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Okadaic-Acid-Induced Inhibition of Protein Phosphatase 2A Produces Activation of Mitogen-Activated Protein Kinases ERK1/2, MEK1/2, and p70 S6, Similar to That in Alzheimer's Disease

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In Alzheimer's disease (AD) brain the activity of protein phosphatase (PP)-2A is compromised and that of the extracellular signal-regulated protein kinase (ERK1/2) of the mitogen-activated protein kinase (MAPK) family, which can phosphorylate tau, is up-regulated. We investigated whether a decrease in PP-2A activity could underlie the activation of these kinases and the abnormal hyperphosphorylation of tau. Rat brain slices, 400-μm-thick, kept under metabolically active conditions in oxygenated (95% O_2, 5% CO_2) artificial CSF were treated with 1.0 μmol/L okadaic acid (OA) for 1 hour at 33°C. Under this condition, PP-2A activity was decreased to ~35% of the vehicle-treated control slices, and activities of PP-1 and PP-2B were not affected. In the OA-treated slices, we observed a dramatic increase in the phosphorylation/activation of ERK1/2, MEK1/2, and p70 S6 kinase both immunohistochemically and by Western blots using phosphorylation-dependent antibodies against these kinases. Treatment of 6-μm sections of the OA-treated slices with purified PP-2A reversed the phosphorylation/activation of these kinases. Hyperphosphorylation of tau at several abnormal hyperphosphorylation sites was also observed, as seen in AD brain. These results suggest 1) that PP-2A down-regulates ERK1/2, MEK1/2, and p70 S6 kinase activities through dephosphorylation at the serine/threonine residues of these kinases, and 2) that in AD brain the decrease in PP-2A activity could have caused the activation of ERK1/2, MEK1/2, and p70 S6 kinase, and the abnormal hyperphosphorylation of tau both via an increase in its phosphorylation and a decrease in its dephosphorylation. ***(Am J Pathol 2003, 163:845–858)***

Microtubule-associated protein tau is abnormally hyperphosphorylated at serines/threonines and aggregated into paired helical filaments (PHF) in Alzheimer's disease (AD) brain.[1–4] To date, neither the exact enzymes involved nor the molecular mechanism leading to the hyperphosphorylation of tau are fully understood. The mitogen-activated protein kinase (MAPK) family might play a role in the hyperphosphorylation of tau in AD brain. This family includes the extracellular signal-regulated protein kinases (ERKs), the stress-activated protein kinase C-jun amino terminal kinase (SAPK/JNK), and p38 kinase. ERK is activated through its phosphorylation at Thr 202 and Tyr 204 by MAP kinase kinase (MEK). The activation of ERK initiates the phosphorylation of p70/85 S6 kinase at Thr 421/Ser 424, Thr 389 and Ser 411 and activates it.[5–7] The p70 S6 kinase, which is also phosphorylated and activated by PDK1 in the PI3 kinase cascade,[8] promotes protein synthesis by enhancing the translation of mRNA of several proteins, especially those involved in cell growth and division.[9] The ERKs, p44 ERK1, p42 ERK2, and PK40erk,[10,11] all are capable of phosphorylating tau *in vitro* at several abnormal hyperphosphorylation sites as seen in PHF-tau.[11–15] The activated ERK1/2,[16–19] JNK,[20] and p38[20–22] have all been found in NFT-bearing neurons. Thus, the MAPK cascade appears to be activated in neurons affected by Alzheimer neurofibrillary degeneration.

The phosphorylation level of tau is also regulated by phosphoseryl/phosphothreonyl protein phosphatases (PPs). The activity of PP-2A, which is present in neurons[23] and regulates tau phosphorylation in brain tissue,[24,25] is specifically decreased in AD brain.[26,27] A recent study has shown a decrease in the mRNA expression of this

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Table 1. Antibodies Employed in This Study

Antibody	Specificity*	Phosphorylation site	Dilution	Supplier [reference]
Phospho-p44/42 MAP kinase	P, active ERK1/2	Thr202/Tyr204	1:100	Cell Signaling†
p44/42 MAP kinase	total ERK1/2		1:100	Cell Signaling
Phospho-MEK1/2	P, active MEK1/2	Ser217/221	1:100	Cell Signaling
MEK1/2	total MEK1/2		1:100	Cell Signaling
Phospho-p70 S6 kinase	P, active p70 S6	Thr421/Ser424	1:100	Cell Signaling
p70 S6 kinase	total p70 S6		1:100	Cell Signaling
Anti-GSK-3α/β	P, active GSK-3α/β	Tyr279/216	1:800	Biosource‡
Phospho-JNK MAP kinase	P, active JNK	Thr183/Tyr185	1:100	Cell Signaling
Phospho-p38 MAP kinase	P, active p38	Thr180/Tyr182	1:100	Cell Signaling
92e	Total tau		1:5000	[68]
Tau-1	NP tau	Ser198/199/202	1:50,000	[69]
12E8	P tau	Ser262/356	1:500	[70]
PHF-1	P tau	Ser396/404	1:500	[71, 72]
R145	P tau	Ser422	1:3000	[67]

*P, phosphorylated epitope; NP, nonphosphorylated epitope;
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enzyme in AD brain.[28] Unlike the activity of PP-2A, the activity of calcineurin/PP-2B, another major PP in the brain, is not significantly affected in AD brain.[26] Since the MAPK pathway is dynamically regulated by the phosphorylation of each component kinase of the cascade and these kinases can be dephosphorylated by PP-2A *in vitro* and in cultured cells,[29–33] the activated MAPK pathway might possibly result from a decrease of PP-2A activity in AD brain.

In the present study, we investigated the regulation of the MAPK pathway and phosphorylation of tau by PP-2A in metabolically competent rat brain slices as a model. We found that the inhibition of PP-2A by okadaic acid (OA) induced a dramatic increase in the phosphorylation/activation of ERK1/2, MEK1/2, and p70 S6 kinase as well as the phosphorylation of tau at several of the sites seen in PHF-tau. The topography of the activation of these kinases differed markedly from one another. The selective inhibition of PP-2B by cyclosporin A (CsA) in the brain slices did not significantly change the phosphorylation/activation of any of the three kinases studied.

Materials and Methods

Materials

The catalytic subunit of PP-2A was isolated from bovine brain according to Cohen et al.[34] Phosphorylase kinase was purified from the skeletal muscle of White New Zealand rabbits by the method of Cohen.[35] Inhibitor-1 was also isolated from the rabbit skeletal muscle and phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (Sigma, St. Louis, MO) according to the method of Cohen et al.[36] Antibodies to different enzymes and tau are listed in Table 1. OA (ammonium salt) was bought from Calbiochem (San Diego, CA), and CsA from Alexis Corp. (San Diego, CA).

For immunohistochemical studies of AD brain, formalin-fixed frozen 100-μm sections of hippocampi from AD cases at Braak stage V[37] were obtained from Dr. Heiko Braak of J.W. Goethe University, Frankfurt, Germany.

Preparation of Rat Brain Slices and Treatment with Protein Phosphatase Inhibitors

CD rats (Caesarean derived from a Wistar rat in Charles River Lab, Wilmington, MA), male, 2 to 3 months old, were injected intraperitoneally with 75 mg/kg Nembutal. The animals were decapitated when deeply anesthetized. The brains were immediately removed and cooled down in ice-cold (4°C) oxygenated artificial cerebrospinal fluid (CSF) consisting of 126 mmol/L NaCl, 3.5 mmol/L KCl, 1.2 mmol/L NaH_2PO_4, 1.3 mmol/L $MgCl_2$, 2.0 mmol/L $CaCl_2$, 11 mmol/L D(+)glucose, 25 mmol/L $NaHCO_3$ (pH 7.4) for 7 to 8 minutes. Each brain was then divided sagittally and 400-μm-thick coronal slices were made with a Camden Vibraslicer (WP Inc., Sarasota, FL). The slices were transferred into a chamber containing the oxygenated artificial CSF and incubated at room temperature for 1 hour, followed by incubation at 33°C for 1 to 3 hours. The PP inhibitors were included in the artificial CSF during incubation at 33°C. Typically eight slices were prepared from one brain within 10 minutes. Half of them were treated with PP inhibitors and the other half with artificial CSF alone as controls. The oxygenation of the artificial CSF was carried out by bubbling the solution with a mixture of 95% O_2 and 5% CO_2 during the entire procedure.

At the end of incubation, the brain slices were either homogenized at 4°C for biochemical analyses or fixed for immunocytochemical studies (see below). For biochemical analyses, the brain slices were rinsed briefly with homogenizing buffer [50 mmol/L Tris-HCl (pH 7.0), 10 mmol/L β-mercaptoethanol, 1.0 mmol/L ethylenediaminetetraacetate (EDTA), 0.1 mmol/L phenylmethyl sulfonyl fluoride, 2.0 mmol/L benzamidine and 2.0 μg/ml each of aprotinin, leupeptin, and pepstatin A] and homogenized at a ratio of 9.0 ml buffer/1.0-g tissue slices. The homogenates were divided into two parts. One was centrifuged at 16,000 $\times$ g for 10 minutes and the resulting supernatant was used for PP activity assays. Into the other half, an equal volume of phosphatase-inhibitor cocktail (20 mmol/L β-glycerophosphate, 2.0 mmol/L Na_3VO_4 and 100 mmol/L NaF, pH 7.0) was

added immediately and the samples were stored at −80°C for Western blotting. Protein concentrations of all samples were quantitated by the method of Bradford[38] using the Protein Assay Reagent from Bio-Rad (Hercules, CA) and bovine serum albumin as a standard.

Involvement of MEK1/2-ERK1/2 Cascade in Phosphorylation of Tau

Some rat brain slices were prepared by using a McIlwain Tissue Chopper (Brinkmann, Westbury, CT) as described previously.[24] The tissue slices (400-μm-thick) were immediately washed twice with oxygenated artificial CSF, and the slices from each brain were evenly divided into 5 portions for parallel treatments with various compounds. After incubation of the tissue slices in oxygenated artificial CSF with or without specific inhibitors at 33°C for 2 hours, the tissue was harvested, washed twice with ice-cold homogenizing buffer (20 mmol/L β-glycerophosphate, pH 7.0, 10 mmol/L β-mercaptoethanol, 1.0 mmol/L EDTA, 0.1 mmol/L phenylmethyl sulfonyl fluoride, 2.0 mmol/L benzamidine, 2.0 mmol/L $NaVO_4$, 100 mmol/L NaF, and 2.0 μg/ml each of aprotinin, leupeptin, and pepstatin A) and then homogenized in 9 volumes of the same homogenizing buffer. The homogenates were stored at −80°C until further analysis.

Assays of Protein Phosphatases and Protein Kinases

The activities of PP-1 and PP-2A in the extracts of brain slices were assayed using [^{32}P]phosphorylase *a* as a substrate as described by us previously.[39] A PP-1 specific inhibitor, Inhibitor 1,[40] was added in the assays for PP-2A activity. PP-1 activity was calculated by subtracting the PP-2A activity from the total phosphorylase phosphatase activity (PP-1 + PP-2A) assayed in the absence of Inhibitor-1. PP-2B activity was assayed using [^{32}P]phosphorylase kinase as a substrate as described previously[41] except that 1.0 μmol/L calyculin A was added into the reaction mixture to inhibit PP-1 and PP-2A. The activation of ERK1/2, MEK1/2, and p70 S6 kinase were determined by immunostaining with antibodies specific to the activated/phosphorylated forms of these enzymes (see Table 1).

Western Blot Analyses

The levels of tau phosphorylation and the immunoreactivities of different enzymes were analyzed by Western blots using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described originally by Laemmli.[42] The protein bands were transferred to Immobilon-P membrane (Millipore, Bedford, MA) and probed with different antibodies listed in Table 1. The blots were developed with alkaline phosphatase-conjugated secondary antibodies, and 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt and *p*-nitro blue tetrazolium chloride as substrates.

Immunohistochemistry

Some of the rat brain slices were fixed in periodate/lysine/paraformaldehyde solution for 3 to 5 hours and embedded in paraffin.[24] Paraffin-embedded sections (6 μm) were cut from the tissue slices. Before processing for immunostaining, the sections were placed in 0.01 mol/L citric acid (pH 6.0) and microwaved as described.[43] To eliminate nonspecific bindings, the sections were treated with 5% H_2O_2/methanol for 10 minutes, then 3% normal goat serum in 50 mmol/L TBS (pH 7.4), for 30 minutes at room temperature. The sections were then incubated with primary antibodies listed in Table 1 at 4°C overnight, followed by incubation with biotinylated anti-mouse or anti-rabbit IgG at a dilution of 1:200 for 2 hours, and visualized using the avidin-biotin-peroxidase complex Elite kit (Vector, Burlingame, CA) and 3–3′-diaminobenzidine-4 HCl/H_2O_2 (DAB; Sigma) as a substrate.

Double-Immunofluorescent Staining and Confocal Microscopy

Autopsied brain tissue blocks including the entorhinal, hippocampal, and temporal cortices and/or amygdala from two normal controls and two individuals with stage V neurofibrillary degeneration according to Braak and Braak[37] were used in this study. The tissue was fixed by immersion in a mixture of 4% paraformaldehyde and picric acid at pH 7.0 for 48 hours.[44] The frozen tissue blocks were sectioned at 100 μm.

Double-immunofluorescent staining of floating sections was carried out using CY3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) to visualize bound antibodies to active forms of MEK1/2, ERK1/2, and p70 S6 kinase, and CY2-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) to label-bound mAb AT8. Radiance PLUS, a Bio-Rad laser-scanning confocal imaging system, equipped with a Nikon Eclipse inverted microscope (TE300) was used to determine co-localization of the CY3 (red)-labeled active forms of ERK1/2, MEK1/2, and p70 S6 kinase with the CY2 (green)-labeled hyperphosphorylated tau (AT8 staining). An argon ion laser that excites at 488 nm with a dichroic beamsplitter 560DCLP and a bandpass filter HQ515/30 was used to detect CY2 (green)-labeled abnormal tau (AT8 staining). A HeNe laser that excites at 543 nm with E570LP emission filter was used to measure the active forms of ERK1/2, MEK1/2, and ERK1/2 labeled by CY3 (red). A Nikon 60×/1.4 NA oil immersion objective was used. Images scanned on the two channels (red and green) were merged to produce a single profile. In this mode, all structures exhibiting colocalization display yellow fluorescence. Fluorescence images were collected at 1× or 2.5× zoom using the Bio-Rad Lasersharp software package and processed using Adobe Photoshop 5.0.

Dephosphorylation with Purified PP-2A

In some cases, tissue sections and blots were dephosphorylated with purified PP-2A before incubation with

primary antibody. The dephosphorylation was carried out by overlaying the sections and blots with PP-2A (20 μg/ml) in buffer containing 60 mmol/L Tris (pH 7.0), 0.1% β-mercaptoethanol, and 1.0 mmol/L $MnCl_2$, at room temperature overnight. For the purpose of comparison, the PP-2A-treated and untreated tissue sections and blots were immunostained in parallel.

Results

Active ERK1/2, MEK1/2, and p70 S6 Kinase Are Associated with Tangle-Bearing Neurons in AD Brain

Previous studies have revealed no significant differences in the immunoreactivity of total ERK1/2[23] or p70 S6 kinase (Pei et al, in preparation) between AD and control/normal neurons. Since ERKs, MEKs, and p70 S6 kinase are activated when phosphorylated at specific sites, we used phosphorylation-dependent antibodies specific to the activated kinases to study immunocytochemically the relationship between the activation of these kinases and the abnormal hyperphosphorylation of tau in AD brain sections. We observed that ERK1/2 was activated in most of the neurons containing AT8-positive/abnormally hyperphosphorylated tau (Figure 1, A1 to A3). Similarly, antibodies against the active form of MEK1/2 or p70 S6 kinase also stained a majority of neurons that were positive with AT8 (Figure 1, B1 to B3 and C1 to C3). However, no significant immunostaining was obtained with these antibodies when control human brain tissue sections were stained in parallel (data not shown). These data suggest that the MAPK pathway is activated in many

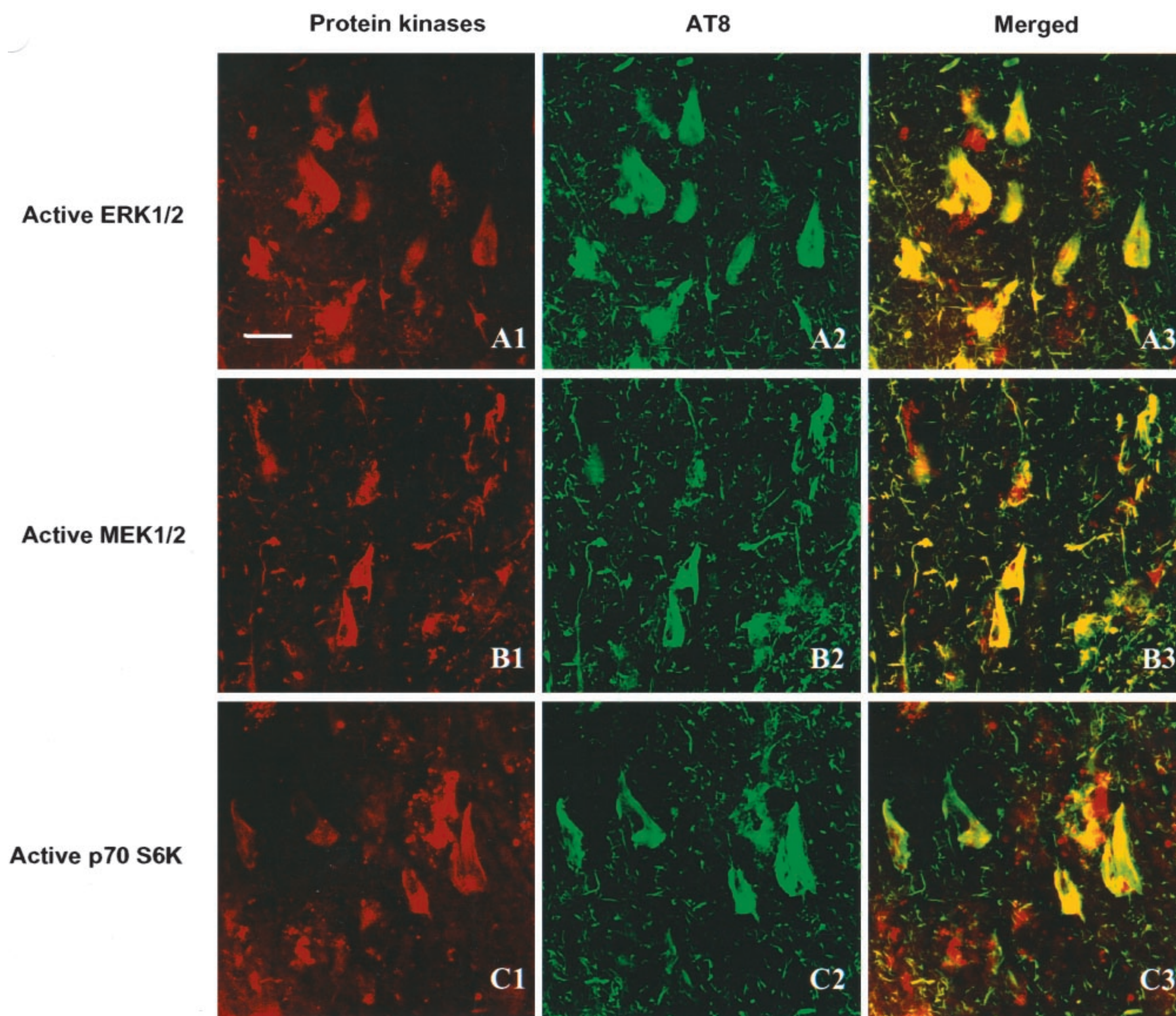

Figure 1. Confocal microscopy demonstrating the partial colocalization of active ERK1/2, MEK1/2, and p70 S6 kinase immunoreactivities with AT8-positive abnormally hyperphosphorylated tau in hippocampal CA1 pyramidal neurons in brains at Braak stage V/C. Formalin-fixed frozen sections, 100-μm thick, were incubated with rabbit polyclonal antibodies to active forms of ERK1/2, MEK1/2, or p70 S6 kinase, and mouse monoclonal antibody AT8 to PHF-tau, and visualized with CY3 (red)-conjugated anti-rabbit IgG and CY2 (green)-conjugated anti-mouse IgM. Active ERK1/2 (**A1–3**), MEK1/2 (**B1–3**), or p70 S6 kinase (**C1–3**) was found in many neurons that were AT8-positive. Scale bar, 20 μm.

Table 2. Levels of Phosphorylated/Activated ERK1/2, MEK1/2, and p70 S6 Kinase Induced by Inhibition of PP-2A in Rat Brain Slices

Kinase	Level (% of control)
ERK1/2	203 ± 43
MEK1/2	299 ± 25
p70 S6	434 ± 31

Homogenate of control and OA-treated (1.0 μmol/L, 1 hour) rat brain slices were analyzed by Western blots developed with antibodies to active ERK1/2, MEK1/2, and p70 S6, and [^{125}I]-labeled secondary antibody, and quantitated by a phosphorimager. Data are mean ± SD of three experiments. The increases in the levels of the activated kinases in the OA-treated brain slices are highly significant ($P < 0.01$).

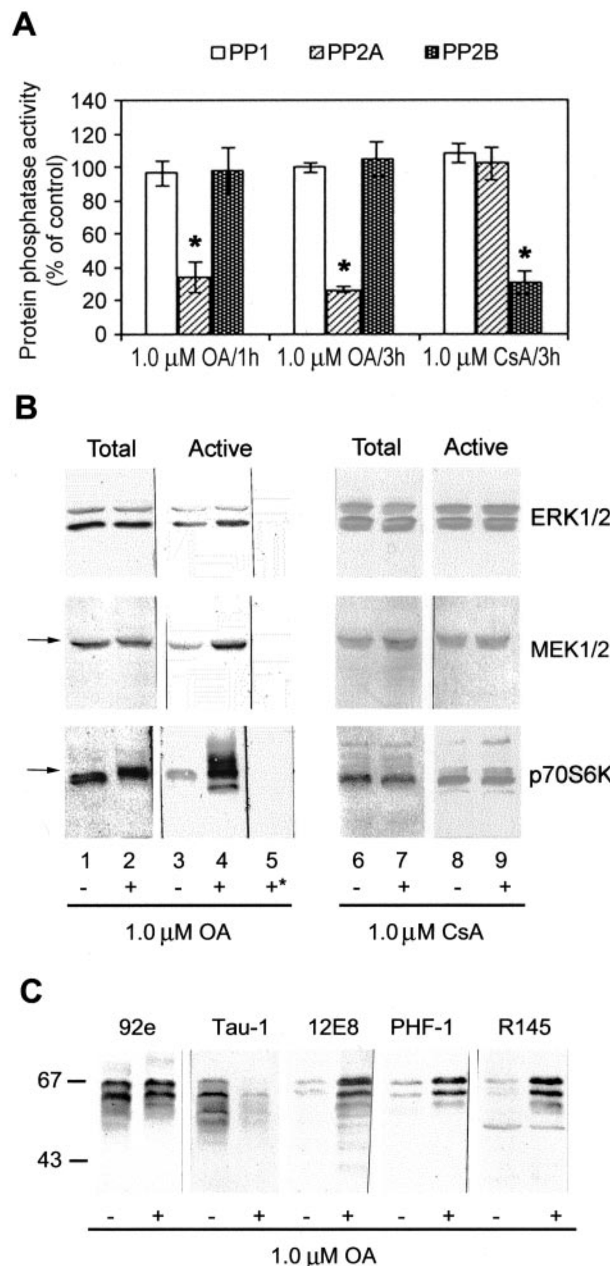

Figure 2. Inhibition of PPs, activation of protein kinases, and phosphorylation of tau in rat brain slices treated with OA or CsA. Rat brain slices were incubated in either oxygenated artificial CSF alone as controls, or in the artificial CSF plus either 1.0 μmol/L OA or 1.0 μmol/L CsA for 1 to 3 hours, followed by homogenization and centrifugation at 16,000 × *g* for 10 minutes. **A:** The resulting tissue extracts were assayed for the activities of PP-1, PP-2A, and PP2-B. The phosphatase activities of the inhibitor-treated samples were expressed as the percentage of the activities of the control samples of each set where the artificial CSF alone was used for incubation for the same period. Bars represent means ± SD of three to four independent assays. *, $P < 0.01$ as compared with controls by Student's *t*-test. Note that under these conditions, OA selectively inhibited PP-2A activity by 65% to 70%, and CsA selectively inhibited PP-2B activity by ~70%. **B:** The homogenate samples (20 μg protein per lane) with (+) or without (−) the treatment of 1.0 μmol/L OA for 1 hour or 1.0 μmol/L CsA for 3 hours were analyzed by Western blots for the levels and the activities of ERK1/2, MEK1/2, and p70 S6 kinase. Representative blots of three independent experiments with similar results are shown. Note that the levels of these kinases were not affected by the inhibitor treatments as detected with antibodies against the kinases (compare **lane 2** with **1**, and **7** with **6**). When detected with antibodies against the phosphorylated/active form of these kinases, increased activation of all three kinases was observed in the OA-treated (compare **lane 4** with **3**), but not the CsA-treated (compare **lane 9** with **8**) brain slices. A slight up-shift of electrophoretic mobility of MEK1/2 and p70 S6 kinase is also seen in OA-treated samples (**arrows**). The staining of these kinases with phosphorylation-dependent antibodies disappeared after the blots were incubated with PP-2A before probing with the primary antibodies (**lane 5**). **C:** Homogenates (10 μg protein per lane) of the brain slices after incubation in the oxygenated artificial CSF in the presence (+) or absence (−) of 1.0 μmol/L OA for 1 hour were analyzed by Western blots for the phosphorylation of tau. The blots were developed with a phosphorylation-independent anti-tau antibody 92e to detect total tau, and phosphorylation-dependent anti-tau antibodies Tau-1 (which detects tau unphosphorylated at Ser198/199/202), 12E8 (which recognizes tau phosphorylated at Ser262/356), PHF-1 (which recognizes tau phosphorylated at Ser396/404), and R145 (which recognizes tau phosphorylated at Ser422), respectively. The molecular mass markers (67 and 43 kd, respectively) are shown at the left of the blots. The blots are representative results of three independent experiments with similar results. Note that the treatment of rat brain slices with 1.0 μmol/L OA for 1 hour induced phosphorylation of tau at all of the above sites studied.

neurons undergoing neurofibrillary degeneration in AD brain. The colocalization of MAPK with phosphorylated tau was also observed previously.[45–47]

PP-2A Inhibition Induces Activation of ERK1/2, MEK1/2, and p70 S6 Kinase and Hyperphosphorylation of Tau in Rat Brain Slices

To investigate whether the activation of the MAPK pathway could have resulted from an inhibition of PP-2A in AD brain, we used the metabolically active rat brain slice model in which PP-2A was selectively inhibited with OA, and as a control the inhibition of PP-2B by CsA.[24] After metabolically active rat brain slices were treated with 1.0 μmol/L OA for 1 to 3 hours, 65% to 70% of the PP-2A activity was inhibited while the activities of PP-1 and PP-2B, the other two major phosphoseryl/phosphothreonyl PPs, remained unchanged (Figure 2A). Similarly, treatment of the brain slices with 1.0 μmol/L CsA selectively inhibited PP-2B activity by ~70% with no effect on the activities of PP-1 or PP-2A (Figure 2A). The expression of ERK1/2, MEK1/2, and p70 S6 kinase was examined with Western blots using phosphorylation-independent antibodies against these kinases. We found that the inhibition of either PP-2A or PP-2B did not change the expression of these kinases in the rat brain tissue. However, the inhibition of PP-2A, but not of PP-2B, induced mobility shift (MEK1/2 and p70 S6 kinase) and as well as significantly activated the kinases, as determined with

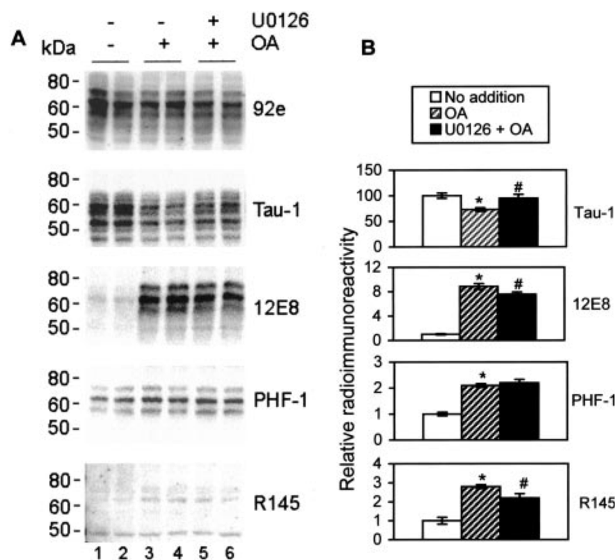

Figure 3. Phosphorylation of tau in rat brain slices after treatments with OA and U0126. **A:** Rat brain slices were incubated in oxygenated artificial CSF for 2 hours (**lanes 1** and **2**), artificial CSF for 1 hour, followed by 1.0 μmol/L OA in artificial CSF for 1 hour (**lanes 3** and **4**), or 50 μmol/L U0126 in artificial CSF for 1 hour, followed by 50 μmol/L U0126 plus 1.0 μmol/L OA in artificial CSF for 1 hour (**lanes 5** and **6**). The homogenates (10 μg protein/lane) of the treated slices were then analyzed by Western blots to assess the level of tau by using a phosphorylation-independent antibody 92e and phosphorylation levels of tau at the specific sites by using phosphorylation-dependent and site-specific anti-tau antibodies Tau-1, 12E8, PHF-1, and R145. **B:** The blots as shown in **A** were scanned and the radioimmunoreactivity was quantitated by using a phosphorimager. The radioimmunoreactivity obtained with each antibody was normalized by that of 92e in each case. The bar graphs are presented as means ± SD ($n = 4$). *, $P < 0.05$ as compared with no addition control; #, $P < 0.05$ as compared with that treated with OA only.

Western blots developed with antibodies against the phosphorylated active forms of these kinases (Figure 2B, lanes 1 to 4 and 6 to 9). When the blots were treated with purified PP-2A before probing with the primary antibodies, the staining with the antibodies to the phosphorylated kinases was inhibited (Figure 2B, lane 5). These results indicated that the staining was specific to the phosphorylated active kinases and that PP-2A could dephosphorylate these kinases. We also developed the blots with [^{125}I]-labeled secondary antibody and quantitation of the signals with a phosphorimager revealed a marked increase in the levels of the activated ERK1/2, MEK1/2, and p70 S6 kinase in the OA-treated slices (Table 2). Phosphorylation of tau at several abnormal hyperphosphorylation sites, as seen in AD brain, was also observed in the OA-treated brain slices (Figure 2C). The activation of the kinases studied was unlikely due to a direct effect of OA on the kinases because it did not affect ERK1/2-catalyzed *in vitro* phosphorylation (unpublished observation) and this phosphatase inhibitor itself is not a catalyst for phosphorylation.

We further investigated whether the OA-induced hyperphosphorylation of tau in rat brain slices could result from the activation of ERK1/2, MEK1/2, and p70 S6 kinase in addition to the direct effect of PP-2A inhibition. When the activation of MEK1/2-ERK1/2-p70 S6 kinase cascade was blocked by incubating the brain slices with specific MEK1/2 inhibitor U0126[48] in addition to OA, we found that tau was hyperphosphorylated to a lesser extent at Ser 198/Ser 199/Ser 202 (Tau-1 sites), Ser 262/Ser 356 (12E8 sites), and Ser 422 (R145 site) as compared to when the slices were treated with OA alone (Figure 3). These results indicate that the activation of MEK1/2-ERK1/2 cascade partially contributes to the hyperphosphorylation of tau at some phosphorylation sites in the OA-treated rat brain slices.

PP-2A Inhibition Induces Marked Differences in the Topography of the Activated ERK1/2, MEK1/2, and p70 S6 Kinase

The antibody against the active form of ERK1/2 faintly stained tissue sections of control-treated rat brain slices (Figure 4F). The staining was mainly found in neuronal cell bodies in the cornu ammonis and dentate gyrus of the hippocampus (Fig. 4A1, B1, C1) and layer III pyramidal neurons of the cerebral cortex (Figure 4, D1). This suggested that the bulk of the ERK1/2 was constitutively inactive. When PP-2A in the brain slices was inhibited by OA, a dramatic increase in staining with this antibody was observed in neuronal processes of the pyramidal neurons of cornu ammonis, leaving stratum pyramidale almost unstained (Figure 4E). The strongest staining was in the stratum lucidum of CA3 sector (Figure 4E, arrowhead). At higher magnification, only some of the neurons in the stratum pyramidale were positively stained for active ERK1/2 (Figure 4, A2 and B2). In dentate gyrus, the fibers in the polymorphic layer had strong immunoreactivity. Moderate immunostaining was observed in granule cell layer and the stratum moleculare (Figure 4, C2 and E). In the adjacent cerebral cortex, intensive staining was found in the pyramidal cell bodies of layer III (Figure 4, D2) and V, and in the neurons of molecular layer (data not shown).

Immunostaining with the antibody against the active form of MEK1/2 indicated a weak to moderate staining only in cell bodies and nuclei of pyramidal neurons in the cornu ammonis (Figure 5, A1 and B1), of granular cells in the dentate gyrus (Figure 5, C1), and of layers III and V pyramidal neurons (Figure 5, D1) of the adjacent cerebral cortex in the control-treated rat brain slices. In contrast, in brain slices treated with OA, a significant increase in immunostaining of neurons in different areas of the hippocampus was observed (Figure 5, compare Exp with Con and E with F).

In the OA-treated tissue sections the antibody against the active p70 S6 kinase stained the pyramidal cells of CA1, CA2, and CA3 strongly and of CA4 weakly (Figure 6, A2, B2, E). The molecular layer and granular layer of dentate gyrus were also strongly immunostained (Figure 6, C2 and E). Strong immunoreactivity was seen in some of the pyramidal neurons of layers III and V of the adjacent cerebral cortex (Figure 6, D2). In the control sections processed in parallel, the active p70 S6 kinase staining was weak as compared with the OA-treated sections (Figure 6, compare Exp with Con and E with F).

The immunohistochemical studies revealed marked differences in the topography of the activated ERK1/2, MEK1/2, and p70 S6 kinase in brain slices in which PP-2A

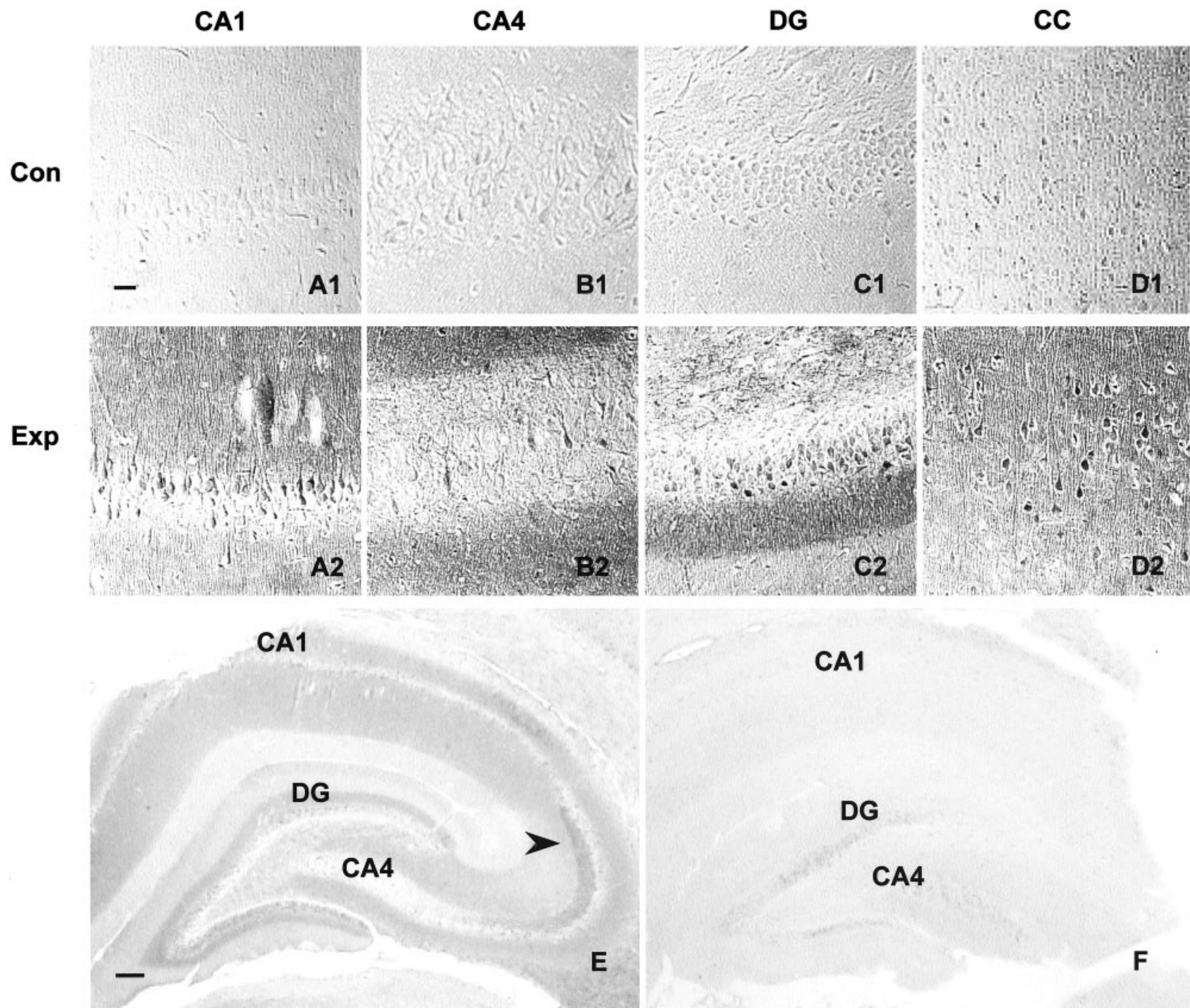

Figure 4. Immunohistochemical staining showing topography of active ERK1/2 in rat brain slices treated with OA. Rat brain slices were incubated in either oxygenated artificial CSF alone as controls (Con) or in the artificial CSF plus 1.0 μmol/L OA for 1 hour (Exp), followed by immunohistochemical staining with antibody against the active ERK1/2. The representative staining of CA1, CA4, and dentate gyrus (DG) of the hippocampal complex, layer III of the adjacent primary sensory cortical cortex (CC), and of the whole section (**E**, Exp; **F**, Con) are shown. **Arrowhead** in **E** indicates the CA3 stratum lucidum. The bar represents 20 μm for **A1** to **D2** and 200 μm for **E** and **F**, respectively.

activity was inhibited by OA (compare Figures 4E, 5E, and 6E). While the activation of ERK1/2 was most pronounced in CA3 and in the neuropil, the activation of MEK1/2 was mostly in the neuronal cell bodies in CA1, CA4, and dentate gyrus. The activation of p70 S6 kinase was observed both in neuronal cell bodies and as well as neuropil, and was most pronounced in CA1, CA2, CA3, and dentate gyrus.

We examined the immunohistochemical distribution of hyperphosphorylated tau in OA-treated brain slices. When stained with antibody R145 that reacts with tau phosphorylated at Ser 422, the pattern of tau staining (Figure 7) was similar to that of activated ERK1/2 (Figure 4).

We also carried out immunostaining of the OA-treated and control-treated tissue sections with antibodies against the phosphorylated (active) p38, JNK, and glycogen synthase kinase-3 (GSK-3). No difference in the immunostaining was detected with any of these antibodies between the OA-treated and control-treated sections (data not shown).

Activated MEK1/2 and p70 S6 Kinase Are Relatively Localized in Neuronal Nuclei in the Okadaic-Acid-Treated Brain Slices

The immunohistochemical studies also showed that active MEK1/2 and p70 S6 kinase in the OA-treated slices were concentrated in the nuclei of neurons, especially of pyramidal cells of layers III and V in the cerebral cortex. Without OA treatment, the weak staining of the active MEK1/2 was mainly located in neuronal perikarya [Figure 8, A (A)]. Inhibition of PP-2A with OA induced a strong immunostaining of the active MEK1/2 in the nuclei of most of the neurons [Figure 8A(B)]. When stained with an antibody that recognizes both the active and inactive

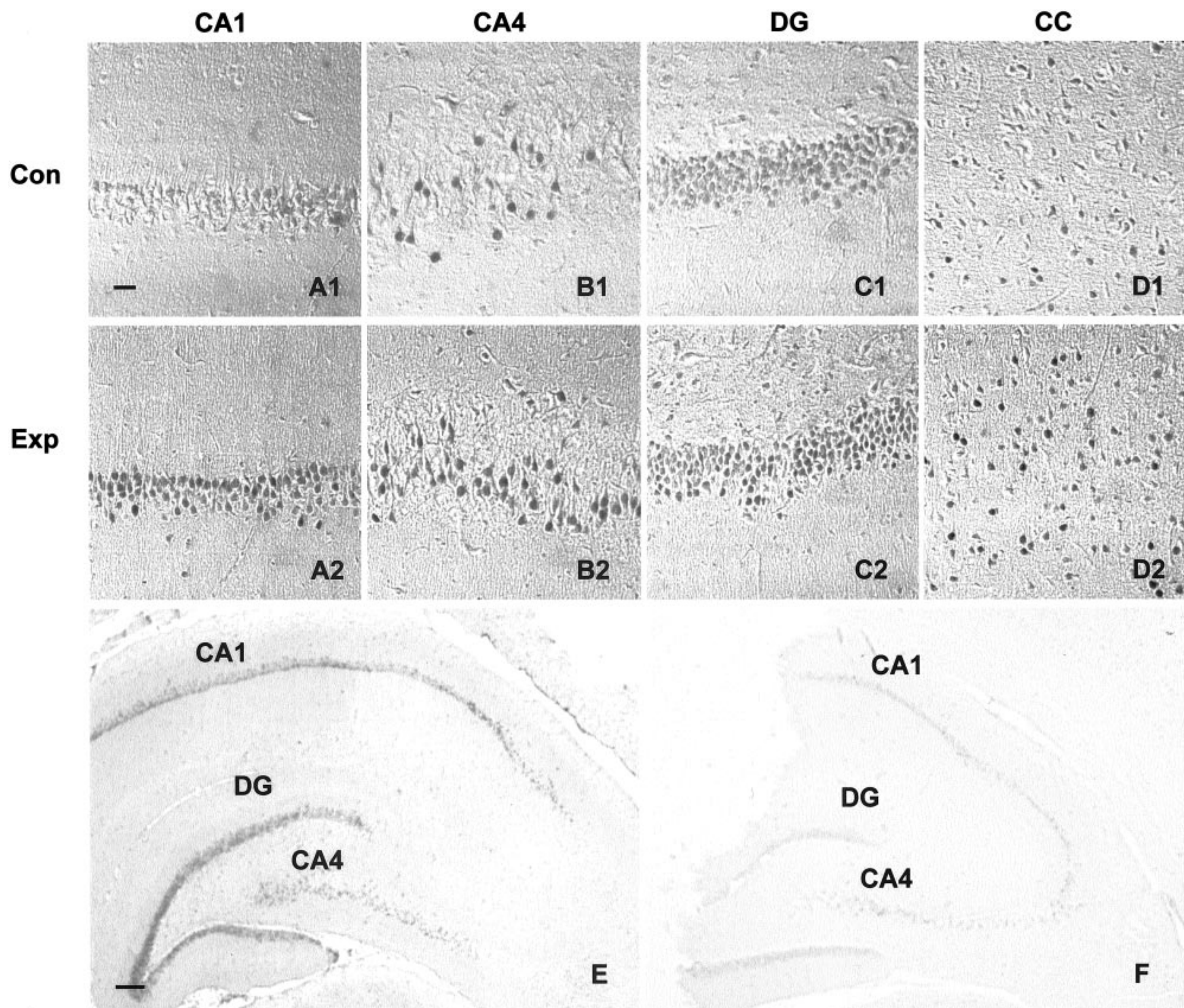

Figure 5. Immunohistochemical staining showing topography of active MEK1/2 in rat brain slices treated with OA. Rat brain slices were incubated in either oxygenated artificial CSF alone as controls (Con), or in the artificial CSF plus 1.0 μmol/L OA for 1 hour (Exp), followed by immunohistochemical staining with antibody against the active MEK1/2. The representative staining of CA1, CA4, and DG of the hippocampal complex, layer III of the adjacent primary sensory CC, and of the whole section (**E**, Exp; **F**, Con) are shown. The magnification is indicated as scale bar of 20 μm for **A1** to **D2** and 200 μm for **E** and **F**.

MEK1/2, the immunoreactivity was mainly located in the cell cytoplasm rather than the nuclei, and was similar in tissue treated with or without OA [Figure 8A (C and D)]. The weak staining in nuclei by antibody against total MEK1/2 was probably due to the low affinity of this antibody. A similar phenomenon was observed with p70 S6 kinase (Figure 8B).

Okadaic-Acid-Induced Activation of ERK1/2, MEK1/2, and p70 S6 Kinase and Phosphorylation of Tau Are Reversed by PP-2A

To confirm that the increase in the immunohistochemical staining of the OA-treated brain slices with antibodies to phosphorylated (active) kinases and to hyperphosphorylation of tau was specifically due to inhibition of PP-2A activity, and that the antibodies used were specific to the phosphorylated enzymes, we incubated some of the OA-treated tissue sections with purified PP-2A before immunostaining. The treatment with PP-2A dramatically reduced the immunoreactivity of tau phosphorylated at Ser-262/356 and of phosphorylated/active ERK1/2, MEK1/2, and p70 S6 kinase as compared with the sections stained in parallel but without PP-2A treatment (Figure 9). These results indicated that PP-2A could dephosphorylate tau at Ser-262/356 and the three kinases, and therefore further support our above observation that the inhibition of PP-2A-induced phosphorylation and activation of ERK1/2, MEK1/2, and p70 S6 kinase and phosphorylation of tau in rat brain slices.

Discussion

The abnormal hyperphosphorylation of tau is a likely cause of neurodegeneration and consequently is a promising rational therapeutic target for AD and other tauopa-

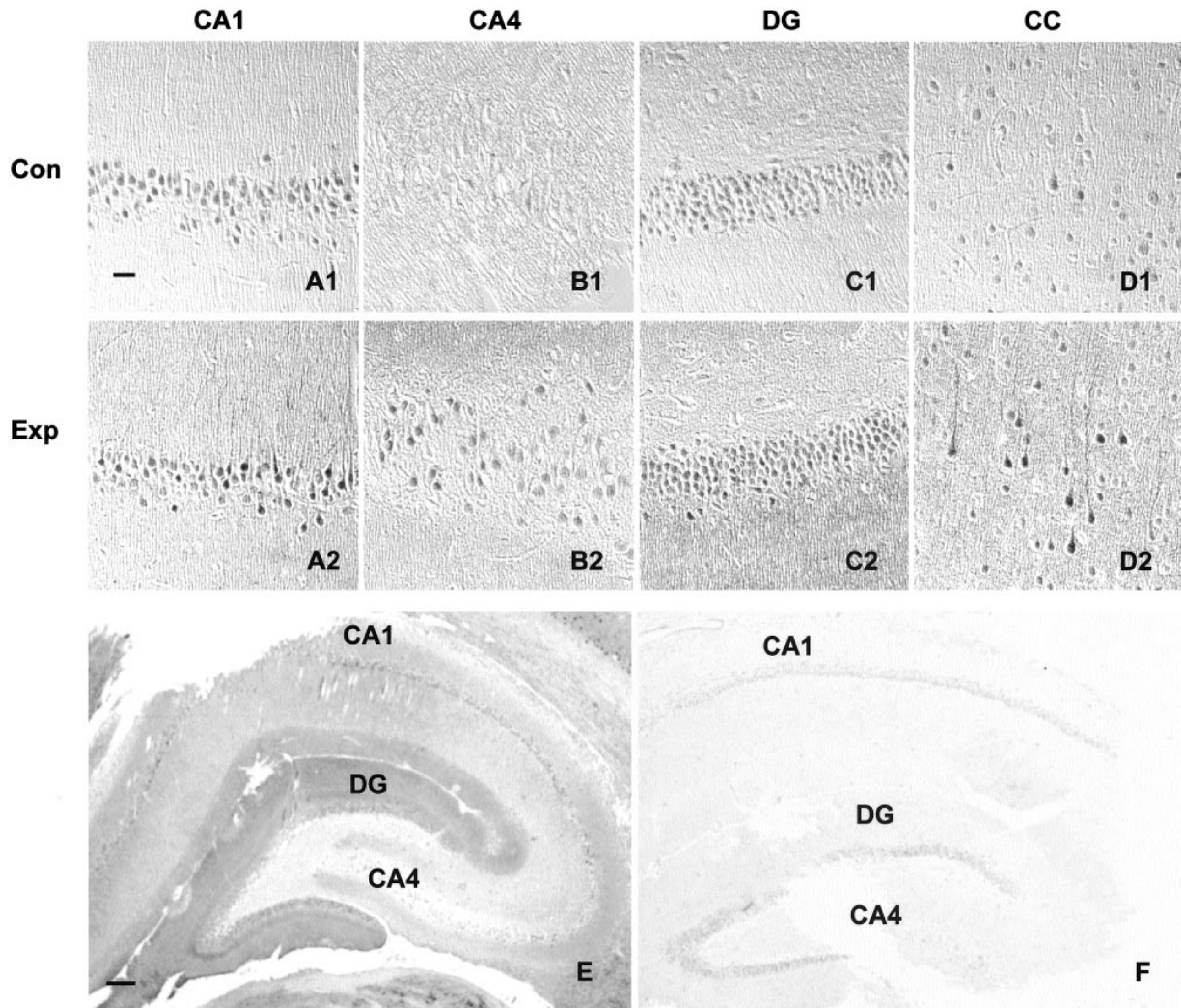

Figure 6. Immunohistochemical staining showing topography of active p70 S6 kinase in rat brain slices treated with OA. Rat brain slices were incubated in either oxygenated artificial CSF alone as controls (Con) or in the artificial CSF plus 1.0 μmol/L OA for 1 hour (Exp), followed by immunohistochemical staining with antibody against the active p70 S6 kinase. The representative staining of CA1, CA4, and DG of the hippocampal complex, layer III of the adjacent primary sensory CC, and of the whole section (**E**, Exp; **F**, Con) are shown. Bar represents 20 μm for **A1** to **D2** and 200 μm for **E** and **F**, respectively.

thies (for review, see[49]). Thus, it is of considerable significance to understand the nature of enzymes and mechanisms involved in the abnormal hyperphosphorylation of tau. In this study we observed localization of activated ERK1/2, MEK1/2, and p70 S6 kinase with hyperphosphorylation of tau in AD brain, and we found that in the metabolically active rat brain slices, selective inhibition of PP-2A induced phosphorylation/activation of ERK1/2, MEK1/2, and p70 S6 kinase, and hyperphosphorylation of tau at several abnormal phosphorylation sites seen in AD brain. To our knowledge, this is the first report showing the activation of three kinases of the MAPK pathway by selective inhibition of PP-2A *in situ* in mammalian brain. These data suggested that the MAPK pathway might be regulated by PP-2A in brain. Since PP-2A activity is compromised in AD brain[26–28] and ERK1/2, MEK1/2, and p70 S6 kinase are activated in NFT-bearing neurons, the present study suggests that the compromised PP-2A activity could result in the activation of the MAPK pathway in selected neurons of AD brain. In the present study, the inhibition of PP-2A resulted in the hyperphosphorylation of tau at Tau-1 (Ser-198/199/202), 12E8 (Ser-262/356), PHF-1 (Ser-396/404), and R145 (Ser-422) sites. All of these sites, except 12E8, a CaM Kinase II site in the brain,[50] have been previously shown to be phosphorylated by ERK1/2 *in vitro*.[51] On the other hand, PP-2A can dephosphorylate most of abnormally hyperphosphorylated sites of tau *in vitro*.[39] Thus, the abnormal hyperphosphorylation of tau might result from a decrease in tau dephosphorylation by PP-2A and as well as from an increase in tau phosphorylation by kinases such as MAP kinases (the present study) and CaM Kinase II,[52] the activities of which are regulated by PP-2A. Although the possibility of contribution by phosphoseryl/phosphothreonyl PP activity(s) other than PP-2A cannot be ruled out in our present study, these findings are in agreement

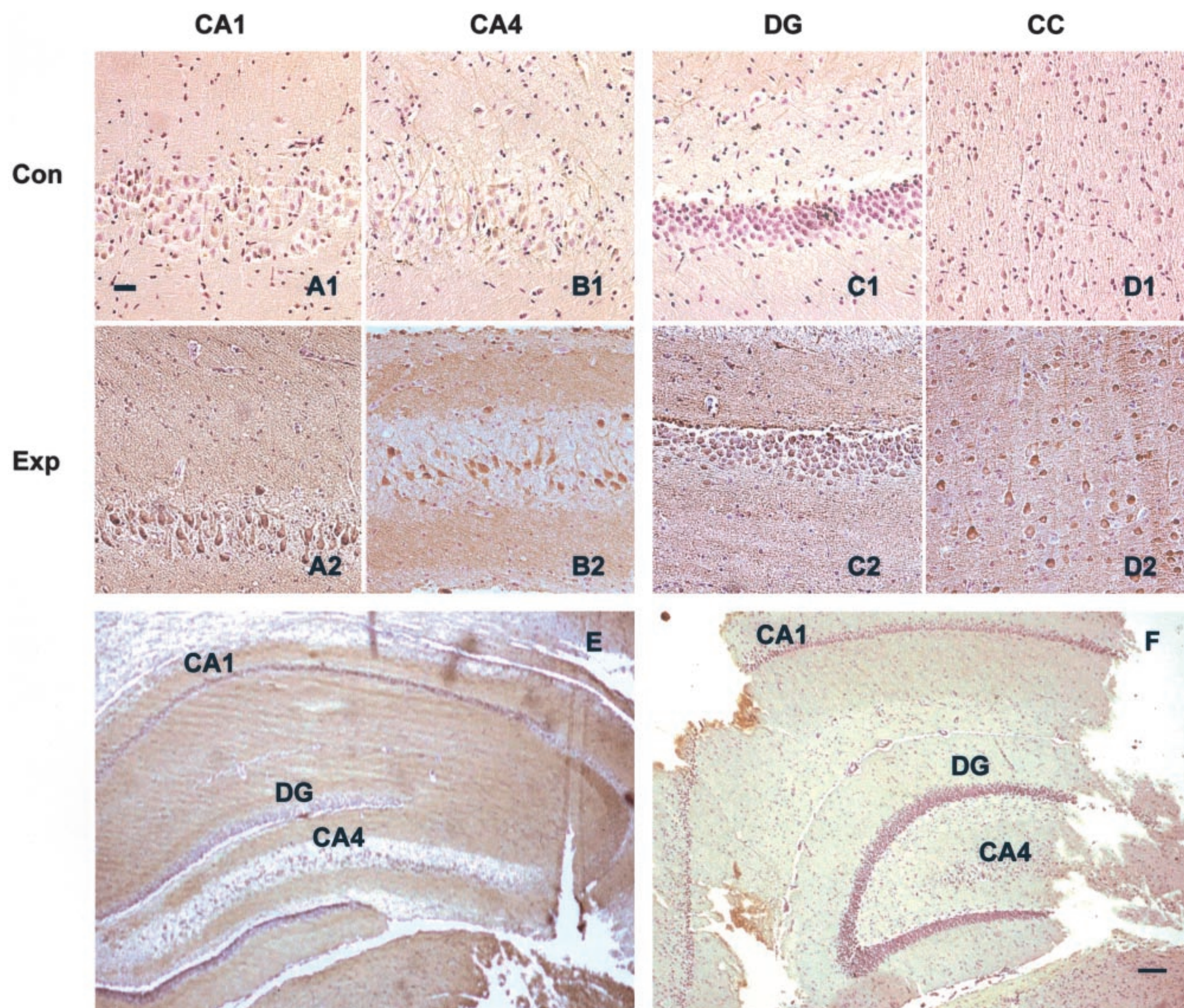

Figure 7. Immunohistochemical staining showing topography of phosphorylated tau in rat brain slices treated with OA. Rat brain slices were incubated in either oxygenated artificial CSF alone as controls (Con) or in the artificial CSF plus 1.0 μmol/L OA for 1 hour (Exp), followed by immunohistochemical staining with antibody R145 against tau phosphorylated at Ser 422. The representative staining of CA1, CA2, and DG of the hippocampal complex, layer III of the adjacent primary sensory CC, and of the whole section (**E**, Exp; **F**, Con) are shown. All sections were counterstained with cresyl violet to demonstrate the cell nuclei. Bar represents 20 μm for **A1** to **D2** and 200 μm for **E** and **F**, respectively.

with those of Kins et al[53] who used the PP-2A dominant negative transgenic mice for the inhibition of this phosphatase activity.

MAPK cascade was originally discovered as a critical regulator of cell division and differentiation. Later discovery that ERKs are abundantly expressed in neurons of mature central nervous system implied their role in mature brain. Several studies have suggested the significance of MAPK cascade in mammalian synaptic plasticity, learning, and memory (for review, see[54]). The ability of ERK1/2 to phosphorylate tau *in vitro* at many abnormal hyperphosphorylation sites as seen in AD brain[12–15] and the activation of ERK1/2, MEK1/2, and p70 S6 kinase in neurons containing abnormally hyperphosphorylated tau suggest that the MAPK cascade might be involved in the pathogenesis of AD. Although overexpression or activation of MAPK alone did not induce tau hyperphosphorylation in cultured cells,[55,56] the activated MAPK cascade in the presence of compromised PP-2A activity in selected neurons in AD brain might still contribute to the abnormal hyperphosphorylation and aggregation of tau. Since MAPK pathway is involved in multiple aspects of cell formation,[57] the activation of MAPK cascade might contribute, in addition to tau hyperphosphorylation, to some other aspects of the pathogenesis of AD.

The activation of MAPK cascade is regulated through the phosphorylation of each component kinase by the appropriate upstream kinase. MEK1/2 is activated through phosphorylation at Ser 217/Ser 221 catalyzed by Raf. The activated MEK1/2 then phosphorylates ERK1/2 at Thr 202/Tyr 204 and thereby activates it.[58] The activated ERK1/2 in turn activates p70 S6 kinase by phosphorylating it at Ser 421/Thr 424.[5–7] Although the inactivation of MAPK was generally thought to be mainly mediated by a family of dual-specificity phosphatases, named MAPK phosphatases that are transiently synthe-

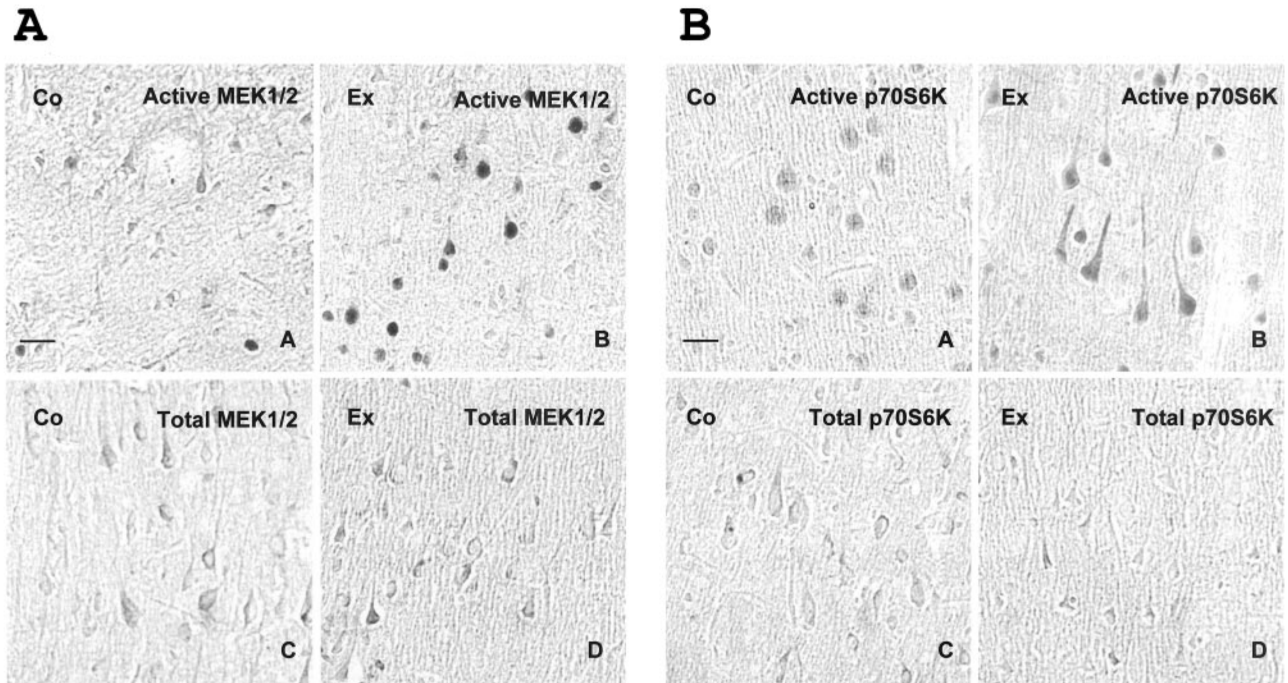

Figure 8. Immunohistochemical staining showing the Intracellular distribution of MEK1/2 and p70 S6 kinase in layer V of the cerebral cortex of rat brain slices treated with OA. Rat brain slices were incubated in either oxygenated artificial CSF alone as controls (Co) or in the artificial CSF plus 1.0 μmol/L OA for 1 hour (Ex), followed by immunocytochemical staining with antibodies to active MEK1/2 and total MEK1/2 (**A**) or to active p70 S6 kinase and total p70 S6 kinase (**B**). The magnification is indicated as 20 μm scale bar.

sized after activation of MAPKs,[59] several lines of evidence have suggested the role of PP-2A in the inactivation of MAPKs. First, the phosphorylated MEK1/2, ERK1/2, and p70 S6 kinase can be dephosphorylated and thereby inactivated *in vitro* by PP-2A ([30,31] and the present study). Second, adrenomedullin decreases ERK activity through activation of PP-2A, and selective inhibition of PP-2A with OA, which is not known to inhibit MAPK phosphatases, reverses the ERK inhibition.[33] Third, PP-2A co-immunoprecipitates with p70 S6 kinase, suggesting a close association and functional relationship of the two enzymes.[60,61] Fourth, transient suppression of

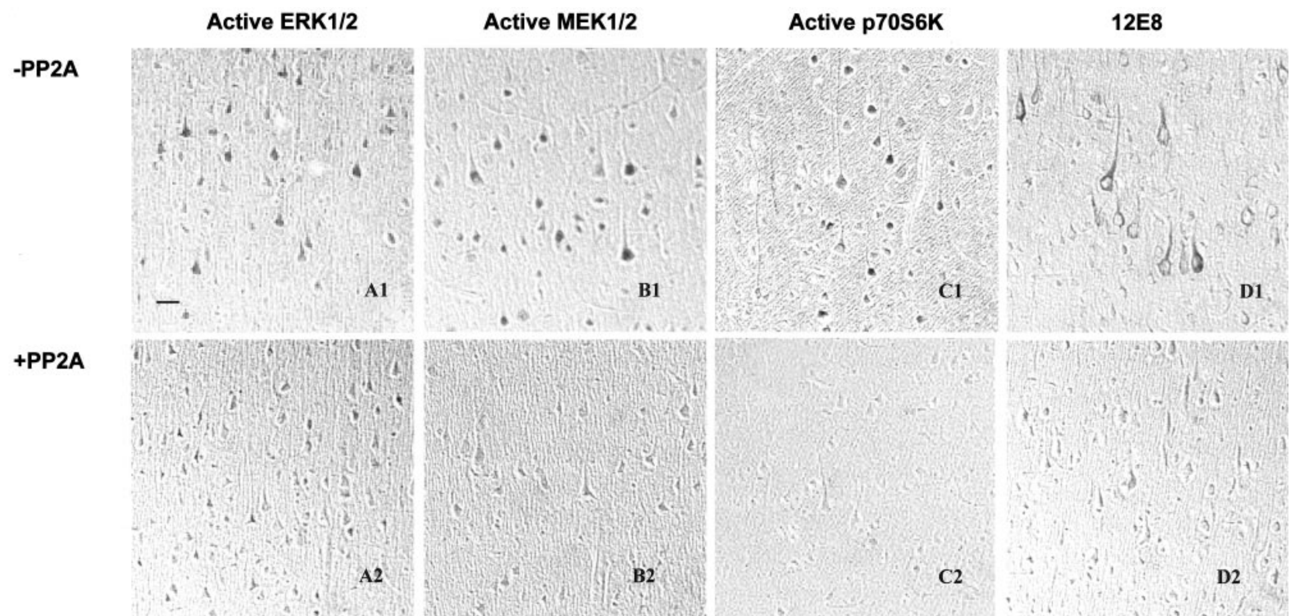

Figure 9. Immunohistochemical staining of active ERK1/2, MEK1/2, and p70 S6 kinase and of tau phosphorylated at Ser-262/356 in OA-treated tissue sections after pretreatment with PP-2A. Tissue sections from the OA-treated (same as in Figures 3 to 5) rat brain slices were incubated either with purified PP-2A in dephosphorylation buffer (+PP2A) or buffer alone (−PP2A), followed by immunohistochemical staining with antibodies to the active forms of ERK1/2 (**A1**, **A2**), MEK1/2 (**B1**, **B2**), or p70 S6 kinase (**C1**, **C2**), or with monoclonal antibody 12E8 to hyperphosphorylated tau (**D1**, **D2**). The sections of primary sensory CC are shown. The bar indicates 20 μm.

PP-2A activity in cultured cardiomyocytes induces a rapid phosphorylation and activation of MAPK.[29] The present study demonstrated the activation of MEK1/2, ERK1/2, and p70 S6 kinase by inhibition of PP-2A in metabolically active rat brain slices, suggesting the regulation of MAPK cascade by PP-2A in mammalian brain.

The functional targets of MAPK signaling depend on the precise location of the kinases within different subcellular compartments. The present study revealed marked differences in both the topography and the subcellular distribution among activated ERK1/2, MEK1/2, and p70 S6 kinase in OA-treated rat brain slices. The activation of ERK1/2 was seen mostly in the neuropil in CA3, CA1, dentate gyrus, and in layer III of the cerebral cortex and that of MEK1/2 was in the neuronal cell bodies of CA1, CA4, dentate gyrus, and layers III and V of the cerebral cortex. Unlike ERK1/2 and MEK1/2, the activated p70 S6 kinase was seen both in the neuropil and in the neuronal cell bodies and had different topographical distribution in the OA-treated brain slices. These findings suggest 1) that PP-2A regulates the activities of MEK1/2, ERK1/2, and p70 S6 kinase directly and individually and 2) that phosphorylation of a protein by these MAP kinases might depend not only on the activation of a specific signaling pathway(s) but also the subcellular localization of the substrate protein.

Translocation from cytoplasm to nucleus of some kinases of the MAPK cascade such as MEK, ERK, p70 S6, and JNK on activation has been reported previously.[62–66] The present study demonstrated that in rat brain neurons, MEK1/2 and p70 S6 kinase are primarily located in cytoplasm, while ERK1/2 is located in both cytoplasm and nucleus. Activation of ERK1/2 by inhibition of PP-2A did not significantly change the subcellular distribution of the kinase. However, a dramatic translocation of MEK1/2 and p70 S6 kinase from cytoplasmic compartment to nuclei was observed after they were activated in rat brain slices by the inhibition of PP-2A with OA. Nuclear translocation of MEK1/2 and p70 S6 kinase might be important for transcription factor activation, whereas cytosolic activated ERK1/2 may play a different role such as cytoskeletal modification. The mechanism of this PP-2A inhibition-induced translocation of MEK1/2 and p70 S6 kinase remains to be understood. The inhibition of PP-2A activity might promote the binding of these kinases to the nuclear proteins and thus prevent their relocalization to the cytoplasm as has been observed for MEK previously.[65]

Unlike MAP kinases, GSK-3, p38, and JNK were not found to show any detectable increase in phosphorylation on inhibition of PP-2A by OA in the brain slices. In agreement with the present study, Kins et al[53] also found activation of the ERK signaling in a PP-2A dominant negative transgenic mouse model. These authors also observed the activation of JNK signaling in the transgenic mouse model, whereas the inhibition of PP-2A in the normal rat brain slices in the present study did not result in any detectable change in either JNK or p38, a stress-activated signaling. These differences in the activation of the JNK signaling between the two systems might either indicate a requirement for an *in vivo* as opposed to an *ex vivo* system or it might represent some secondary effect of the dominant negative transgene unspecific to the inhibition of PP-2A. Two previous studies, one in cultured SY5Y human neuroblastoma cells[67] and another in metabolically active rat brain slices[25] in which PP-2A activity was inhibited by OA, also failed to observe any increase in the activity of GSK-3β. The latter study, however, found that PP-1 regulated the activities of GSK-3β as well as of cdc2/cdk5.

In conclusion, the present study shows that inhibition of PP-2A activity leads to the hyperphosphorylation of tau, not only due to a reduction in its dephosphorylation but probably also by phosphorylation through activation of MEK1/2, ERK1/2, and p70 S6 kinase among possibly other kinases. ERK1/2 is activated in a specific topographic and subcellular distribution pattern and tau is hyperphosphorylated at some of the same sites known to be phosphorylated by this enzyme *in vitro*. Thus, MAP kinases can play a direct role in the abnormal hyperphosphorylation of tau in PP-2A compromised brain such as in AD.

Acknowledgments

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