Influence of okadaic acid on hyperphosphorylation of tau and nicotinic acetylcholine receptors in primary neurons

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Abstract

The aim of the study was to investigate the influence of hyperphosphorylation of tau induced by okadaic acid on the expression of nicotinic acetylcholine receptors and the neurotoxicity of β -amyloid peptide. Primary cultures of neurons isolated from the hippocampus of the brains of neonatal rats were exposed to okadaic acid or/and $A\beta_{1-42}$. Tau phosphorylated at Ser404 and Ser202, and the protein expressions of $\alpha 7$, $\alpha 4$ and $\alpha 3$ nAChR subunits were quantified by Western blotting, and their corresponding mRNAs by real-time PCR. Superoxide dismutase activity was assayed biochemically and malondialdehyde by thiobarbituric acid-reactive substance. As compared to controls, phosphorylations of tau at Ser404 and Ser202 in the neurons were elevated by exposure to 20 nM okadaic acid for 48 h but not by 1 or 2 μ M $A\beta_{1-42}$. Treatment with 20 nM okadaic acid or 1 μ M $A\beta_{1-42}$ for 48 h resulted in the reduced $\alpha 7$, $\alpha 4$ and $\alpha 3$ proteins, and $\alpha 4$ and $\alpha 3$ mRNAs, as well as the decreased activity of superoxide dismutase and the increased malondialdehyde. Okadaic acid and $A\beta_{1-42}$ together caused more pronounced changes in the expressions of $\alpha 7$ and $\alpha 4$, superoxide dismutase activity and lipid peroxidation than either alone. When pre-treatment with vitamin E or lovastatin, the neurotoxicity induced by okadaic acid was significantly attenuated. These findings indicate that hyperphosphorylation of tau induced by okadaic acid inhibits the expression of nicotinic acetylcholine receptors at both the protein and mRNA levels, as well as enhances the neurotoxicity of β -amyloid peptide.

Keywords: Okadaic acid, tau protein, nicotinic acetylcholine receptor, β -amyloid peptide, neurotoxicity, primary neurons

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Introduction

Alzheimer's disease (AD), the most common cause of dementia, is characterized by the presence of extracellular senile plaques in the brain, consisting of aggregated β -amyloid peptide ($A\beta$) and intracellular neurofibrillary tangles (NFTs) containing hyperphosphorylated tau protein.¹ As a neuronal microtubule-associated protein (MAP), tau contains 80 serine/threonine and 5 tyrosine residues that can be potentially phosphorylated.² Normally, tau in the brain has 2–3 moles of phosphate per mole, and whereas in the brain of patients with AD, this stoichiometry is at least threefold greater than that of normal, in which the hyperphosphorylated protein aggregates into paired helical filaments.^{2,3}

This hyperphosphorylation not only eliminates the ability of tau to promote the assembly of stabilizing microtubules but also causes this abnormal protein to sequester normal tau, as well as MAP1A/MAP1B and MAP2, thereby

inhibiting and disrupting microtubules.⁴ Protein phosphatase 2A (PP2A) is primarily responsible for dephosphorylation of tau in the human brain,⁵ and the expression and activity of the enzyme are reduced in selected regions of brains with AD.⁶

Okadaic acid (OA), a polyether C38 fatty acid extracted from the black sponge *Halichondria okadai*, is a potent selective inhibitor of the serine/threonine (Ser/Thr) protein phosphatase 1 (PP1) and PP2A,^{7–9} which may have rapid metabolic consequences that lead to cell death by altering rates of phosphorylation/dephosphorylation.¹⁰ Moreover, in experimental animals, OA may cause deposition of $A\beta$ and tau hyperphosphorylation, and subsequent neuronal degeneration, loss of synapses and memory impairment, all of which resemble the pathology of AD.^{10–13} Consequently, OA is a powerful tool for unraveling the various regulatory mechanisms underlying the neurotoxicity associated with AD.¹¹ Although this inhibitor is known to

give rise to neurotoxicity by a number of pathways, the exact mechanism remains unclear.

One important feature of AD is loss of cholinergic neurons and alterations in cholinergic receptors.¹⁴ For example, expression of nicotinic acetylcholine receptors (nAChRs), which belong to a super-family of ligand-gated ion channels and mediate a variety of important physiological functions, is attenuated in the hippocampus and cerebral cortex, both in patients and animal models with AD.^{15–17} In the central nervous system, α ($\alpha 2$ – $\alpha 10$) and β ($\beta 2$ – $\beta 4$) subunits combine to form both hetero- and homo-pentameric neuronal nAChRs. OA administration resulted in memory impairment with the decreased cerebral blood flow and ATP level, the increases in mitochondrial (Ca^{2+})_i, neuroinflammation, oxidative-nitrosative stress, Caspase-9 and cholinergic dysfunction in the cerebral cortex and hippocampus of mice brain.¹⁸

In the present study, we exposed primary cultures of neurons to OA to determine whether hyperphosphorylation of tau induced by OA influences the expression of nAChRs and thereby potentially enhances the neurotoxicity of A β .

Materials and methods

Materials

Lipid Peroxidation Assay Kit for detecting malondialdehyde (MDA), superoxide dismutase (SOD) Determination Kit, poly-L-lysine hydrobromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lovastatin, vitamin E and other general chemicals were purchased from Sigma (USA). Rabbit polyclonal anti- $\alpha 7$ nAChR (SC5544) antibody, goat polyclonal anti- $\alpha 3$ (SC1771) and - $\alpha 4$ (SC1772) antibodies, rabbit polyclonal anti-tau phosphorylated at Ser404 antibody (SC12952), mouse monoclonal anti- β -actin antibody, donkey anti-goat IgG (SC2020), anti-mouse IgG (SC2005) and Cruz Marker Molecular Weight Standards (SC2035) conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology Inc. (USA). OA ($\geq 98\%$ pure, as determined by HPLC) was procured from Enzo Life Sciences (USA). Neurobasal-A Medium, Hibernate-E Medium, B-27 Serum-Free Supplement, GlutaMAX Supplement, Dulbecco's MEM medium (DMEM), fetal bovine serum (FBS) and streptomycin-penicillin (Gibco Life Technologies, USA); anti-tau phosphorylated at Ser202 antibody (ab108378) (Abcam, USA); anti-mouse IgG labeled with CY-3, anti-rabbit IgG labeled with 488, a cDNA synthesis kit and TRIzol reagent (Thermo Scientific, USA); SYBR green master (ROCHE, Switzerland); mouse anti-NeuN antibody (Merck Millipore, Germany) and rabbit anti-GFAP antibody (Dako, Denmark) were purchased from the sources indicated.

Cell cultures and treatments

Primary hippocampal neurons were prepared from the brains of neonatal Sprague-Dawley (SD) rats according to a published procedure^{19,20} with slight modifications. Briefly, the hippocampal regions were separated from the

brains of neonatal rats within 2–3 min after sacrifice and maintained in Hibernate A Medium chilled on ice. After removing the meninges, the hippocampus was washed three times with Hank's buffered saline solution and then digested with 0.25% trypsin for 10 min in 37°C. Subsequently, the incubation medium was discarded and DMEM containing 10% FBS was added to terminate digestion.

After washing twice more with Hank's buffered saline solution, the digested tissue was resuspended in 2 ml of Neurobasal/B27 complete medium (Neurobasal A Medium with 2% B27, 1% GlutaMAX Supplement, 100 U/ml penicillin and 100 mg/ml streptomycin) and broken apart with fire-polished glass pipettes. The upper single-cell suspension was transferred into a new tube and the cells were counted by trypan blue exclusion, and thereafter placed on 96-, 12- or 6-well on PLL-coated plates at a cell density of approximately $5.0 \times 10^4/\text{cm}^2$. The neurons were maintained in a humidified atmosphere containing 5% CO_2 at 37°C, with half-replacement of the medium every three days. The primary neurons isolated in this manner were approximately 90% pure, as determined by immunofluorescent double staining with mouse anti-NeuN antibody (diluted 1:50) and anti-mouse IgG labeled with CY-3 (red), and with rabbit anti-GFAP (glialfibrillary acidic protein) antibody (diluted 1:300) and anti-rabbit IgG labeled with 488 (green). After 10 days of incubation, the medium was replaced with neurobasal medium without B27, and the neurons then were treated with OA or/and A β_{1-42} for 48 or 24 h. To investigate the neuroprotective effect of vitamin E or lovastatin against OA, the cultured neurons were pre-treated with 20 μM vitamin E or 0.1 μM lovastatin for 24 h before treatment with OA for 48 h.

All experiments described here were approved by the regional ethical committee of Guizhou Province of China.

Assay of MTT reduction

Following treatment with OA and/or A β_{1-42} for 48 h, 10 ml of MTT stock (1 mg/ml) was added to each well and the incubation continued for another 4 h. Thereafter, 100 μl of a solution containing 20% SDS and 50% dimethylformamide (pH 4.8) was then added to each well. After incubation overnight, the absorption at a 570-nm was determined.²¹

Analysis of phosphorylated tau protein by Western blotting

For analysis of tau protein phosphorylated at Ser404 and Ser202, proteins extracted from the neurons²² were separated with 10% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) followed by incubation with primary antibody for 120 min. After washing, the membranes were incubated with HRP conjugated anti-rabbit IgG (1:5000) for 60 min and, finally, with ECL Plus reagent for 5 min. The signals were visualized by exposure to Hyper Performance Chemiluminescence film.

Table 1 Primers used for amplification of cDNA encoding for different subunits of rat nAChRs or for cyclophilin

nAChR	subunits or CP	5'-3' Nucleotide sequence	Size (bp)	T _m (°C)
α3		Upper-TTCAGCCGTGCAGACTCCA Lower-GGCAACGTACTTCCAATCATC	271	55
α4		Upper-TAATACGACTCACTATAGGGAGGGCGAGGCCGGCATCTTGAGT Lower-GCTGGGCACATGCTGGACAC	274	60
α7		Upper-TAATACGACTCACTATAGGGAGGAAGAGGCCCGGAGAGGACAA Lower-CGCCACATACGACCCAGAG	207	60
CP		Upper-GACAAGGTCCCAAAGACAGC Lower-GTCCAGCATTGTCATGGAC	235	C

CP: cyclophilin; T_m: temperature of denaturation.

Quantification of α7, α4 and α3 nAChR proteins by Western blotting

The levels of α7, α4 and α3 nAChR proteins were quantitated by Western blotting as described previously.²³ Briefly, equal amounts of the solubilized membrane fractions prepared from cultured cells were separated on 10% SDS-PAGE and then blotted onto PVDF membranes. These membranes were then incubated with rabbit polyclonal anti-α7, goat polyclonal anti-α4 or -α3 antibody (0.25 mg/ml in each case) for 120 min, and after washing, with HRP-conjugated anti-goat or anti-rabbit IgG (0.04 mg/ml) for 60 min. Finally, the membranes were incubated in ECL Plus reagent for 5–15 min and signals visualized by exposure to Hyper Performance Chemiluminescence film.

Quantification of mRNA encoding α7, α4 and α3 nAChRs by real-time PCR

Following of extraction of cellular RNA with Trizol Reagent, 1 μg was subjected to reverse transcription with primers specified for α7, α4 or α3 nAChR mRNA (Table 1). Quantitative real-time PCR was carried out in the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA) in accordance with the manufacturer's protocol and analyzed with GeneAmp7300 SDS software. In brief, the 10 μl reaction solutions contained 1 μl first-strand cDNA, 5 μl 2×SYBR Green Master (Rox) Mix, 0.5 μl each of the forward and reverse primers (10 μM), 3 μl DNase and RNase-free H₂O. The thermal cycling conditions involved 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The levels of α7, α4 and α3 nAChR transcripts were estimated using the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ (threshold cycle) represents the difference in CT values for the target and housekeeping mRNA.

Analysis of the activity of SOD and content of MDA

SOD was assayed by the SOD determination Kit-WST. In brief, the neurons were homogenized on ice in the lysis buffer and then centrifuged at 13,000 × g for 10 min to remove insoluble material. The supernatant, enzyme assay solution and WST buffer were added in the order indicated by the instructions for the kit. Following incubation at 37°C for 20 min, the absorbance at 450 nm was

determined using a microplate reader and SOD activity calculated.

The content of MDA, a major product of lipid peroxidation, was detected with thiobarbituric acid-reactive substance (TBARS) assay employing the Lipid Peroxidation Assay Kit.²⁴ In brief, 200 μl of the supernatant prepared as described above were placed into a micro-centrifuge tube and 600 μl of the TBARS solution then added. This mixture was incubated at 95°C for 60 min and cooled to room temperature in an ice bath for 10 min. Finally, 200 μl was pipetted into each well of a 96-well plate and the absorbance at 532 nm measured.

Statistical analysis

The values are expressed as means ± SD for the different groups. These means were examined for statistically significant differences employing analysis of variance (ANOVA), the Student–Newman–Keul's test or the paired-samples *t* test in the SPSS17.0 software (SPSS Inc., USA).

Results

Purity of the primary cultured neurons

Immunostaining of the primary cultured neurons originating from the hippocampal region of the brains of newborn rats with an antibody directed towards the neuronal marker (NeuN) (Figure 1(a)) and an antibody against GFAP, a marker for astrocytes (Figure 1(b)), revealed that 90.5% of these cells were neurons (Figure 1(c)).

Cell viability and cytotoxicity

The MTT assay was used to detect cell proliferation and cytotoxicity (Liu et al., 1997). After exposure to various concentrations of OA or Aβ_{1–42} for 48 h, MTT reduction was attenuated significantly at more than 30 nM or 1.5 μM, respectively (Figure 2).

Expression of phosphorylated tau

In cells incubated with OA for 24 h, the level of tau phosphorylated at Ser404 or Ser202 did not change significantly (data not shown). However, this levels of tau phosphorylated at Ser404 or Ser202 were increased by 138% and 145%, respectively, upon incubation with 20 nM (but not 10 nM) OA for 48 h (Figure 3(a) and (c)). While, no obvious

Figure 1 Immunostaining of primary cultures of neurons originating from the hippocampus of the brains of neonatal rats. The mouse antibody against the NeuN and rabbit antibody against the GFAP were used. NeuN-positive neurons (a) and GFAP-positive astrocytes (b) were shown. (c) The proportions of neurons and astrocytes was shown as bar graph. (A color version of this figure is available in the online journal.)

Figure 2 MTT levels in the primary neurons treated with OA or Aβ₁₋₄₂. MTT reduction was detected after the primary neurons were treated with 1–40 nM OA (a) or 1–2 μM Aβ₁₋₄₂ (b) for 48 h. The results represent means ± SD (n = 20). *P < 0.05 and **P < 0.01 in comparison to untreated cells, as determined by analysis of variance (ANOVA), followed by the Student–Newman–Keul's test

differences in phosphorylated tau were found when the cultured neurons were treated with 1 or 2 μM Aβ₁₋₄₂ for 48 h (Figure 3(b) and (d)).

Expression of α7, α4 and α3 nAChR subunits at protein and mRNA levels

When the primary cultured neurons were treated with 20 nM OA for 48 h, the levels of α7 was reduced by 37% (Figure 4(a)), α4 by 44% (Figure 4(b)) and α3 by 40% (Figure 4(c)). Treatment with 1 μM Aβ₁₋₄₂ for 48 h resulted in corresponding the significant decreases of 42% (Figure 4(a)), 40% (Figure 4(b)) and 38% (Figure 4(c)), respectively. Interestingly, when the neurons were treated with 20 nM OA and 1 μM Aβ₁₋₄₂ in combination for 48 h, the level of α7 was lowered by 60% (Figure 4(a)) and that of α4 by 70% (Figure 4(b)) but with no further reduction in the level of α3 (Figure 4(c)).

When the cultured neurons were treated with 20 nM OA for 48 h, there was no change in the level of α7 mRNA (Figure 5(a)), whereas the levels of α4 and α3 mRNAs were lowered by 42% (Figure 5(b)) and 45% (Figure 5(c)), respectively. Similarly, incubation with 1 μM Aβ₁₋₄₂ for 48 h reduced the level of α4 mRNA by 28% (Figure 5(b)) and that of α3 by 50% (Figure 5(c)), without affecting α7 mRNA (Figure 5(a)). Clearly, exposure to OA and Aβ₁₋₄₂ together caused greater decreases in the levels of α7 mRNA

(Figure 5(a)) and α4 mRNA (Figure 5(b)) than either OA or Aβ alone; but no further effect on α3 mRNA (Figure 5(c)).

Neurotoxicity induced by OA or Aβ

The activity of SOD was decreased and the content of MDA increased upon exposure of the primary-cultured neurons to 20 nM OA or 1 μM Aβ₁₋₄₂ for 48 h and even more so upon treatment with a combination of both (Table 2). In addition, when the pre-treatment with vitamin E or lovastatin to primary neurons, the neurotoxicity resulted from OA, including the decreases in activity of SOD, MTT reduction and protein expression of α7 nAChR subunit, was attenuated (Table 3).

Discussion

It has been indicated that OA increases tau phosphorylation, β-amyloid cognitive deficiency seen in OA-treated rats without a change in motor function.²⁵ The unilateral micro-infusion of OA into the dorsal hippocampus causes cognitive deficiency, NFTs-like pathological changes (in both the cortex and hippocampus), and oxidative stress (such as enhanced protein carbonylation and lipid peroxidation), which are characteristic features of AD.²⁶ In addition, exposure of rat neurons to OA induced accumulation of autophagosomes, which occurs in AD.²⁷

Figure 3 The level of phosphorylation of tau in primary-cultured neurons exposed to OA or Aβ₁₋₄₂. Tau protein was detected with rabbit polyclonal anti-tau phosphorylated antibody at Ser404 (a and b) or Ser202 (c and d) to OA (10 or 20 nM) and Aβ₁₋₄₂ (1 or 2 μM), respectively, for 48 h. The results are expressed as means ± SD (*n* = 8). **P* < 0.05 in comparison to untreated cells

Figure 4 The levels of α7 (a), α4 (b) and α3 (c) nAChR proteins in primary-cultured neurons exposed to OA or/and Aβ. Immunoblotting was carried out with rabbit monoclonal anti-α7, and goat polyclonal anti-α4 and -α3 antibodies after primary-cultured neurons were exposed to 20 nM OA or/and 1 μM Aβ₁₋₄₂ for 48 h. The results are expressed as means ± SD (*n* = 6). **P* < 0.05 in comparison to controls; and #*P* < 0.05 in comparison to the treatment of OA or Aβ alone. Representative Western blots are depicted beneath the bar graphs

Figure 5 The levels of $\alpha 7$ (a), $\alpha 4$ (b) and $\alpha 3$ (c) nAChR mRNA in primary-cultured neurons exposed to OA (20 nM) or/and $A\beta_{1-42}$ (1 μ M) for 48 h. The mRNA levels were determined by real-time PCR. The results are expressed as means $\pm$ SD ($n = 6$). * $P < 0.05$ in comparison to controls; and # $P < 0.05$ in comparison to the treatment of OA or $A\beta$ alone

Table 2 SOD activity and MDA content in primary-cultured neurons exposed to OA and $A\beta_{1-42}$

Exposure	SOD (U/ mg protein)	MDA (nmol/mg protein)
Control	28.87 $\pm$ 5.42	3.13 $\pm$ 0.36
OA	14.47 $\pm$ 3.62*	3.35 $\pm$ 0.44
$A\beta$	17.14 $\pm$ 4.21*	4.37 $\pm$ 0.89*
OA+A β	9.86 $\pm$ 3.12*#	6.25 $\pm$ 0.44*#

$n = 8$.

* $P < 0.05$ compared to control values.

$P < 0.05$ as compared to cells exposed to OA or $A\beta$ alone.

Table 3 Attenuation of Vitamin E and lovastatin on neurotoxicity of primary cultured neurons exposed to OA

Exposure	SOD (U/mg protein)	MTT reduction (% of control)	nAChR $\alpha 7$ (% of control)
Control	26.99 $\pm$ 3.69	100.00 $\pm$ 5.12	100.00 $\pm$ 1.16
OA	13.22 $\pm$ 4.19*	84.49 $\pm$ 4.61*	78.40 $\pm$ 0.72*
VE	27.88 $\pm$ 1.94	102.70 $\pm$ 3.87	104.8 $\pm$ 1.15
Lov	26.35 $\pm$ 2.80	98.50 $\pm$ 5.48	132.7 $\pm$ 1.25*
OA+VE	21.52 $\pm$ 1.95#	97.74 $\pm$ 5.83#	99.40 $\pm$ 1.06#
OA+Lov	18.45 $\pm$ 2.12#	99.12 $\pm$ 6.01#	97.56 $\pm$ 2.34#

$n = 8$.

* $P < 0.05$ compared to control values.

$P < 0.05$ as compared to cells exposed to OA alone.

Thus, OA-induced neurotoxicity may be a novel tool for unraveling the pathology of AD, as well as developing new therapeutic approaches.⁷

Hyperphosphorylation of tau and subsequent formation of intracellular NFTs are thought to be implicated in the pathogenesis of AD.⁴ The abnormal phosphorylation of tau observed in AD brains is due to an imbalanced regulation of protein kinases and protein phosphatases. The reduced activities of the tau PP-2A and PP-1 in the AD brain would induce the increased phosphorylation of tau either by diminished dephosphorylation of this protein directly or by tau kinases that are activated by phosphorylation.⁶

In these investigations in vitro, the exposure of primary cultured neurons or SH-SY5Y cell line to OA induces

hyperphosphorylation of tau and neurotoxicity.^{26,28-30} Here, we found that both of OA and $A\beta_{1-42}$ at high concentrations are neurotoxic towards the cultured neurons and can attenuate the viability of the cells. The same concentration of OA enhanced phosphorylation of tau at Ser404 or Ser202, in agreement with previous observations.^{26,30}

Although definitive proof is lacking and there is substantial criticism of this hypothesis, accumulating evidence supports involvement of the amyloid cascade and, in particular, changes in $A\beta$ -induced tau-pathology in the development of AD.^{31,32} However, some reports contradict such involvement. For example, exposure of primary cultures of rat embryonic hippocampal cells to 0.5 μ M fibrillary $A\beta_{1-42}$ for three days caused retraction of dendrites and shrinkage of neuronal cell bodies, with no impact on tau phosphorylation at Ser202, Thr231/Ser235, Ser262 or Ser396/Ser404.³³ In the present study, $A\beta_{1-42}$ did not enhance phosphorylation of tau at Ser404 and Ser202, perhaps because the concentration was too low and/or incubation time too short.

Loss of nAChRs is consistently observed in the brains of patients with AD. Significant reduction in the level of acetylcholine, AChE activity and mRNA, and expression of $\alpha 7$ nAChR in rat brain following injection of OA have been reported.³⁴ In the present study, the levels of $\alpha 7$, $\alpha 4$ and $\alpha 3$ nAChR proteins in neurons exposed to OA were lowered significantly. In addition, significant decreases in the levels of $\alpha 4$ and $\alpha 3$ nAChR mRNA were also detected here. However, the level of $\alpha 7$ mRNA was not changed, which might be a compensation for the loss of the receptors.^{35,36}

Immunohistochemical analysis revealed that the selective $\alpha 7$ nAChR agonist A-582941 enhances phosphorylation of glycogen synthase kinase (GSK)3 β in the cingulate cortex and hippocampus, and decreased phosphorylation of tau in hippocampal CA3 Mossy fibers and spinal motoneurons in a hypothermia-induced tau hyperphosphorylation mouse model and double transgenic amyloid precursor peptide (APP)/tau AD mice, respectively, which demonstrates that inactivation of GSK3 β may be associated with $\alpha 7$ nAChR-induced signaling, leading to attenuated hyperphosphorylation of tau.³⁷ Moreover, the selective $\alpha 7$ and $\beta 2$ nAChR agonists PNU 282987 and 5-Iodo-A85380, respectively, in the cultural SH-SY5Y cells could afford neuroprotection against OA-induced neurotoxicity, suggesting that

targeting nAChRs might attenuate neurodegeneration secondary to hyperphosphorylation of tau.³⁸ The results documented here indicate that the relationships between OA, nAChRs and hyperphosphorylation of tau are complex.

The discovery, in which certain forms of early onset familial AD might be caused by enhanced production of A β , gave rise to the hypothesis, that amyloidogenic A β is intimately involved in the pathogenesis of AD.³⁹ A β is generally considered to cause synaptic loss and neurodegeneration in AD⁴⁰ and a belief that has driven the development of drugs for treatment of AD for more than 20 years.⁴¹ However, it was indicated that the amyloid hypothesis may be failing therapeutically due to several unresolved issues in the field including the presence of A β deposition in cognitively normal individuals, the weak correlation between plaque load and cognition.⁴² Previously, we found that A β induced the inhibited expression of nAChR subunits at both protein and mRNA levels.^{43,44} Here, we found that exposure of cultured neurons to A β_{1-42} also lowered the levels of $\alpha 7$, $\alpha 4$ and $\alpha 3$ nAChR proteins, as well as $\alpha 4$ and $\alpha 3$ mRNA, which is consistent with our earlier findings.

The $\alpha 7$ nAChR subunit appears to provide neuroprotection in connection with AD, since selective elevation of its level in the brains of transgenic mice carrying the Swedish APP670/671 mutation can compensate for deficits in synaptic plasticity due to an elevated level of soluble β -amyloid peptide (A β).^{35,36} Interestingly, the level of $\alpha 7$ mRNA in cultured neurons was not altered here by exposure to OA or A β alone but was lowered significantly by both in combination.

At the same time, such combined treatments did not alter the expression of $\alpha 3$ nAChR protein or mRNA to a greater extent than exposure to OA or A β alone. In our earlier reports, $\alpha 3$ nAChR was shown to promote cleavage of APP by α -secretase, enhance antioxidation and inhibit the toxicity of A β by reducing the expression of BACE1 (β -site APP cleaving enzyme 1), suggesting that this subunit might also play an important neuroprotective role in connection with the pathogenesis of AD.^{45,46} Whether $\alpha 3$ nAChR can counteract mixed toxic effects requires, indeed, further investigation. In addition, OA not only inhibits phosphatases acting directly on tau but also elevates oxidative stress and neuroinflammation,⁷ indicating that OA may play more complex roles in connection with the different subtypes of nAChRs.

Interestingly, upon exposure of cultured neurons to A β here, their SOD activity was reduced and MDA content increased, in agreement with earlier observations.^{14,45,47} The effects were more pronounced upon exposure to OA and A β in combination than either alone, indicating that OA may enhance the neurotoxicity of A β towards primary cultured neurons or vice-versa.

OA causes neurotoxicity by various pathways. A dramatic increase in the phosphorylation/activation of the extracellular signal-regulated protein kinase (ERK1/2), mitogen/extracellular signal-regulated kinases (MEK1/2), and p70 S6 kinase was observed in OA-treated slices of rat brains, suggesting that in AD brain the decrease in PP-2A activity could cause 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.⁴⁸ The activation of major kinases, such as mitogen-activated protein kinases, ERK, protein kinase A, c-Jun N-terminal kinase, protein kinase C, Ca²⁺/calmodulin-dependent protein kinase II, Calpain, and GSK3 β , in neurons is associated with AD pathology. These kinases, associated with abnormal hyperphosphorylation of tau, suggest that the cascade of these kinases could exclusively be involved in the pathogenesis of AD.⁷

Interestingly, here we found that the pre-treatment with vitamin E or lovastatin attenuated the neurotoxicity of OA, including the activity of SOD, cellular viability and expression of $\alpha 7$ nAChR, in the primary-cultured neurons. It indicated that vitamin E completely prevented the lipid peroxidation induced by OA in vitro.⁴⁹ In our previous study, we have found that lovastatin may play a neuroprotective role without cholesterol dependent and can stimulate $\alpha 7$ nAChR by a mechanism that enhances production of α -secretase during APP processing.⁵⁰ Further investigations are needed for confirming the neurotoxic effects of OA in molecular mechanism in AD tau pathology.

In conclusion, we show here that OA induces hyperphosphorylation of tau, inhibits expressions of the $\alpha 7$, $\alpha 4$ and $\alpha 3$ nAChR subunits, and results in neurotoxicity; A β did not influence the level of tau hyperphosphorylation in the experiment condition; finally, OA may enhance the neurotoxicity of A β .

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. LZ, XY and X-LW conducted most of the experiments and wrote the manuscript; JP advised the design of the research project and guided young researchers; Z-Z G was responsible for the design of the study, controls of data analyses and paper writing. LZ and YX have contributed equally to this paper.

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