nM of the drug for all subsequent studies.

3.2. Memantine restores the OA-altered PP-2A, CaMKII and PKA activities to the normal level

The activity of CaMKII is stimulated by Ca^{2+}/CaM through its autophosphorylation at Thr-286/287 and is regulated by PP-2A which dephosphorylates this site [9]. Thus, stimulation of CaMKII activity by inhibition of PP-2A provided a very useful non-NMDA pathway model of a protein phosphorylation/dephosphorylation imbalance. Employing this model we

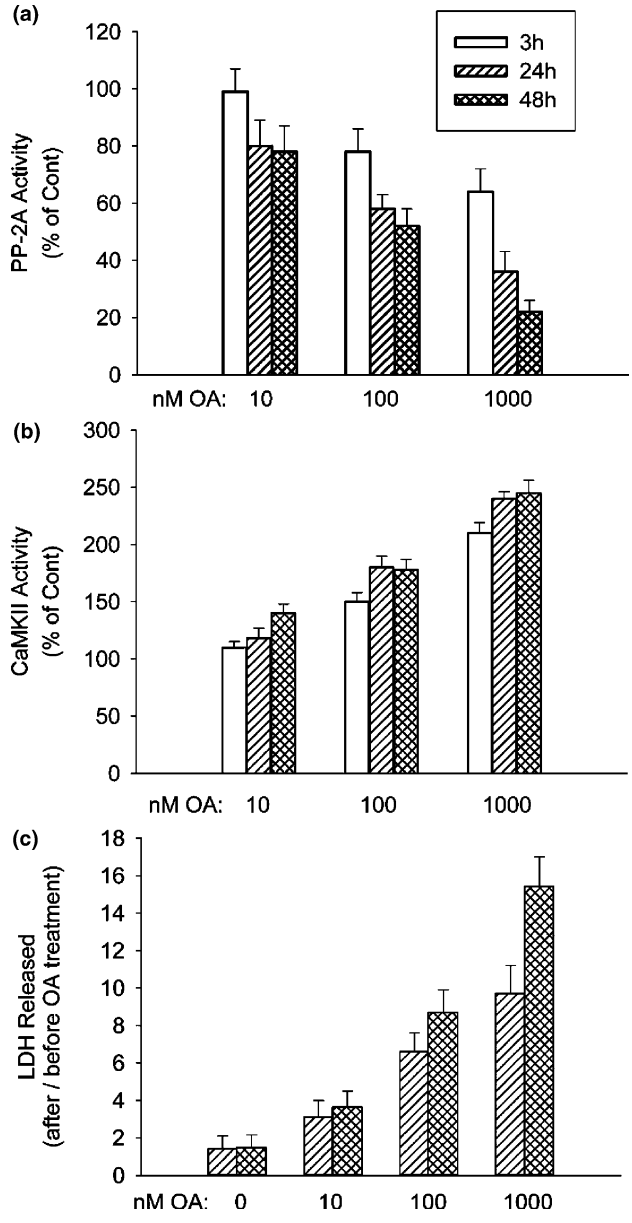

Fig. 1. Inhibition of PP-2A and stimulation of CaMKII activities, and release of LDH by OA in hippocampal slices in culture. Hippocampal slices were treated with either medium alone (control) or with OA. The phosphatase and CaMKII activities were expressed as means ± S.D. obtained from at least three independent assays. (a) PP-2A activity as % of control-treated slices. A decrease of 42% ($P < 0.05$) and 78% ($P < 0.01$) in PP-2A activity was observed in slices treated with 100 nM OA for 24 h and with 1000 nM OA for 48 h, respectively. (b) CaMKII activity increased to 180% ($P < 0.01$) and 240% ($P < 0.01$) in hippocampal slices treated for 24 h with 100 and 1000 nM OA, respectively. (c) The cell death as assayed by LDH activity released in the medium (ratio of LDH activity before/after OA treatment) increased with increase in OA concentration and treatment period ($P < 0.001$).

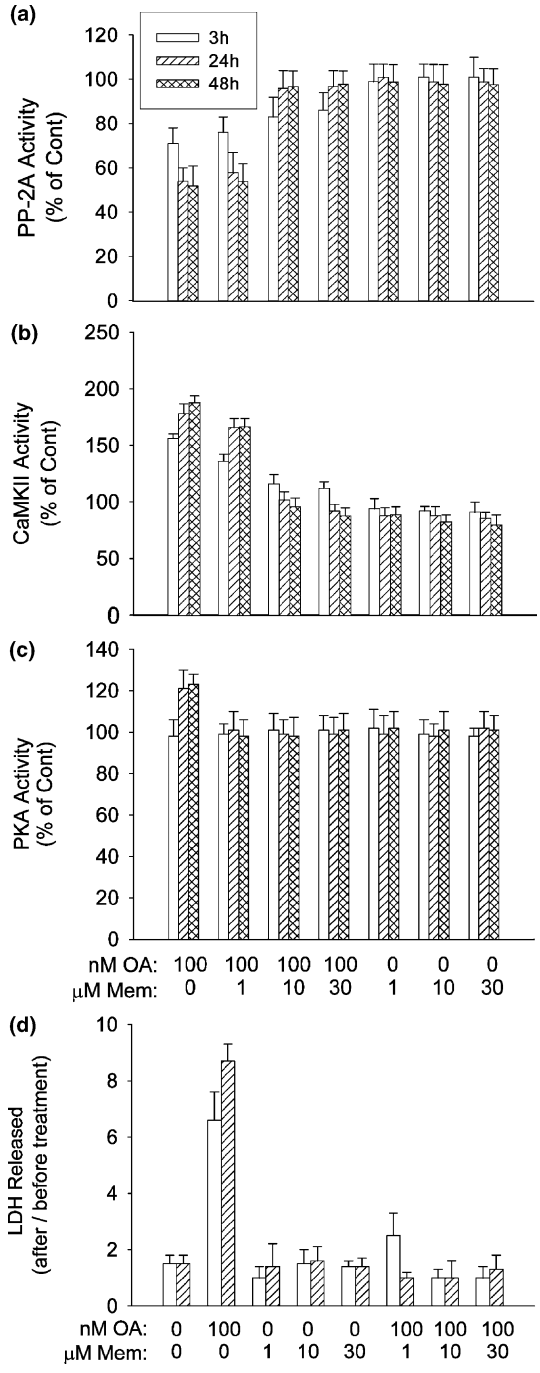

Fig. 2. Restoration of activities of PP-2A and CaMKII to normal level and inhibition of LDH release by memantine in OA-treated hippocampal slices. (a)–(c) The slices in culture were treated without control or with 100 nM OA for 3, 24 or 48 h, followed by wash to remove OA and treatment with 0, 1, 10 or 30 μM memantine for 24 h. The phosphatase and the kinase activities were expressed as the percentage of the corresponding activities of slices treated with medium alone (control) in culture. Bars represent means ± S.D. obtained from at least three independent assays. (a) Memantine restored the PP-2A ($P < 0.02$) and (b) CaMKII activities ($P < 0.02$) to normal level and had no significant effect on activities in control hippocampal slices. (c) As low as 1 μM memantine restored the OA-induced increase in PKA activity to normal level ($P < 0.05$) and (d) completely inhibited the OA-induced cell death ($P < 0.001$).

investigated the effect of memantine on the phosphorylation of tau and the PKA and PP activities involved. We found that 10 μM memantine during 24 h restored the OA-induced changes in the activities of PP-2A, CaMKII and PKA to normal levels (Fig. 2(a)–(c)). Memantine had no significant effect on the activities of cdk5 or GSK-3 in the OA-treated cultures (Fig. not shown), or the activities of PP-2A, CaMKII or PKA in the control/untreated cultures (Fig. 2(a)–(c)). The effect of memantine on the restoration of PP-2A and CaMKII activities could be observed at 1 μM concentration but the full effect was

seen at 10 μM concentration of the drug. Neither increase of memantine from 10 to 30 μM nor duration of the treatment from 24 to 48 h resulted in any significant additional effect on the restoration of either PP-2A or CaMKII activity. The OA-induced cell death in the cultures was completely inhibited by 1 μM memantine during 24 h and by 10 μM of the drug by 3 h (Fig. 2(d)). In the control cultures memantine had no effect on the LDH activity in the medium using 1–30 μM concentrations of the drug investigated.

3.3. Memantine restores tau phosphorylation to normal level

PP-2A downregulates the activity of CaMKII, a major tau Ser-262 kinase in the mammalian brain [9,28]. Since we found in the OA-treated hippocampal cultures a marked increase in CaMKII activity and its restoration to normal level by memantine, we studied in these cultures the effect of these treatments on the phosphorylation of tau at Ser-262 and as a control at Ser-212, Ser-214, Ser-396/404 and Ser-422. Tau Ser-212 is known to be phosphorylated by cdk5 and MAPK, Ser-214 by PKA, Ser-396/404 by GSK-3β and cdk5 and Ser-422 by stress activated PKAs [see 29]. We found a marked increase in the phosphorylation of tau at Ser-262 and Ser-422 and a modest increase at Ser-214, without any significant change in the level of total tau, in the OA-treated cultures (Fig. 3(a)). Further treatment with 10 μM memantine for 24 h restored the tau phosphorylation at Ser-262 and Ser-214 to normal levels (Fig. 3(a) and (b)). However, memantine had no effect on the OA-induced phosphorylation of tau at Ser-422 (Fig. 3(a)). We found that 2 μM memantine inhibited the tau phosphorylation at Ser-262 and that this effect was maximal at 5 μM concentration of the drug (Fig. 3(c)).

Immunocytochemical staining of the untreated and treated cultures with phosphodependent rabbit antibody to phospho tau Ser-262 revealed a marked increase in the p-Ser-262 staining in cells in the area corresponding to stratum oriens and alvus in the OA-treated cultures (Fig. 3(d)). Long processes, presumably axons with irregular contour and short rod-shaped fragments reminiscent of neuropil threads/degenerating neurites were often seen along the outer regions of stratum radiale (Fig. 3(d, ii)). In the cultures treated with 10 μM memantine for 24 h following the OA treatment, the p-Ser-262 immunostaining of the neurons markedly decreased (Fig. 3(d, iii)).

3.4. Memantine inhibits aggregation of MAP2 and NFs

A protein phosphorylation/dephosphorylation imbalance in the neuron might not only affect the phosphorylation of tau but like in AD, might also affect other cytoskeletal proteins. We found that following the OA treatment, the MAP2 immunostaining increased markedly in the somatodendritic compartments of neurons, possibly interneurons, with a corresponding decrease in the neuropil in an area roughly corresponding to stratum oriens (Fig. 4(a, i, ii)). Dystrophic dendrites/neuropil threads with the characteristic of beaded uneven contour, alternating swollen and shrunken segments were seen, suggesting a degeneration of neurons. In the memantine treated cultures a decrease in the degeneration and restoration of the staining of the neuropil were observed (Fig. 4(a, iii)). Western blots revealed a decrease in MAP2 in the OA-treated cultures and a reversal to normal levels by treatment with memantine (Fig. 4(b)).

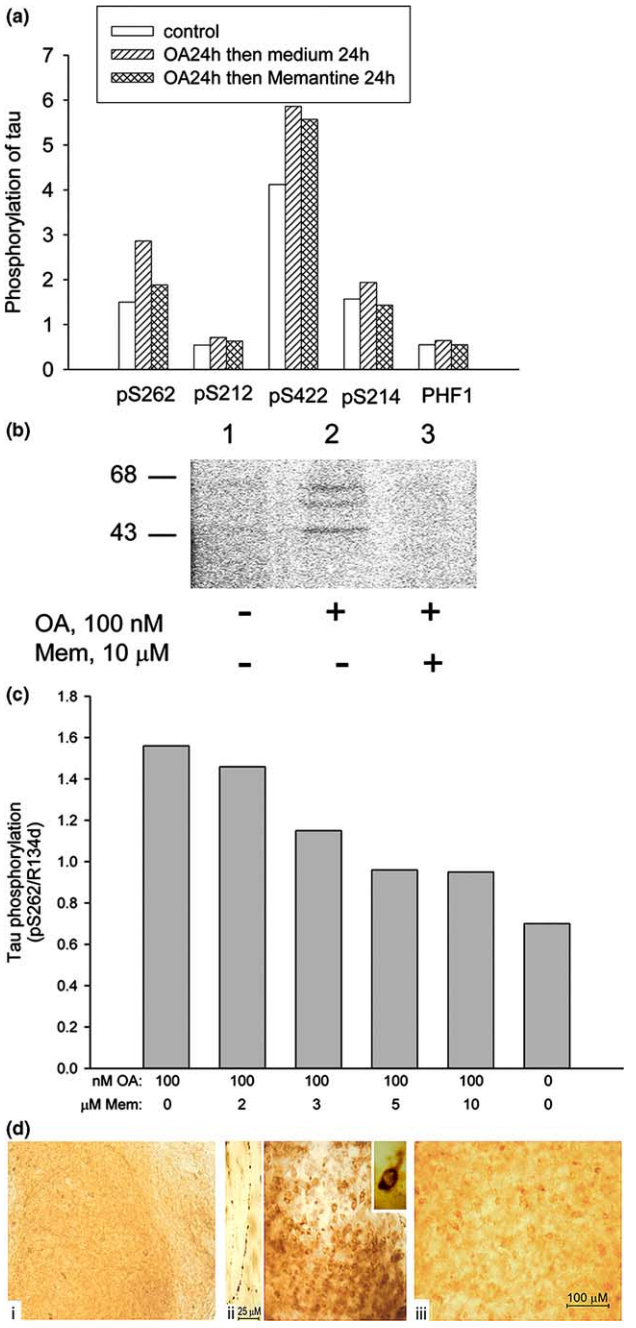

Fig. 3. Restoration of OA-induced tau phosphorylation at Ser-262 to normal level by memantine. The hippocampal slices in culture were treated with or without (control) 100 nM OA for 24 h, washed and then treated with (unless otherwise indicated) or without (control) 10 μM memantine for 24 h. (a) The immunoreactivities at different sites obtained with different antibodies quantitated by a phosphorimager and normalized against the level of total tau detected with pAb R134d. (b) [^{125}I] Western blots of the cultured hippocampal slices developed with pAb pS-262: 1, control; 2, OA 24 h, then medium 24 h; 3, OA 24 h, then memantine 24 h. (c) Effect of different concentrations of memantine on the restoration of the OA-induced phosphorylation of tau at Ser-262 as determined by radioimmunodot-blots as in (a). The data are the averages of two independent assays. (d) Immunocytochemical staining showing tau phosphorylation at Ser-262 in cultured hippocampal slices. i, Control, with medium only; ii, treated with 100 nM OA 24 h, then medium 24 h; iii, 100 nM OA 24 h, then memantine 24 h. The insets (ii) show at high magnification Ser-262 hyperphosphorylation and accumulation of tau in the somatodendritic compartments of a neuron and in axons.

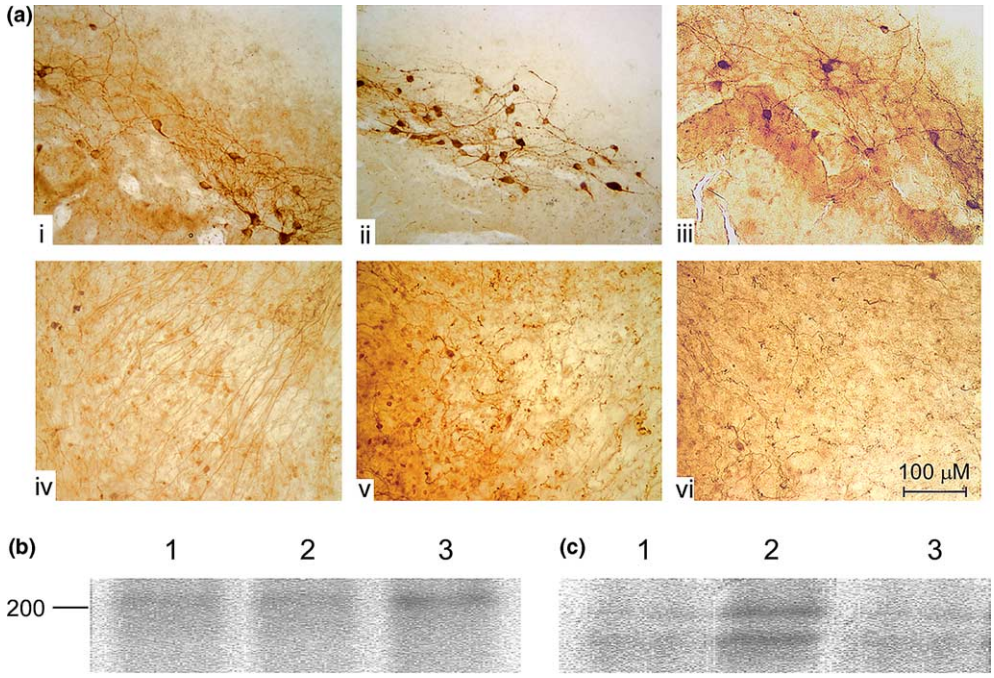

Fig. 4. Restoration of OA-induced changes in MAP2 and NFs by memantine. (a) (i–vi) Immunocytochemical staining of MAP2 (i–iii) and NFs (iv–vi) in cultured hippocampal slices. (i, iv) Control-treated; (ii, v) treated with 100 nM OA for 24 h, followed by medium for 24 h; (iii, vi) 100 nM OA 24 h, followed by 10 μM memantine, 24 h. (b) and (c) [^{125}I] Western blots of slice homogenates showing changes in MAP2 (b) and NFs (c). Lane 1, control; lane 2, treated with OA as in (a); lane 3, treated with OA and then with memantine as in (a).

The immunocytochemical labeling of OA treated cultures with antibodies to pNF-H/M revealed an increase in phosphorylation and accumulation of NF-H/M in the neuronal cell bodies and their neurites in the areas corresponding to stratum oriens and alveus. Thick tortuous, thread-like and beaded fragmented neurites similar to neuropil threads were abundantly seen in the OA treated cultures (Fig. 4(a, v)). Memantine, 10 μM, during 24 h treatment partially reversed these pathological changes (Fig. 4(a, vi)). Western blots of the OA- and OA plus memantine-treated cultures confirmed the reversal of phosphorylation of NF-H/M subunits by memantine (Fig. 4(c)).

3.5. *The restorative effect of memantine on the activities of PP-2A and CaMKII is through PP-2A signalling and not by its interaction with OA*

Since memantine only restored the OA-induced decrease in PP-2A and increase in CaMKII but had no effect on these activities in the control-treated cultures, we investigated whether the memantine effect was due to any direct interaction with OA. We found that the removal of OA after 24 h treatment restored the PP-2A and CaMKII activities slightly, whereas the treatment of the cultures with both OA and memantine for 24 h or with OA for 24 h, wash and then with memantine for 24 h almost completely restored the two enzyme activities (Fig. 5(a) and (b)). These findings suggested that the effect of memantine on PP-2A and CaMKII activities was unlikely to be through any direct interaction with OA and that memantine could both inhibit and reverse these changes. Furthermore, the addition of OA (100 nM) to a $16\,000 \times g$ extract of homogenate of untreated cultures inhibited ~90% of PP-2A activity and the addition of different concentrations of memantine, 1–60 μM had no significant effect on the phosphatase activity (Fig. 5(c)). Similarly the addition of memantine, 1–10 μM to the $16\,000 \times g$ extract of the OA-treated cultures failed to restore the PP-2A activity (Fig. 5(d)). All these data taken together unequivocally demonstrated that memantine restored PP-2A activity and probably as a consequence the CaMKII activity through PP-2A signaling and not by any direct interaction with OA.

3.6. *Effect of memantine on PP-2A and phosphorylation of tau is primarily through modulation of PP-2A signalling*

CaMKII can be activated either by autophosphorylation resulting from inhibition of PP-2A or activation of NMDA receptor. PP-2A is tethered to NR3A subunit of the NMDA receptor. Stimulation of this receptor leads to dissociation and decrease in the activity of PP-2A [12]. The NR3A subunit is, however, greatly down-regulated in the adult and thus, stimulation of the receptor in adult brain might not have any significant effect on the PP-2A activity. We found that the treatment of the hippocampal slice cultures with 0.3 mM glutamate for 1 h (Fig. 6) but not 24 h (data not shown) produced a marked increase in the phosphorylation of tau at Ser-262. However, this change, which was transient, was associated only with an increase in CaMKII activity but not accompanied by any detectable changes in the activities of PP-2A or MAPK the activity of which is regulated by PP-2A (Fig. 6). These studies suggested that in brain the activation of the NMDA receptor by its natural agonist glutamate activated CaMKII without affecting the PP-2A activity, and that the restoration of the OA-induced inhibition of the phosphatase activity by memantine might therefore have been independent of its activity as an NMDA receptor antagonist. To further

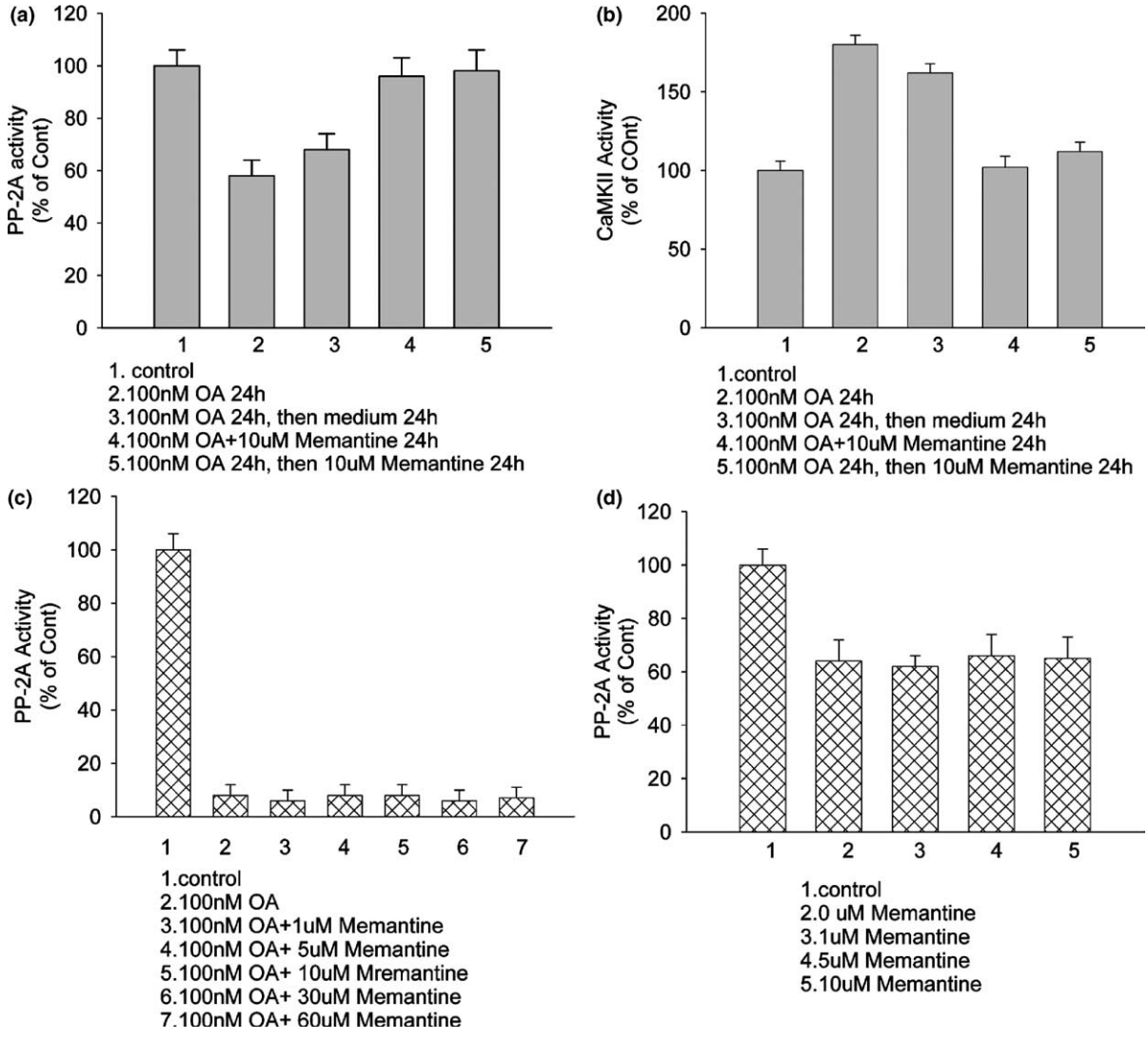

Fig. 5. Restoration of the activities of PP-2A and CaMKII of OA-treated hippocampal slices by memantine both when employed along with OA or followed by OA treatment. The phosphatase and kinase activities of OA or memantine-treated samples were expressed as means ± S.D. obtained from at least three independent assays. (a) Restoration of PP-2A activity by memantine ($P < 0.05$). (b) Restoration of CaMKII activity by memantine ($P < 0.05$). All treatments were the same as in (a). Compare bars 4 with 5 and 4/5 with 2/3. (c) Addition of OA (100 nM) to the hippocampal slices extract resulted in 90% inhibition of PP-2A activity, and addition of memantine to this extract had no significant effect on the phosphatase activity. d. Addition of memantine to the extract of cultured hippocampal slices in which PP-2A activity had been inhibited (~40%) by OA for 24 h, had no significant effect on the phosphatase activity (compare bars 3–5 with bars 1 and 2).

examine this issue, we investigated, in addition to memantine, the effect of another two NMDA receptor antagonists on the restoration of the OA-induced changes in PP-2A and in CaMKII activities and phosphorylation of tau at Ser-262. For these studies we selected DK and AP which antagonize competitively the NMDA receptor through the strychnine-insensitive glycine binding site and the glutamate binding site, respectively [30]. We found that while 5 μM memantine restored the OA-induced changes in the activities of PP-2A and CaMKII and the phosphorylation of tau at Ser-262, neither 5–10 μM DK nor 5–10 μM AP could restore these changes (Fig. 7). These studies suggested that in addition to its activity as an NMDA receptor antagonist, memantine modulates PP-2A signalling, and probably this latter effect is involved in the restoration of PP-2A activity and thereby the CaMKII activity and the phosphorylation of tau to normal level.

4. Discussion

Abnormal hyperphosphorylation of tau/neurofibrillary degeneration is pivotally involved in the pathogenesis of AD and related tauopathies. One of the major goals of research on this family of diseases is to develop drugs that can inhibit and reverse this lesion. We have discovered (i) that memantine can inhibit and reverse the abnormal hyperphosphorylation and aggregation of tau and NF-H/M and loss of MAP2 and the associated neurodegeneration; and (ii) that these effects of this drug probably involve its previously unknown ability to modulate PP-2A signaling, a property not detected in two other NMDA receptor antagonists, i.e., DK and AP examined in the present study.

Treatment of the OA-treated hippocampal slices in culture with memantine practically completely restored the activities

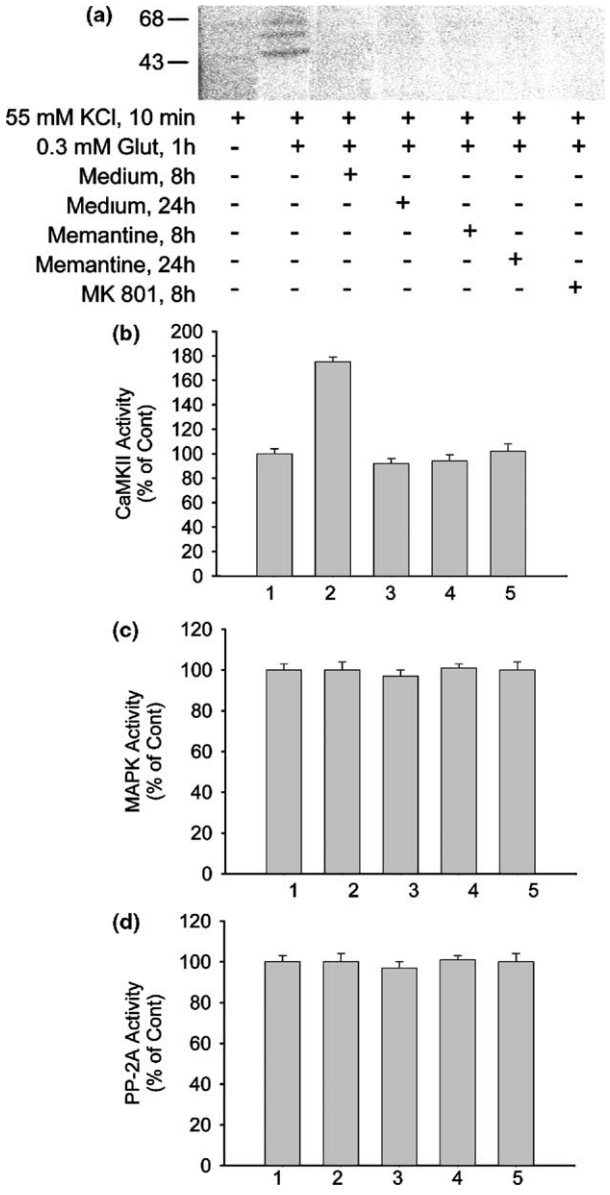

Fig. 6. Effect of glutamate on phosphorylation of tau at Ser-262 and on PP and PKA activities in hippocampal slices in culture. Hippocampal slices in culture were first treated with 55 mM KCl, 10 min, to deblock calcium channels and then with 0.3 mM glutamate 1 h, followed by medium, 10 μM memantine or 15 μM MK801 for 3, 8, or 24 h. The slices were then homogenized and either employed for [^{125}I] Western blots developed with PSer-262 tau antibody (a) or centrifuged at $16\,000 \times g$ for 15 min, and the extracts used for assaying the activities of PP-2A, CaMKII and MAPK (b,c, and d). The phosphatase and kinase activities were expressed as percentage of the activity of control samples incubated in medium alone. Bars represent means ± S.D. obtained from at least three independent assays. (b)–(d) 1, in medium, 3 h; 2, 0.3 mM glutamate, 1 h; 3, 0.3 mM glutamate, 1 h, followed by medium 3 h; 4, 0.3 mM glutamate, 1 h, followed by 10 μM memantine, 3 h; 5, 0.3 mM glutamate, 1 h, followed by 15 μM MK801, 3 h. (b) After 1 h glutamate treatment, CaMKII activity increased to ~180% ($P < 0.001$) and the phosphorylation of tau at Ser-262 increased markedly (a).

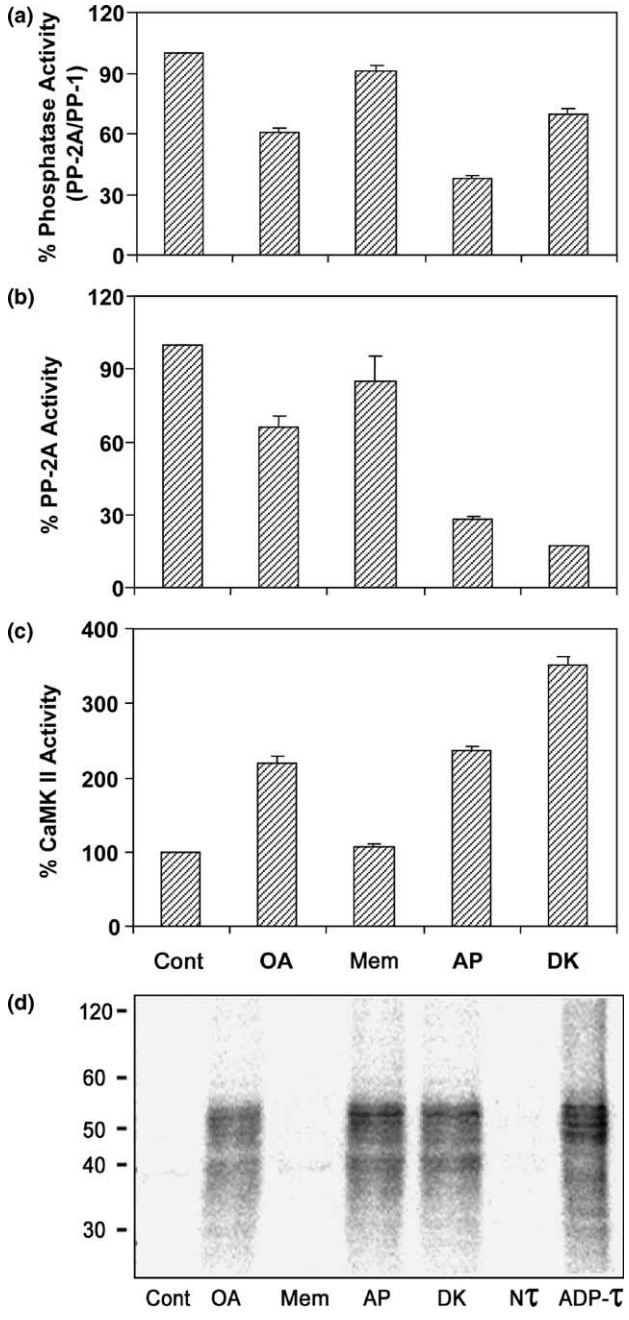

Fig. 7. Effect of memantine, AP and DK on the OA-induced changes in PP-2A and CaMKII activities and phosphorylation of tau at Ser-262. Hippocampal slices in culture were treated with OA or medium alone (cont) for 24 h, followed by a wash and then fresh medium without (control) or with 5 or 10 μM (data not shown) of memantine (Mem), AP or DK for another 24 h. Total phosphatase (a) (PP-2A/PP-1), PP-2A (b) and CaMKII (c) activities as percentage of control and Western blots (d) developed for the phosphorylation of tau at Ser-262 show that only memantine but neither AP nor DK restore the OA-induced changes to normal levels. Nτ, 5 μg/lane, recombinant human brain tau$_{441}$; AD Pτ, abnormally hyperphosphorylated tau isolated from an AD brain. Rest of the details same as in Figs. 1–4.

of PP-2A, CaMKII and PKA, and phosphorylation of tau at Ser-262 and Ser-214 but not of Ser-422 to normal state and inhibited the associated neurodegeneration. Ser-262, Ser-214 and Ser-422, which are major abnormally phosphorylated tau sites in AD, are phosphorylated by CaMKII, PKA and stress-activated PKAs, respectively [9,27,28,31]. Ser-262 is the only site abnormally hyperphosphorylated in the microtubule binding domains and the phosphorylation of this site, which is

believed to be dynamically involved in tau's activity in stabilizing microtubules, results in inhibition of the microtubule assembly-promoting activity of tau [17,32]. In the present study, the reversal of the OA-induced cell death and the abnormal hyperphosphorylation of tau at Ser-262, but not at Ser-422 to normal-like state by memantine is consistent with the critical role of the former site in converting tau into an inhibitor/toxic molecule. The phosphorylation of tau at Ser-422 is apparently a later event because this site is phosphorylated in PHF and not cytosolic AD P-tau, and a recent study has confirmed its association to relatively mature tangles in transgenic mice expressing tau P301L mutation [33]. The fact that memantine treatment which completely reversed the PP-2A-induced cell death had no effect on phosphorylation of Ser-422 suggests that this site might not be involved in cytotoxicity but mainly in promoting tau's self assembly into PHF/neurofibrillary tangles.

The immunocytochemical studies revealed abnormal hyperphosphorylation at Ser-262 and accumulation of tau in the OA-treated cultures. The hyperphosphorylation of tau was found primarily in the cells of the stratum oriens and the alveus and in a focal area close to CA3. The cells of this area, some of which might have migrated to this area in culture, showed especially intense immunostaining. Abnormally hyperphosphorylated tau was found to be aggregated in neurites resembling neuropil threads seen in AD brain. Treatment of these cultures with memantine restored in large part the hyperphosphorylation and aggregation of tau to normal-like state during 24 h.

The OA-induced protein phosphorylation/dephosphorylation imbalance not only affected tau but also revealed fragmented MAP2 staining in dendrites and hyperphosphorylation and aggregation of NF-M/H subunits. Memantine reversed the hyperphosphorylation of NF-H/M and increased the levels of MAP2, consistent with the inhibition of neurofibrillary degeneration.

The reversal of the OA-induced protein phosphorylation/dephosphorylation imbalance by memantine was most likely through its effect on PP-2A signaling pathway and neither solely as an NMDA antagonist nor by any direct interaction between OA and memantine. Memantine, which had no significant effect on the activities of PP-2A, CaMKII and PKA on normal control cultures, restored the activities of these enzymes and the abnormal hyperphosphorylation of tau both when administered along with OA or after removal of OA from the culture medium. In contrast, in vitro addition of memantine to an extract of the cultured slices had no effect on the PP-2A activity inhibited with OA. Similarly, memantine had no significant effect in vitro on the PP-2A activity of the extract of hippocampal slices which were cultured in the presence of OA. These in vitro findings showed the absence of any direct interaction between memantine and PP-2A or OA.

We found that the treatment of the hippocampal slice cultures with glutamate resulted in a marked increase in CaMKII activity without any detectable effect on the activities of PP-2A and MAPK, suggesting that the stimulation of glutamate receptors, which include the NMDA receptors, produced an intracellular Ca^{2+} influx which stimulated CaMKII activity, but had no effect on either PP-2A activity or MAPK activity which is regulated by PP-2A. Furthermore, unlike memantine, DK and AP, two other NMDA receptor antagonists up to 10 μM concentrations studied, failed to restore the OA-induced changes in PP-2A and CaMKII activities and the phosphorylation of tau at Ser-262. Thus, the restoration of the activities of PP-2A and the abnormal hyperphosphorylation of tau to normal-like state by memantine probably involves the modulation of PP-2A signaling, the exact nature of which remains to be understood. Memantine has been reported to substitute for the function of magnesium in blocking the overactivated NMDA receptors in hippocampal slices [34,35]. Such an effect of memantine could also be involved in inhibiting the abnormal hyperphosphorylation of tau through modulation of CaMKII activity.

In conclusion, the OA-induced decrease in PP-2A activity and increase in abnormal hyperphosphorylation of tau and consequent neurodegeneration in adult rat hippocampal slices in culture is a promising ex vivo model of tauopathies/neurofibrillary degeneration. Memantine modulates PP-2A signaling and both inhibits and reverses neurofibrillary degeneration in this model. Memantine is known for its anti-excitotoxic and enhanced neurotransmission activities and has been found to inhibit the deterioration of the activities of daily living in moderate to severe cases of AD [13,14]. This drug might also be useful for AD and other tauopathies because of its ability to inhibit neurofibrillary degeneration through the restoration of PP-2A, CaMKII and PKA activities and hyperphosphorylation of tau to normal levels.

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