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An okadaic acid induced Alzheimer's model and the neuroprotective effects of estrogen

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Alzheimer's disease (AD) is a neurodegenerative disease that causes progressive cognitive and behavior deterioration in the elderly over age 65, although the less-prevalent early-onset AD can occur much earlier. AD is the most common cause of dementia, accounting for 50 to 70 percent of all dementia cases. Affected brains of AD are characterized by the presence of senile plaques (SP), neurofibrillary tangles (NFTs) and the loss of cholinergic neurons in the basal forebrain. To date, not only is there no cure for AD, but also the cause and the factors that underlie the progression of AD are not well known.

It is critical to develop useful animal models to study the pathology of AD for preclinical testing of drugs. It is well known that very few species develop the behavioral, cognitive and neuropathological symptoms of AD spontaneously. To date, no ideal animal AD tau models have been described. Most of the commonly used tau transgenic models are associated with the development of motor impairments, which significantly limit the use of these models in behavioral tests. In the present study, in an attempt to characterize a new experimental *in vivo* AD model, okadaic acid (OA), a protein

phosphatase inhibitor, was microinfused unilaterally, via an osmotic pump, into the dorsal hippocampus area of ovariectomized female adult rat. After 14 days of OA infusion, rats were subjected to behavior tasks, including spatial learning and memory learning (as assessed by Morris Water Maze) and motor function (by Rotarod). Then the rat brain was subject to Bielschowsky's silver staining and immunohistochemistry for testing tau pathology. Meanwhile, right after the OA infusion, the model was subjected to testing the levels of phosphorylated tau, tau protein phosphatases and certain tau kinases without behavioral tests. Our data showed that the unilateral microinfusion of OA into the dorsal hippocampus could contribute to a cognitive deficiency as well as NFTs-like pathological changes evidenced by the significant increase of tau hyperphosphorylation. Further, our data revealed that cdk5 may be involved in OA induced tau hyperphosphorylation. Our data also showed that the unilateral microinfusion of OA into dorsal hippocampus induced oxidative stress in both cortex and hippocampus.

Epidemiological studies showed that AD is three times more prevalent in women than men and estrogen protects against AD. To investigate the effect of estrogen on tau phosphorylation, SH-SY5Y cell line was treated with OA to induce tau phosphorylation and the neuroprotective effects of estrogen were observed by co-treatment with estrogen. We found OA induced in vitro tau hyperphosphorylation, which was prevented by estrogen in a dose dependent manner. This preventive effect could be partially blocked by ICI 182,780, an estrogen receptor (ER) antagonist. Meanwhile, an OA induced ugregulation of cdk5 and inactive GSK3β (p-Ser 9) levels were also observed. Estrogen

was able to block this effect but counteracted by ICI 182,780. Our results suggest that cdk5 may be involved in OA induced tau hyperphosphorylation and estrogen can prevent the tau hyperphosphorylation via re-establishing the balance between tau kinases and phosphatases. This effect may be mediated by an ER.

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LIST OF ABBREVIATIONS

Aβ	beta amyloid peptide
ABC	avidin-biotin complex
AD	Alzheimer's disease
APP	amyloid precursor protein
BHT	butylated hydroxyl toluene
Cdk5	cyclin-dependent kinase 5
DAB	3,3'-diaminobenzidine
DMSO	dimethyl sulfoxide
DNPH	2,4-dinitrophenylhydrazine
E2	17β-estradiol
ECL	enhanced chemiluminescence
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
GSK3β	glycogen synthase kinase 3 beta
HYPO	sodium thiosulfate

MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MWM	Morris water maze
NFTs	neurofibrillary tangles
OA	okadaic acid
PHF	paired helical filaments
PMSF	phenylmethyl sulphonyl fluoride
PP	protein phosphatase
PVDF	polyvinylidene difluoride
SD	Sprague Dawley
Ser	serine
SP	senile plaque
TCA	trichloroacetic acid
Thr	threonine
TPK	tau protein kinase

WHI	Women’s Health Initiative

Chapter 1: Introduction

Alzheimer's disease

Alzheimer's disease (AD) is a brain disorder named for German physician Alois Alzheimer, who first described it in 1906 (1). AD is a neurodegenerative disease that causes progressive cognitive and behavior deterioration in the elderly over age 65 (2, 3), although the less-prevalent early-onset AD can occur much earlier. AD is the most common cause of dementia, accounting for 50 to 70 percent of all dementia cases (3). Affected brains of AD are characterized by the presence of senile plaques (SP), neurofibrillary tangles (NFTs) and the loss of cholinergic neurons in the basal forebrain (4-6). To date, not only is there no cure for AD, but also the cause and the factors that underlie the progression of Alzheimer's disease are not well understood. However, it is generally postulated that beta amyloid peptide (Aβ) and tau protein play a key role in the progressive neurodegeneration observed in AD (7, 8).

The etiology of AD

The etiology of AD remains elusive. Although various hypotheses have been proposed to explain the molecular pathogenesis of AD, two major competing hypotheses exist to explain the cause of the disease: amyloid hypothesis (9) and tau hypothesis (10). The supporters of each side have argued long and hard about whether one of the

pathological hallmarks is necessary and sufficient to induce dementia, or the lesion is the cause of another lesion. One of the most challenging areas of research is to elucidate the relationship between these AD hallmarks. Intracellular NFTs and extracellular SP are the two well defined pathological hallmarks of Alzheimer's disease (AD). There are still many questions remained unanswered, although over 100 years' effort have been put in the study of the lesions of SP and NFTs since the first description by Dr. Alois Alzheimer. It is still a mystery how the NFTs and SP are formed, though the components of both are well studied.

Amyloid hypothesis

The "amyloid hypothesis" was first proposed in 1991 ant states that Aβ deposition is the central event and the fundamental cause of AD (9). Aβ, a peptide of 39-43 amino acids, is the major component of SP in the brains of AD patients (11). With the help of β and γ secretases (12-15), Aβ is the product of sequential cleavage of the amyloid precursor protein (APP), a transmembrane glycoprotein (16-18). The most common isoforms are Aβ40 and Aβ42, of which Aβ42 is the more fibrillogenic due to its more hydrophobic nature (19). Mutations in APP associated with early-onset AD have been found to increase the production of either total Aβ or the relative concentration of Aβ42 (15, 20).

This theory is supported by the fact that people with Down syndrome, which has triplication of chromosome 21 on which APP gene located (21), develop sufficient neuropathology for a diagnosis of early-onset Alzheimer disease by the age of 40 years (22, 23). Moreover, associated with late-onset AD, a locus on chromosome 10 is also associated with increase of Aβ generation (24, 25). APOE4 is another major genetic risk factor for late-onset AD, which results in the accumulation of Aβ in the cerebral cortex before clinical AD (26). More evidence for the amyloid hypothesis comes from the study with human APP gene mutant transgenic mice that display amyloid plaques with spatial learning deficits (27, 28). Further evidence is from the study of amyloid vaccine. With passive and active immunization against Aβ, both plaque pathology and behavior phenotypes in the transgenic mice are arrested and even reversed (29-31).

Although the *amyloid hypothesis* has proposed incredible explanations for etiology of AD and has received few serious challenges, it is not fully supported by all the evidence, of which some actually are against it. For example, amyloid vaccine has been considered a promising therapy for AD, but recent clinical studies showed that it failed to improve dementia, though it showed the effect on clearing the amyloid plaques on early human trials (32). Moreover, it was found that deposition of amyloid plaques had no correlation with hippoampal neuronal loss in a transgenic mice model of AD (33). Then, the tau hypothesis is proposed to solve the puzzle of AD (10).

Tau hypothesis

Tau hypothesis is the idea that tau pathology is the central event of AD pathogenesis. NFT is another hallmark of AD. Consisting mainly of hyperphosphorylated tau protein, NFTs are pathological tau protein aggregates, also refered as paired helical filaments (PHF), found within neurons in cases of AD (34, 35). Tau proteins are microtubule associated proteins that are abundant in neurons at central nervous system (36). Under physiological condition, tau plays a key role in microtubules stabilization, axonal transportation and neurite outgrowth (36-39). Under pathological conditions, deposits of abnormally hyperphosphorylated tau protein are found in many neurodegenerative disorders like AD (37, 39-42). Meanwhile, tau pathology is also found in the aging brain (40, 43). However, the relationship between tau pathology in brain aging and the development of tauopathy, neuronal disorders resulting from the pathological aggregation of tau protein in the brain, and cognitive deficits is unclear.

The *tau hypothesis* is supported by the clinical studies showing that it is the numbers of NFTs, rather than the degree of plaques, that correlate closely with the severity of dementia (44, 45). Evidence also shows that NFTs are found in the neurons, aggregate to disintegrate the neuron's transport system and result in neuronal death (10, 46-48). Studies from transgenic mice have showed that mice expressing a repressible human tau variant results in progressive age-related NFTs, neuronal death and behavioral

impairment which could be reversed by the suppression of transgenic tau except for the accumulation of NFTs (49, 50). Although *tau hypothesis* is supported by many studies, it remains unclear whether NFTs are the initiating factor or merely markers of the disease process and whether NFTs crosstalks with other AD hallmarks.

Tauopathy and AD

Tauopathies are a group of diverse neurodegenerative diseases, such as AD, which have a common pathological feature, the presence of intracellular accumulations of abnormal filaments of tau protein (NFTs). About 100 years since NFTs are first described by Dr. Alois Alzheimer in one of his patient suffering from AD, the breakthrough discovery of tauopathy was the finding that the main component of PHFs, which making up the NFTs, is aberrant phosphorylated tau (41, 42, 51-53). Within the last 20 years, a great deal of efforts had been put into the study of tau and tau phosphorylation in both physiological and pathological settings. However, there is still much left to be learned.

Tau protein kinases

Tau protein is a phospho-protein whose expression and phosphorylation is well regulated (42, 54, 55). The longest tau isoform in the human central nervous system contains 441 residues, including 79 putative serine or threonine residues and 5 tyrosine residues located in two proline-rich regions. The phosphorylation of these residues influences the binding ability of tau to microtubules (39, 48, 56). These phospho-sites

have been divided into two groups: proline-directed and non-proline-directed groups (57). In the proline-directed tau protein kinases, three major families have been characterized, including MAPK, GSK3 and CDC2-like kinases (cdk2 and cdk5), and among non-proline-directed tau kinases, there are PKA, PKC, CaM Kinase II and MARK kinases (37). Inappropriate phosphorylation of tau on these sites will lead to tau dysfunction and further result in cell death.

Although many kinases have been considered as potential tau protein kinases, so far, only a few are thought to be good candidates in vivo. GSK3β and cdk5 are two of the candidates receiving strong supports.

GSK3 has many in vivo substrates and plays a very important role in energy metabolism, neuronal cell development and body pattern formation (58). The initial physiological function is recognized to phosphorylate glycogen synthase and thus inactivating glycogen synthase. Two isoforms, α and β, have a high degree of amino acid homology (58). GSK3β, a proline-directed serine-threonine kinase, is highly expressed in the brain (59). It was found that tau protein kinase I, which could generate a PHF epitope on tau from microtubule proteins of bovine brain (60), is actually identical to GSK3β (61). In vitro studies showed that GSK3β transfection into mammalian cells increased GSK3β activities, decreased the mobility of tau protein and facilitate the formation of PHF (62). Moreover, studies showed that lithium, a GSK3 inhibitor, led to a reduction of tau

phosphorylation, enhancement of tau binding to microtubules and further promoting microtubule assembly (63). More evidence from transgenic mice studies showed that overexpression with GSK3β in transgenic mice induced tau hyperphosphorylation, neuronal death and spatial learning deficits (64). All these findings provide strong evidence that GSK3β is one of the kinases phosphorylate tau in vivo.

Another in vivo candidate for tau kinase is cdk5, which is abundant in brain tissue and has been known to associate with tau (65, 66). One of unique cdk family members, cdk5 is activated by coupling with the non-cyclins, p35 and p39, which are regulatory proteins exclusively expressed in postneurons (67). Cdk5 is also recognized as a subunit of tau protein kinase II and is associated with its activator p25, a proteolytic cleavage product of p35 (65). The p25 subunit accumulates in the brain and promotes the long-lasting activation and mislocation of cdk5 in AD patients (68). Evidence supporting cdk5 as an in vivo tau kinase also came from the study showing that the p25 is found in the neurons containing NFT in AD brain and p25/cdk5 induces in vivo tau phosphorylation (68). Research with transgenic mice overexpressing human p25 suggested that there are abnormally hyperphosphorylated tau and neurofillament found in the brain of p25 transgenic mice, accompanied with cytoskeletal disruption (69). In vitro, tau has been identified as one of the substrated of cdk5 (70). Meanwhile, in human tau, many physiological relevant Ser/Thr sites have been identified as cdk5 sites, including Ser202,

Thr205, Thr212, Ser235, Ser396 and Ser404 (71, 72). Considering the fact that these in vitro cdk5 sites are also phosphorylated in vivo (73), it is proposed that cdk5 may be involved in tau pathology.

Tau protein phosphatases

Tau is a phospho-protein whose dephosphorylation is mediated by protein phosphatases (PPs). Highly expressed in mammalian brains, five different phosphoserine/phosphothreonine PPs are found, including PP1, 2A, 2B, 2C and 5 (74). It has been showed that PP1, PP2A/B and PP5 dephosphorylate tau in vitro (75), but the role of each of them in the regulation of site-specific phosphorylation of tau in the human brain is unclear. Quantitative and kinetic analyses showed that PP2A, PP1, PP5 and PP2B accounted for 71%, 11%, 10% and 7%, respectively, of the total tau phosphatase activity of human brain (74). In AD brain, it is reported that the expression and activities of some PPs decline (76-78). PP2A has been reported the major tau phosphatase in brain (74), whose activity is reduced in AD brain and dephosphorylation of tau can be blocked in vitro by okadaic acid, a PP1/2A inhibitor (76, 79, 80). A hypothesis has been proposed that PP2A plays a central role in the regulation of tau hyperphosphorylation in AD (80).

Animal models of AD

It is critical to develop useful models to study the pathology of AD for pre-clinical testing of drugs. Very few species are known to develop the behavioral, cognitive and neuropathological symptoms of AD spontaneously. To date, no ideal animal AD model has been described, but a variety of animal models have been produced from invertebrates to mammals (81). The most widely used animal models of AD is transgenic mice induced by single, double or triple mutation of certain genes related to AD, which have made remarkable breakthroughs about the pathology of AD (81-83). Also, some conventional pharmacological methods are used to produce AD models which are still valuable for studying certain pathological pathways, such as chemical lesion induced AD models (79, 84). Although the transgenic models have yielded certain important advancement in the understanding of pathological pathways, the successes of the preclinical study hadn't been translated into much needed therapeutic improvements (81-83). It happens because AD in human is much more complicated and transgenic mice can only mimic partial symptoms of AD. In spite of the limitations of each animal model, the urgent progress toward a cure for AD depends on the strengths of animal models.

AD and estrogen

To date, there is no cure for AD and available treatments only offer relatively palliative but little symptomatic benefits. Four drugs are currently approved by FDA to

treat the cognitive symtoms of AD: three are acetylcholinesterase inhibitors and one NMDA receptor antagonist. None of these drugs has been shown to delay or halt the progression of the disease. So, it is urgent to explore new drug for AD treatment.

Estrogen, a well known neuroprotectant, has been considered as a potential treatment for AD. AD is three times more prevalent in women than man, and epidemiological studies have indicated that estrogen protects against AD (85). Clinical studies show that postmenopausal women, whose estrogen levels are dramatically decreased, are at risk for neurodegenerative diseases (86) and postmenopausal estrogen replacement therapy reduces the risk or delays the onset of AD (87). Estrogens are also shown to affect cognitive function during aging and estrogen replacement therapy is reported to reverse the decline of cognitive function due to menopause (88, 89). Study also shows that cerebrospinal estradiol levels are lower in an AD group versus control, and among the AD group, estradiol levels are inversely correlated with Aβ levels (90). All evidence show that estrogen declining in the brain may be a contributor for AD pathology and estrogen replacement therapy may be a potential treatment for AD.

Estrogens have been recognized as neuroprotectants which have been shown to be effective for protecting against different cellular dysfunction and/or damage. A growing body of evidence from both *in vitro* and *in vivo* studies has addressed the neuroprotective effects of estrogen against different insults, such as serum deprivation, Aβ-induced

toxicity, glutamate-induced excitotoxicity, mitochondria toxins, and hydrogen peroxide (91). Evidences from our laboratory shows that estrogen may protect neuronal cells against oxidative stress and excitotoxicity by activating a combination of PPs, which play an important role in hyperphosphorylating tau protein (92, 93). Estradiol has also been shown to protect brain from insult-induced AD neuropathology, including activation of apoptosis, stimulation of Aβ production, hyperphosphorylation of tau, activation of cyclin-dependent kinases, and activation of catastrophic attempts at neuronal mitosis (94). However, the mechanisms of estrogen exerting neuroprotective effects still remain unclear. There are several possible pathways have been proposed, including estrogen receptor-mediated neuroprotection, mitochondria-mediated neuroprotection, regulation of signal transduction pathways and antioxidative effects (91). Collectively, these data indicate that estrogens could be valuable candidates for brain protection.

Given the growing body of evidence for the potent neuroprotective effects of estrogens in cell and animal models and the epidemiological evidence of early postmenopausal treatment, it seems to be reasonable that some of the estrogen compounds would be tested in clinical trials for neurodegenerative conditions. However, in 2002, investigators from the Women's Health Initiative (WHI) studies halted a clinical trial in part because hormone therapy was bad for the heart, and they announced that it might be bad for the brain as well ten months later. In these studies, two hormone

preparations, Premarin (95, 96) and PremPro (97-99), were assessed after years of continuous daily administration. However, due to the preparations of hormone, the route of administration, the regimen of hormone administration and the age of subjects, the interpretation of WHI studies is limited. Averaged 65 years of age and approximately 12 years postmenopausal at the entrance of WHI studies, the subjects would have had significant asymptomatic atherosclerosis (100). Substantial data demonstrate atheropreventive effects of estrogen before vascular damage occurs, whereas adverse effects of oral estrogen on thrombosis and inflammation may predominate once complex atheromas are present (100).

Given that we now know more about the estrogen-mediated signaling pathway and structure-activity relationships for estrogen-induced neuroprotection (101, 102), new preparations and delivery methods for estrogen-induced neuroprotection should be investigated in future clinical trials. In brief, there are safe and effective means to administrate estrogens or their analogues for the treatment of neuronal loss associated with chronic neurodegenerative disease.

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Chapter 2

An okadaic acid induced cognitive deficiency and oxidative stress in an experimental model of tauopathy

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease that causes progressive cognitive and behavior deterioration in the elderly. Affected brains of AD are characterized by the presence of senile plaques (SP), neurofibrillary tangles (NFTs) and the loss of cholinergic neurons in the basal forebrain. Clinical research shows that the severity of dementia in AD patients is positively related to the number of NFTs in neocortex, but not to the degree of SP deposition. In an attempt to characterize a new experimental *in vivo* AD model, okadaic acid (OA), a protein phosphatase inhibitor, was microinfused into the dorsal hippocampus area of ovariectomized female adult rat unilaterally via an osmotic pump. After 14 days of OA infusion, rats were subjected to the behavior tasks, including spatial learning and memory by Morris Water Maze and motor function by Rotarod. The high dose group (OA, 70ng/day 14 days) failed to perform the learning and memory tasks in Water Maze, but were not affected in visible water maze and rotarod test. High dose OA increased the protein carbonyl and MDA content in both side of hippocampus and cortex. By using Bielschowsky's silver staining, NFTs-like pathological changes were observed in both cortex and hippocampus of high dose OA group. Our data suggest that the microinfusion of OA into unilateral dorsal hippocampus could contribute to a cognitive deficiency as well as NFTs-like pathological changes and it may be related to oxidative stress.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that causes progressive cognitive and behavior deterioration in the elderly (1, 2). Affected brains of AD are characterized by the presence of senile plaques (SP), neurofibrillary tangles (NFTs) and the loss of cholinergic neurons in the basal forebrain (3-5). Currently, the etiology and pathogenesis of AD remains unknown. However, it is generally postulated that beta amyloid peptide (Aβ) and tau protein play a key role in the progressive neurodegeneration observed in AD (6, 7). One of the most challenging areas of research is to elucidate the relationship between these AD hallmarks. One hallmark is the formation of SP, deposition of Aβ derived from the proteolytic processing of larger amyloid precursor protein (8, 9). Another major pathological hallmark of AD is the presence of NFT and their constituents, paired helical filaments, consisting mainly of hyperphosphorylated tau (10-12). Deposition of amyloid plaques does not correlate well with neuron loss (13). Several studies have showed that the numbers of NFT correlate closely with the degree of dementia (14, 15). These observations support the tau hypothesis, the idea that tau protein abnormalities initiate the disease cascade (16-18). In AD, tau protein undergoes chemical changes and becomes hyperphosphorylated, then begins to pair with other threads to form paired helical filaments and further to create NFTs, which disintegrate the neuron's transport system (18, 19).

Several protein kinases, including glycogen synthase kinase-3 (GSK-3), PKC, MAPK and cdk5 phosphorylate tau protein at some but not all those sites that are found in AD tau (20-24), whereas its dephosphorylation is mainly catalyzed by protein phosphatase 2A (PP2A) (25, 26). It is proposed that an imbalance between tau phosphorylation and dephosphorylation is critical to AD (27-29). This disturbance might be the result of either higher activities of tau kinases, lower activities of tau protein phosphatases, or both.

Besides the pathological hallmarks of AD, a growing body of evidence suggests that an imbalance between free radical formation and destruction is involved in AD pathogenesis (30, 31). In order to obtain chemical energy, a large pool of biomolecules is oxidized. Some chemically unstable, highly reactive intermediates are formed, refered as free radicals, which can attack surrounding biological environment leading to lipid peroxidation, protein oxidation or DNA oxidation (32, 33). When the body produces free radicals beyond the ability of endogenous antioxidant system to destroy them, oxidative stress occurs (32, 33). Although the initiating events of AD are still unknown, numerous studies reported that oxidative stress-mediated injuries are elevated in the AD brain as well as in cellular and animal models of AD (34, 35).

Together, these findings have helped to establish the hypothesis that breaking the balance between tau protein phosphorylation and dephosphorylation will lead to AD-like

tauopathy. A potent protein phosphatase blocker is okadaic acid (OA). OA is a cell permeable and sensitive inhibitor of protein phosphatase 1 and 2 (PP1/2), the only well defined molecular targets (36, 37). Studies from our laboratory also showed that OA could induce oxidative stress (38, 39). Therefore, we hypothesized that the inhibition of protein phosphatases by OA could produce AD-like symptoms, including cognitive impairment and oxidative stress.

To determine the possibility of using OA infusion as an AD tauopathy model, adult ovariectomized female rats received microinfusion of OA into the dorsal hippocampus unilaterally. In the present study, we observed the effects of OA on learning and memory as well as on pathological changes in the brain of OA rats, and the protein carbonyl and MDA content in the hippocampus and cortex.

Materials and methods

Animals

Female Sprague Dawley (SD) rats were purchased from Charles Rivers (Wilmington, MA) and maintained in our animal facility in a temperature-controlled room (22-25 °C) with 12-hour dark-light cycles. All rats will have free access to laboratory chow and tap water. All animal procedures are reviewed and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee.

Materials

Okadaic acid (Cat #: 495604) was purchased from Calbiochem (Gibbstown, NJ). ALZET osmotic pumps (model 1002) and brain infusion kits were purchased from DURECT Corporation (Cupertino, CA). Lipid peroxidation assay kit (Cat #: 437634) was from EMD Chemicals, Inc. (Gibbstown, NJ). Protein carbonyl assay kit (Cat #: 10005020) was purchased from Cayman Chemical Company (Ann Arbor, Michigan). Silver nitrate and other reagents were purchased from Sigma-Aldrich (St Louis, MO).

Ovariectomy

To avoid the influence of estrogens on the model, all the rats used in this study were received ovariectomized bilaterally and were maintained on a phytoestrogen-free diet.

Four week old female SD rats were anesthetized with ketamine (60mg/kg) and xylazine (10mg/kg). A small cut was made through skin and muscle. Ovaries were externalized and removed. Ovariectomies were performed at least two weeks before further procedure.

Implantation of brain infusion kit and osmotic pump

Adult ovariectomized female Sprague-Dawley rats (250-300g) were anesthetized by intraperitoneal ketamine (60mg/kg) and xylazine (10 mg/kg) and immobilized on a stereotaxic apparatus. A small cut was made through the scalp and a small hole was made through the exposed skull. A stainless steel brain infusion cannula of 0.36 mm outer diameter was embedded into the right dorsal hippocampus of animals by using stereotaxic surgery and fixed on the skull. The cannula terminal co-ordinates, with the incisor bar set at –3.3 mm, were in mm from bregma and dura: posterior 3.8, lateral ±2.5, depth –3. For microinfusion of OA, a brain infusion kit and ALZET Osmotic Pumps (Cupertino, CA) was used and a catheter tube is used to attach the cannula to the flow moderator of the ALZET pump, which is implanted subcutaneously. Before the placement of cannula and ALZET Osmotic Pump, the brain infusion assembly and osmotic pump was prepared and filled with the solution to be delivered. Desired OA solution in artificial spinal-cerebral fluid (contain 1% DMSO for dissolving OA) was filled into the pump and allow infusion at an appropriate rate into unilateral dorsal

hippocampus. The control received the same surgery and microinfusion with the same volume of vehicle. This pump (model 1002, ALZET) has a 100 µl reservoir and two weeks duration. The cannula position was verified by injection of 10 µl of a solution of pontamine sky blue via the cannula. Ten minutes later, the rat was decapitated and its brain was removed for checking the validity of the dorsal hippocampus injection. H&E staining was used to confirm the cannula position after OA infusion (Figure 5J).

Morris water maze (MWM)

The Morris water maze is a behavioral procedure designed to test spatial learning and memory. It is commonly used to explore the role of the hippocampus in the formation of spatial learning and memory. The test apparatus MWM was a large circular pool (180 cm in diameter by 80 cm high) filled with water to a depth of 60 cm. The water was made opaque by the addition of approximately 0.5 liter of blue dye powder and thermostatically maintained at 24±1°C. An 11 by 11 cm transparent platform 59 cm high is placed in a fixed location in the tank, 1 cm below the water surface. Many extra-maze cues surrounded the maze and were available for the rats to use in locating the escape platform. The experimenter conducting the behavioral tests was always unaware of the group assignments of animals. On each trial, the rat was placed in the water close to and facing the wall of the pool in one of four equally spaced locations. The rat was allowed to swim

freely around the pool until it found the platform, onto which it promptly climbed to escape from the water. If a rat fails to locate the platform within 90 seconds, it was then placed there by the experimenter. The intertribal interval was 10 minutes. Each rat received 3 trials (rat swimming from a different starting point from each trial) per day for 5 days, called the **acquisition test**, which was used to assess the spatial learning performance. During the acquisition test, the platform remained in a constant location equidistant from the center and the edge of the pool, in the center of the fourth quadrant. On each trial one measure was recorded: the swimming distance to escape onto the platform, measured by a movement tracking software. After two days break, two sessions of the **retention test**, which was used to assess the spatial memory performance by recording the swimming distance to escape onto the platform, was conducted with the platform in the same location.

Visible MWM test

Following the retention test, a visible platform test was conducted. The platform was identified by a visible flag that was elevated above the surface of the water. Three sessions were administered, each consisting of 3 trials at a 10-min intervals, 90 seconds per trial. On each trial, the rat had to swim to the platform from a different starting point

in the tank and the platform was also moved to a different location before each trial. The swimming distance to escape onto the platform was recorded.

Motor function test by rotarod test

This task was used to assess motor coordination, balance, and motor learning. Rat were trained, three trials per day for 3 days, to balance on a rotating rod at 10, 20, and 30 rpm for the first, second, and third days, respectively. Then motor coordination and sustainability were assessed by the time to falling off the rod rotating at 30 rpm, averaged from three successive trials.

Bielschowsky's silver staining

Silver staining is one of the methods extensively used in histological identification of pathological deposit in the brain. The principal of silver staining is that the brain slices were treated with ammoniacal silver, and then reduced to visible metallic silver. After dorsal hippocampal infusion and behavior tests, rats received transcardiac perfusion with 4% formaldehyde in PBS, followed by immersion fixation of removed brain, for at least another 24 hours before paraffin-embedding. For neuropathological diagnosis, tissue blocks were embedded in paraffin and sectioned at 10 µm. To start the silver staining, formalin-fixed and paraffin-embedded sections were deparaffinizated with xylene and rehydrated through a series of graded ethanol (start from pure ethanol) to water. After 3

times washing, the slides were placed in pre-warmed (40ºC) 10% silver nitrate solution for 15 minutes' staining till sections became light brown color. Then the slides were put in distilled water and washed for 3 times. Meanwhile, concentrated ammonium hydroxide was added to the silver nitrate solution drop by drop until precipitate formed is just clear. Slides were placed back in this ammonium silver solution and stained in 40ºC oven for 30 minutes or until sections became dark brown. Without washing, the slides were placed directly into a developer working solution for 1 minute or less (exact incubation time need to be checked under microscopy). The developer working solution was freshly made of 1% concentrated ammonium hydroxide and 1% developer stock solution, which comprised of 20% formaldehyde, 0.5% citric acid and 0.1% concentrated nitric acid. After developing, the slides were dipped into 1% ammonium hydroxide solution for 1 minute to stop the silver reaction and followed by 3 changes of washing in distilled water. After 5 minutes incubation in 5% sodium thiosulfate (HYPO) solution and 3 times washing in distilled water, the slides were dehydrated and cleared through 95% alcohol, absolute alcohol and xylene, followed by mounting with resinous medium.

Lipid peroxidation assay

Lipid peroxidation is well-established mechanism of cellular injury in both plants and animals. This process leads to the production of lipid peroxides and their by-products and

ultimately the loss of membrane function and integrity. Malondialdehyde (MDA) is one of the end products derived from the peroxidation of polyunsaturated fatty acids and related esters. Measurement of such aldehydes provides a convenient index of lipid peroxidation (40). A colorimetric assay kit (Cat#: 437634) from Calbiochem was used to assay MDA content in the brain of model rats. This assay takes advantage of a chromogenic reagent, N-methyl-2-phenylindole in acetonitrile, which reacts with MDA at 45 °C. Condensation of one molecule of MDA with 2 molecules of reagent N-methyl-2-phenylindole yields a stable chromophore with maximal absorbance at 586 nm. For tissue homogenates preparation, cortex and hippocampi were separated from the rat brain and put into ice-cold Tris buffer (20mM, pH 7.4) at 1:10 (W/V). Prior to homogenization, butylated hydroxyl toluene (BHT) was added to a final concentration of 5 mM to the buffer in order to prevent sample oxidation during the homogenization. After homogenization, homogenate were centrifuged (3,000 g at 4 °C for 10 min). The clear supernatant was collected for the lipid peroxidation assay and protein assay. For MDA measurement, 200 μl of sample was added into a clean test tube followed by adding 650 μl of solution containing one volume of ferric ion in methanol and 3 volumes of N-methyl-2-phenylindole in acetonitrile, and then 150 μl of 12 N HCl was added. After mixing, the testing tube was incubated at 45 °C for 60 min. Samples were cooled on ice and absorbance was measured at 586 nm. For each sample, there was a sample blank

where the 650 µl of solution (25% ferric ion in methanol / 75% N-methyl-2-phenylindole in acetonitrile) was replaced by 650 µl of 25% methanol / 75% acetonitrile), and a reagent blank where the sample was replaced by water. A standard curve was created by measuring the absorbance of a series of concentration of MDA at 586 nm. The color yielded was a linear function of the MDA concentration over the range from 0 to 20 µM.

Protein carbonyl assay

The most general indicator and by far the most commonly used marker of protein oxidation is protein carbonyl content. The most convevient procedure is the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically (41). A protein carbonyl assay kit (Cat#: 10005020) from Cayman Chemical was used to assay protein carbonyl content in the brain of rats. For tissue homogenates preparation, cortex and hippocampi were separated from the rat brain and put into ice-cold buffer (50 mM phosphate, pH 6.7 containing 1 mM EDTA) at 1:10 (W/V). After homogenization and centrifugation at 10,000 g for 15 min at 4 °C, the supernatant was collected for the assay. Supernatant absorbance was determined at 280 nm and 260 nm to assess contamination by nucleic acids. The homogenization buffer was used as a blank. If the ratio 280/260 nm was less than 1, a

further step to remove nucleic acids with 1% streptomycin sulfate was used. 200 µl of sample was transferred to two 2.0 ml plastic tubes. One tube was the sample tube and the other was the control tube. 800 µl of DNPH was added to the sample tube while adding 800 µl of 2.5 N HCl to the control tube. Both tubes were incubated in the dark at room temperature for one hour with vortexing briefly every 15 min. After incubation, 1 ml of 20% trichloroacetic acid (TCA) was added to each tube on ice. 5 min later, tubes were centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 ml of 10% TCA on ice for 5 min. Tubes were centrifuged again at 10,000 g for 15 min at 4 °C and the pellet was saved and resuspended in 1 ml of (1:1) ethnol / ethyl acetate mixture. This step was repeated two more times. After the final wash, the protein pellets was resuspended in 500 µl of guanidine hydrochloride by vortexing. Tubes were centrifuged at 10,000 g for 15 min at 4 °C to remove any left over debris. 220 µl of supernatant was transfered from tubes to a 96 well plate and absorbance was measured at 360 nm. The carbonyl content was calculated using a molar extinction coefficient of 22/mM/cm for DNPH and was expressed as nmoles DNPH/mg protein. Values were normalized to percentage of untreated control groups.

Statistics

The results were analyzed with one-way ANOVA with prism software (Graphpad Inc.,

San Diego, CA). The significance of differences among groups was determined by Tukey's multiple comparison tests. All results were expressed as mean ± SEM.

Results

Effect of OA on the behavior performance of model rats

To examine the effect of OA on the behavior performance of rats, OA was microinfused into the dorsal hippocampal area of adult ovariectomized female SD rats unilaterally via an osmotic pump. After 14 days of OA infusion, different groups of rats were subjected to the behavior tasks, including spatial learning and memory by Morris Water Maze and motor function by Rotarod.

Body weight

The body weight of rats was monitored from the first day of infusion to the end of behavior test (Figure 1). Overall the body weights of all the rats in different groups was increasing during the experimental period from the start of microinfusion of OA or vehicle into the unilateral hippocampus of the adult ovariectomized female SD rats, throught the behavioral tests after microinfusion, to sacrifice of the rats, except a drop on second day and sixteen day; the former is associated with the surgery of implantation of pumps and brain infusion kits and the latter is associated the start of water maze training. The recovery of body weight took one to two days. The body weights of rats in both the low dose (OA 7 ng/day for 14 days) and the high dose (OA 70 ng/day for 14 days) groups was slightly lighter than control group, but there were no difference among groups.

Microinfusion of OA or vehicle into unilateral dorsal hippocampus of adult ovariectomized female rats has little influence on the body weight.

Spatial learning and memory

14 days after the microinfusion of OA in the unilateral dorsal hippocampus, all rats were subject to behavior test starting with MWM. In this test, the rats were trained to locate the escape platform with reference to its location in space. The swimming distance was recorded to assess the performance of rats in the water maze.

During the first three training sessions of acquisition sessions (session 1 to 5), the rats from control group and low dose (OA 7 ng/day for 14 days) group swam shorter distance as evidenced by decrease in path length over sessions (Figure 2). In contrast, the high OA dose group showed little improvement in behavior over the 5 sessions (Figure 2). Results from the acquisition sessions showed that microinfusion of high dose OA (70 ng/day for 14 days) into unilateral hippocampus of rats causes a decline of spatial learning ability.

The retention sessions (session 8 and 9) were started two days after the acquisition training. In the control group and low OA dose group, the rats swam the same of shorter distances to find the platform. But there no significant changes in the high OA dose group

(Figure 2). This retention test showed that microinfusion of high OA dose into unilateral hippocampus of rats causes a spatial memory deficiency.

Visible MWM

After those tests, the rats were re-tested in the Morris water maze with a visible platform. This test permits the assessment of motivational and/or sensorimotor factors rather than spatial learning per se and to rule out group differences in sensorimotor ability and/or motivation. The rats from all three treatment groups learned the path to the platform with the training. Although high OA dose group rats traveled longer path to locate the platform at session 3, there was no difference in the ability of the different treatment groups to locate the visible escape platform at every single session (Figure 3).

Rotarod

In order to rule out the group differences in motor coordination, all the rats were subjected to rotarod test. Learning of coordinated running was measured by the latency to fall over the 3 training sessions and maximum performance was estimated by performance on the final session (Figure 4). A one-way ANOVA on latency to fall for the final session failed to indicate any effect of OA treatment on motor function.

Silver staining of pathological changes in OA induced experimental AD tauopathy model

To further characterize the pathological changes of the experimental AD model induced by OA, after a series of behavioral tests, half of rats from each treatment group were submitted to prepare paraffin sections of rat brain samples. The rats received transcardiac perfusion with 4% formaldehyde in PBS, followed by immersion fixation of removed brain, for at least another 24 hours before paraffin-embedding. For silver staining, tissue blocks were embedded in paraffin and sectioned at 10 μm. After deparaffinization and rehydration, the brain sections were stained in silver nitrate solution following the Bielschowsky's protocol. As Figure 5 and 6 showed, there were NFTs-like pathological changes (silver positive staining) discovered in both sides of cortex and the ipsilateral hippocampus received high dose OA infusion, as well as in the ipsilateral cortex of lose dose OA group, but not seen in other area of low dose group and control group. It suggests that OA infusion can induce NFTs-like pathological changes.

Oxidative stress induced by OA in rats

After a series of behavioral tests, the other half of the rats was decapitated and the brains were removed. The cortex and hippocampus from both contralateral and ipsilateral brain were separated and used for MDA and protein carbonyl content assay.

MDA assay

As Figure 7A showed, there was an OA dose-dependent increase of MDA content in both contralateral and ipsilateral cortex. The MDA content was significantly increased in the cortex of high OA dose group in both contralateral and ipsilateral side ($p<0.01$). Further, there was also a significant increase of the MDA content in ipsilateral hippocampus ($p<0.01$) and contraletal hippocampus ($p<0.05$) (Figure 7B) of high OA dose group. Although, there was no significance of MDA content between low OA dose group and control in both sides of hippocampus, a dose-dependent increase in MDA content was observed.

Protein carbonyl content assay

As Figure 8 showed, the protein carbonyl concentrations in both cortex and hippocampus increased in an OA dose-dependent manner. In the cortex (Figure 8A), the protein carbonyl content in both side of the brain of the low OA dose group was increased but was not significantly different from control group ($p>0.05$). There were a significant increase of the protein carbonyl content in contralateral cortex ($p<0.01$) and ipsilateral cortex ($p<0.05$) of the high OA dose group. In the hippocampi (Figure 8B), significant increases of protein carbonyl content were observed in both contralateral side

($p<0.05$) and ipsilateral side ($p<0.01$) of high OA dose group, but not in the low OA dose group.

Discussion

It is critical to develop useful animal models to study the pathology of AD for pre-clinical testing of drugs. It is well known that very few species develop the behavioral, cognitive and neuropathological symptoms of AD spontaneously. To date, no ideal animal AD models have been described, but a variety of animal models have been produced from invertebrates to mammals (42). The most widely used animal model of AD is transgenic mice, which have made certain significant breakthroughs about the etiology of AD (42-44). There are also some conventional methods widely used to produce animal models for AD, such as chemical lesion and immune-mediated damage of the basal forebrain cholinergic system (27, 45-47). Although the transgenic models have yielded some important advancement in the understanding of pathological pathways, the successes of the preclinical study hadn't been translated into much needed therapeutic improvements (42-44). It happens because AD in human is much more complicated and transgenic mice can only mimic partial symptoms of AD. In spite of the limitations of each animal model, the rapid progress toward a cure for AD relies on the strengths of animal models.

The present study aimed at the characterization of a rat model of AD induced by OA, which is a PP1/2A inhibitor. Although tau has received less attention than Aβ in AD research, some clinical studies had shown that the numbers of NFT, which are mainly

made of hyperphosphorylated tau, correlate closely with the degree of dementia, but not the degree of SP deposition (14). We proposed that disturbance of the balance between tau protein kinases and phosphatases will lead to abnormal hyperphosphorylation of tau, which further induces neurofibrillary degeneration of AD and other tauopathies. To determine whether phospho-tau is central to the neurotoxic cascade in AD, OA was administrated to inhibit the PPs and to break the balance between tau protein kinases and phosphatases in our models. There are five different phosphoserine/phosphothreonine PPs, including PP1, PP2A, PP2B, PP2C and PP5, which are highly expressed in mammalian brains (26). In AD brain, it is reported that the expression and activities of some PPs decline (26, 29, 48, 49). PP2A is reported the major tau phosphatase in brain (26), whose activity is reduced in AD brain and dephosphorylation of tau can be blocked in cells by OA (25, 29, 50). Our data showed that microinfusion of OA (70 ng/day for 14 days) into the dorsal hippocampus of ovariectomized adult female SD rats unilaterally induced a significant learning and memory deficiency without changes of body weight, motor coordination and sensorimotor ability. These data are consistent with previous studies (51-53) and suggest that the dorsal hippocampal infusion of OA could induce AD-like cognitive impairment *in vivo*.

The hippocampus is known to play a major role in long term memory and spatial navigation (54, 55). Although the precise role the hippocampus in memory is not clear, it

is known to be critical for spatial memory in rodents (56). Some studies had shown that unilateral hippocampus is sufficient for executing a spatial task (57), but other studies show that a unilateral hippocampal lesion led to spatial memory impairment (53). Certain clinical studies also reported that a cognitive decline was observed in temporal lobe epilepsy patients due to unilateral hippocampal sclerosis (58). Meanwhile, it was reported that spatial memory deficits were found (across working and reference memory components) in patients with right hippocampal damage, with intact spatial memory in patients with corresponding left hippocampal damage (59). Some researches found that spatial learning impairment was parallel to the magnitude of dorsal hippocampal lesions but not ventral lesions and even a small block of the dorsal hippocampus could support spatial learning in water maze (60, 61). Based on these studies, we microinfused OA (7ng/day or 70ng/day for 14 days) into dorsal hippocampus unilaterally. Our data showed that a significant spatial learning and memory deficiency in Morris water maze performance was observed in the group receiving infusion of OA at 70ng/day. It is accordance with the previous studies showed spatial memory deficits in both rats and patients with unilateral damage to the right hippocampal formation (53, 59). Therefore, it suggests that unilateral dorsal hippocampal damage is sufficient to induce spatial memory impairment *in vivo*. But the underlying mechanism still remains elusive. Is there some compensatory biochemical changes happened in the contralateral side of brain after the

ipsilateral damage induced by OA? We further examined the pathological changes in the brain of OA treated rats and found that there was silver positive staining of NFTs-like formation in both cortex and hippocampus, especially contralateral side of the brain, which may be responsible for the cognitive deficits due to unilateral hippocampal lesions induced by OA. Our data also showed that there were more profound silver positive NFT-like conformational changes observed in cortex than hippocampus, which may due to the massive neuronal death in hippocampus induced by OA.

In our AD models, oxidative stress was also observed via measuring of protein oxidation and lipid peroxidation. Oxidative stress is known to play an important role in AD pathogenesis. Associated with NFT and SP, two hallmark pathologies of AD, a great deal of oxidative damage had been found in AD brains (34, 35). Although the initial events of AD are still unknown, numerous studies reported there are oxidative stress mediated injuries elevated in the AD brain as well as in cellular and animal AD models (35). OA is also known to be neurotoxic. We have also found that OA induced cell death via increased reactive oxygen species, protein carbonylation, lipid peroxidation, caspase-3 activity, and mitochondrial dysfunction (39). The increase of protein carbonyl and MDA content in both sides of cortex and hippocampus found in our study is consistent with the previous clinical studies (34, 62, 63). This suggests that our model could not only mimic the cognitive impairment of AD, but also generate certain oxidative stress,

which is an important contributing factor to AD pathogenesis.

In summary, we have demonstrated that the microinfusion of OA into dorsal hippocampus of ovariectomized female SD rats unilaterally induces spatial learning and memory impairment and NFTs-like pathological formation associated with the increase of protein carbonyl and MDA content in both hippocampus and cortex of the brain. These data support the hypothesis that the inhibition of protein phosphatases by OA could produce AD-like cognitive impairment and pathological changes, which may be associated with oxidative stress.

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Figure legends

Figure 1. Body weight of experimental AD rats induce by OA

The body weight of the rats in different treatment group was monitored daily from the beginning of microinfusion of OA or vehicle, through the behavioral tests, to sacrifice of the models. Control group (n=20) received microinfusion of vehicle (1% DMSO in artificial spinal-cerebral fluid) into unilateral dorsal hippocampus for 14 days and treatment groups (n=10) which received microinfusion of OA (7 or 70 ng OA/day for 14 days) into unilateral dorsal hippocampus. The data were shown as mean ± S.E.M.

Figure 2. The MWM performance of experimental AD rats induce OA

The behavior test at Morris water maze started after 14 days' microinfusion of OA or vehicle into the dorsal hippocampus of the rats from different treatment groups. The swimming path length to the escape platform of rats was recorded to assess learning ability (acquisition test) and memory ability (retention test). Control: 1% DMSO in artificial spinal-cerebral fluid, n=20. Treatment groups: OA (7 or 70ng/day) was microinfused into unilateral dorsal hippocampal area for 14 days, n=10. Data were represented in mean ± S.E.M. ** $p < 0.01$ between control and OA (70ng/day) groups.

Figure 3. Performance of experimental AD rats induced by OA in the visible MWM

After regular MWM test, the rats were re-tested in the Morris water maze with a visible platform to rule out group differences in sensorimotor ability and/or motivation. Learning to find a visible platform was measured by distance traveled to locate the platform associated by a visible rectangular flag. Control: 1% DMSO in artificial spinal-cerebral fluid, n=20. Treatment groups: OA (7 or 70ng/day) was microinfused into unilateral dorsal hippocampal area for 14 days, n=10. Data were represented in mean ± S.E.M.

Figure 4. The motor coordination of experimental AD rats induced by OA in Rotarod test

After the MWM test, all the rats were subject to Rotarod test. After two sessions' training, the latency to fall off the rotating rod was measured at a rate of 30 rpm. Control: 1% DMSO in artificial spinal-cerebral fluid, n=20. Treatment groups: OA (7 or 70ng/day) was microinfused into unilateral dorsal hippocampal area for 14 days, n=10. Data were represented in mean ± S.E.M.

Figure 5. The Bielshowsky silver staining of the hippocampus in OA treated rats.

After the behavioral tests, half of rats received transcardiac perfusion with 4% formaldehyde in PBS, followed by immersion fixation of the removed brain. Then brain

tissue was processed to paraffin sectioning and 10 µm slides were prepared for silver staining. There was no positive silver staining found in the hippocampus of either control group (A, B, C) or low dose OA (7ng) group (D, E, F). A few silver positive staining was found in the dentate gyrus area of ipsilateral hippocampus rececived high dose infusion of OA (70ng) (I) but not in the contralateral side (G, H). H&E staining showed the cannula position at dorsal hippocampal area (J).

Figure 6. The Bielshowsky silver staining of the cortex in OA treated rats.

There was no positive staining found in either contralateral (A, B) or ipsilateral (C) cortex of control group. A few silver positive staining was found in the ipsilateral side of the cortex received low dose OA (7ng) infusion (F) but was not seen in the contralateral cortex (D, E). Much more silver positive staining was found in the both sides of cortex in high OA dose group (G, H, I).

Figure 7. The MDA content in the brain of experimental AD rats induced by OA

After behavioral tests, all the rats from different treatment groups were subject to decapitation and the brains were removed. Cortex and hippocampus were separated and homogenized, and then the supernatants were collected for the lipid peroxidation assay as described in the methods. Control: 1% DMSO in artificial spinal-cerebral fluid, n=4.

Treatment groups: OA (7 or 70ng/day) in vehicle, n=4. Data were represented in mean ± S.E.M. * means $p<0.05$ and ** means $p<0.01$.

Figure 8. The protein carbonyl content in the brain of experimental AD rats induced by OA

The cortex and hippocampus tissue from different treatment groups were homogenized, and the supernatants were collected for the protein carbonyl assay as described in the methods. Control: 1% DMSO in artificial spinal-cerebral fluid, n=4. Treatment groups: OA (7 or 70ng/day) in vehicle, n=4. Data were represented in mean ± S.E.M. * $p<0.05$; ** $p<0.01$ between OA (70ng/day) group and control group.

Figure 1

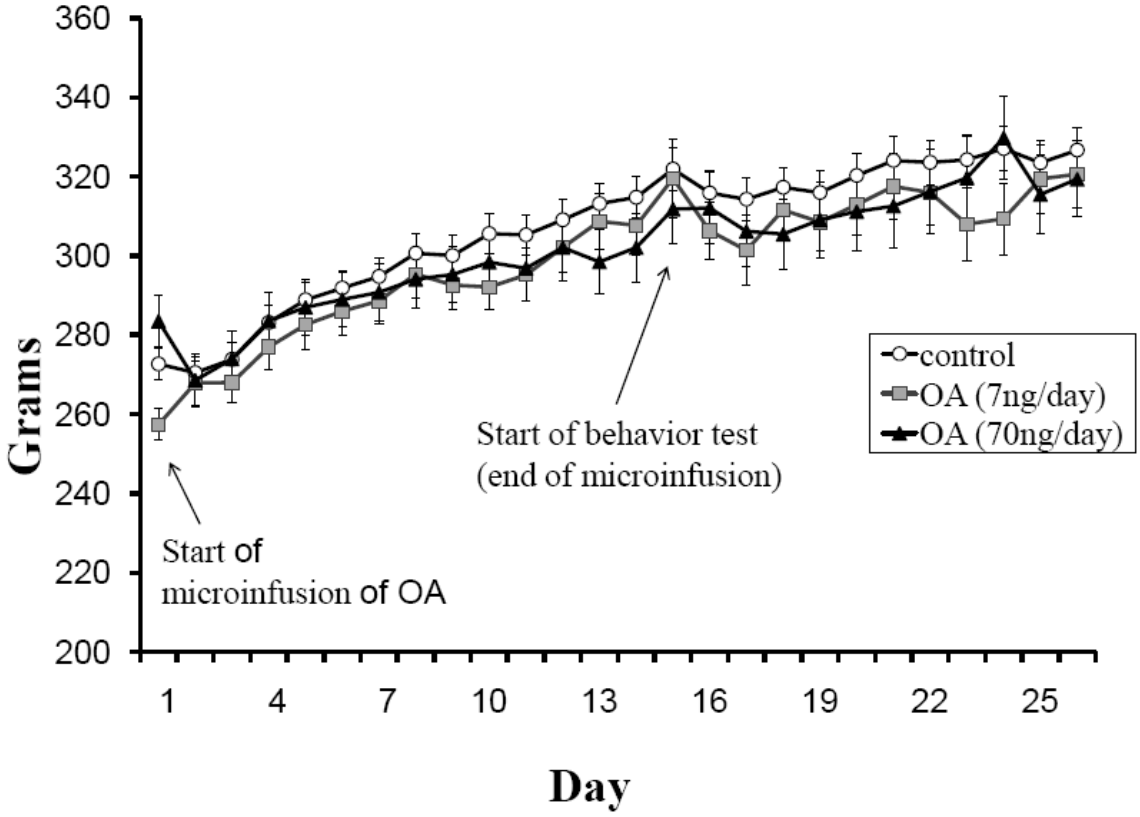
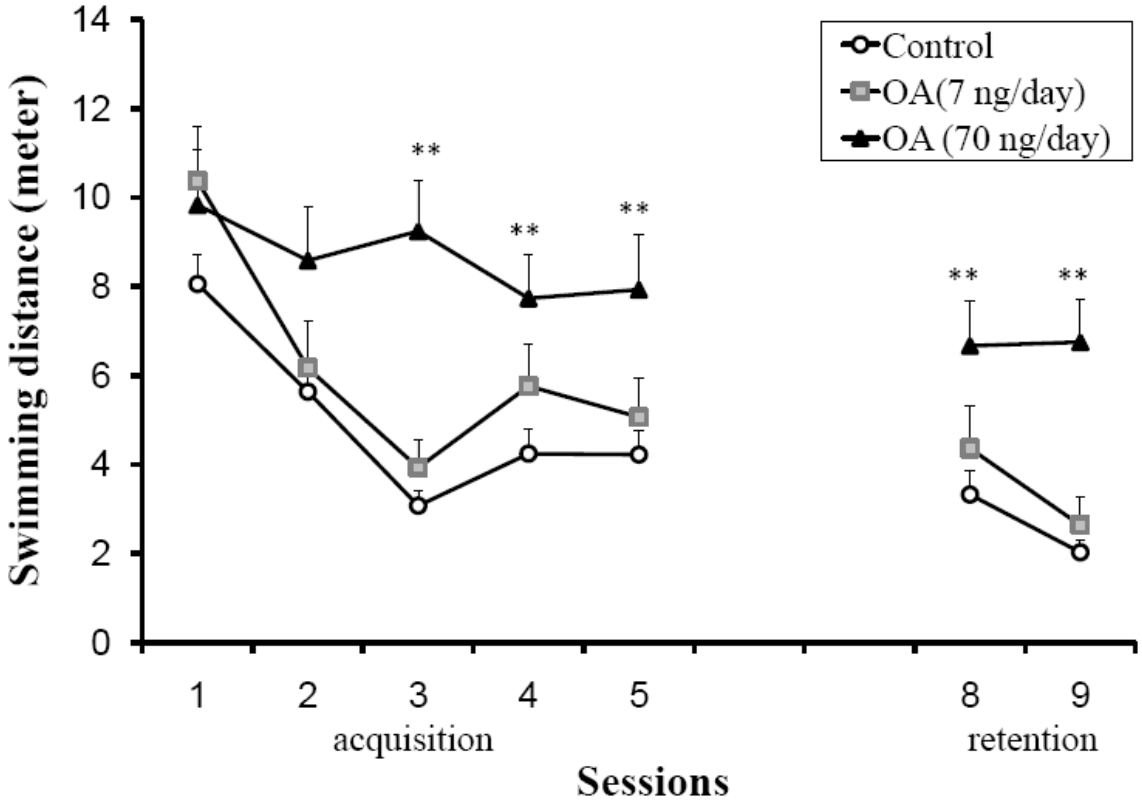

360
340
320
300
280
260
240
220
200
Grams
1
4
7
10
13
16
19
22
25
Day
control
OA (7ng/day)
OA (70ng/day)
Start of behavior test
(end of microinfusion)
Start of
microinfusion of OA

Figure 2


Swimming distance (meter)
14
12
10
8
6
4
2
0
Control
OA(7 ng/day)
OA (70 ng/day)
**
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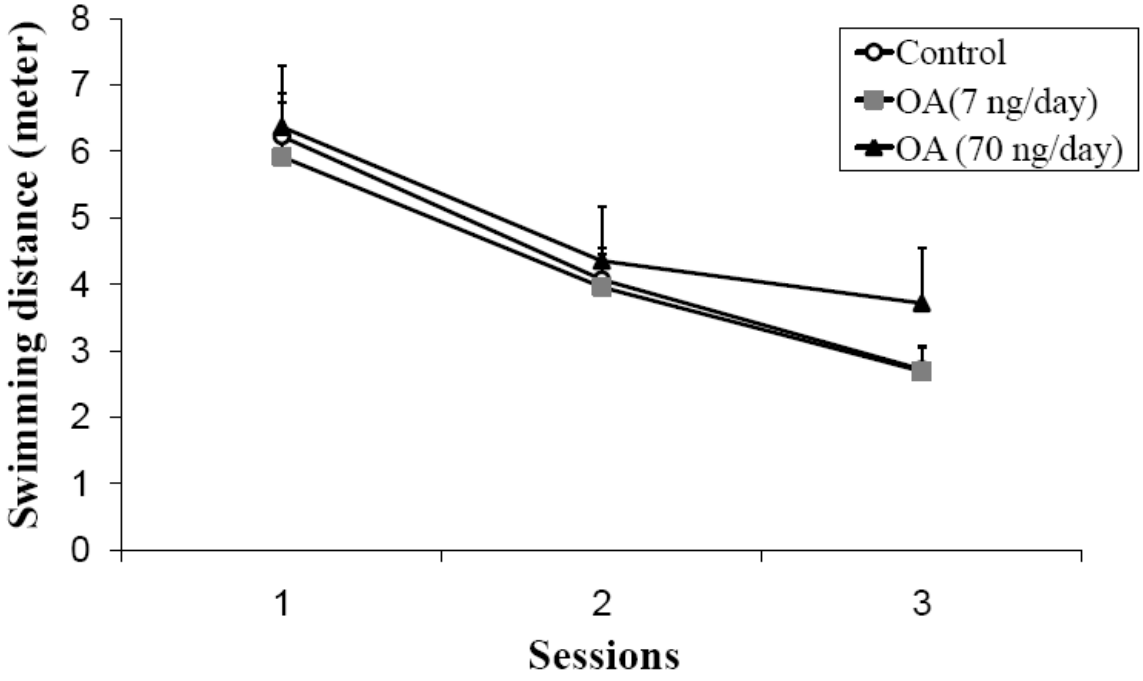
1
2
3
4
5
8
9
acquisition
retention
Sessions

Figure 3


Swimming distance (meter)
0
1
2
3
4
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6
7
8
1
2
3
Sessions
Control
OA(7 ng/day)
OA (70 ng/day)

Figure 4

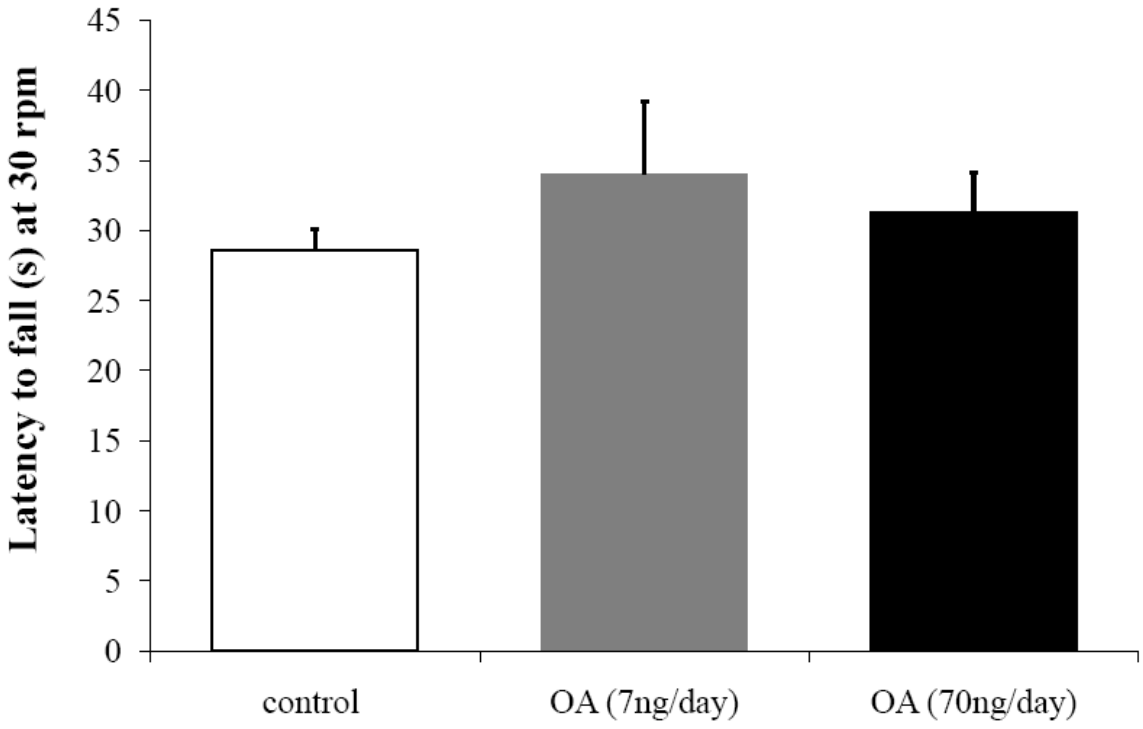

Latency to fall (s) at 30 rpm
45
40
35
30
25
20
15
10
5
0
control
OA (7ng/day)
OA (70ng/day)

Figure 5.

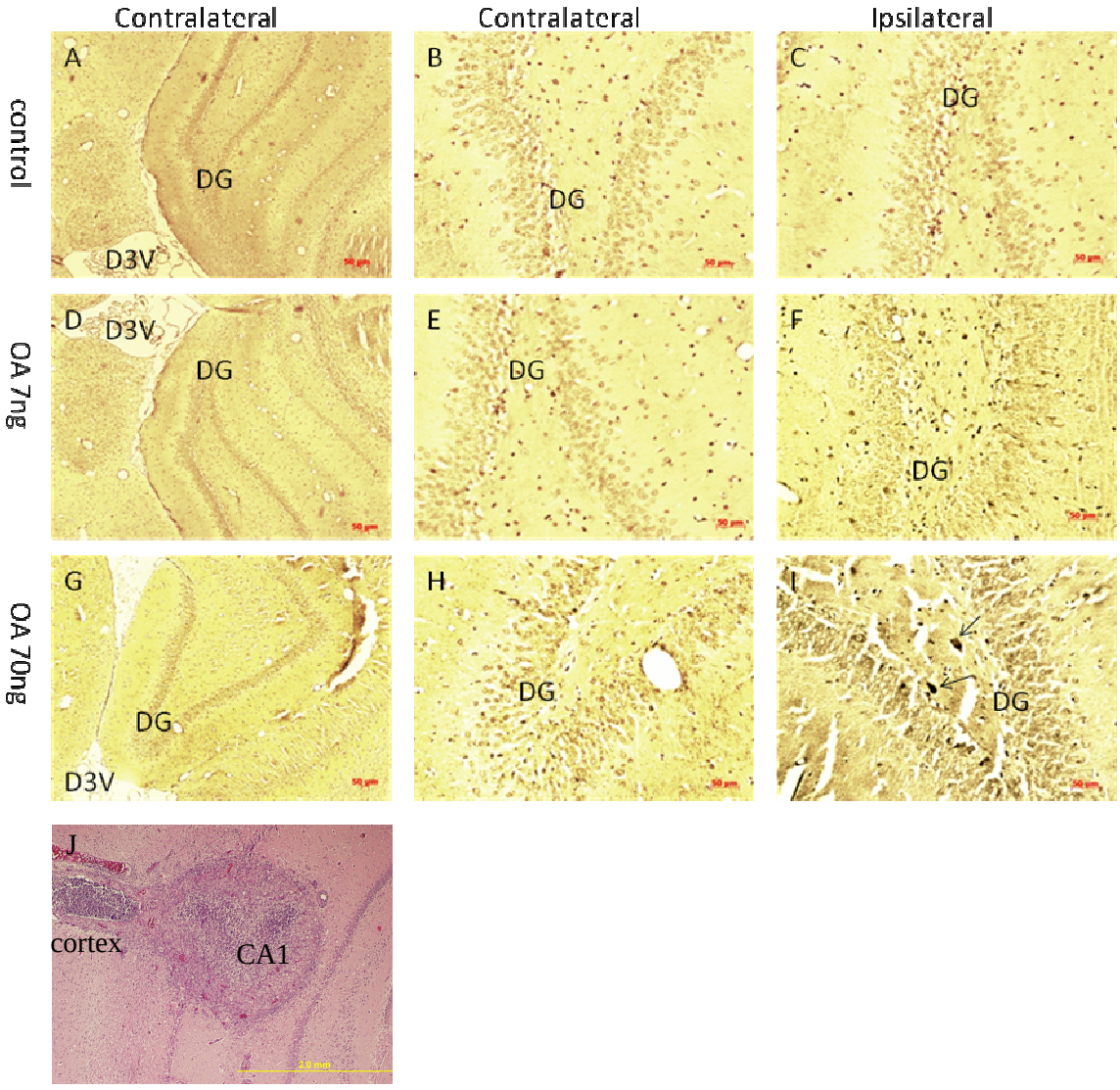
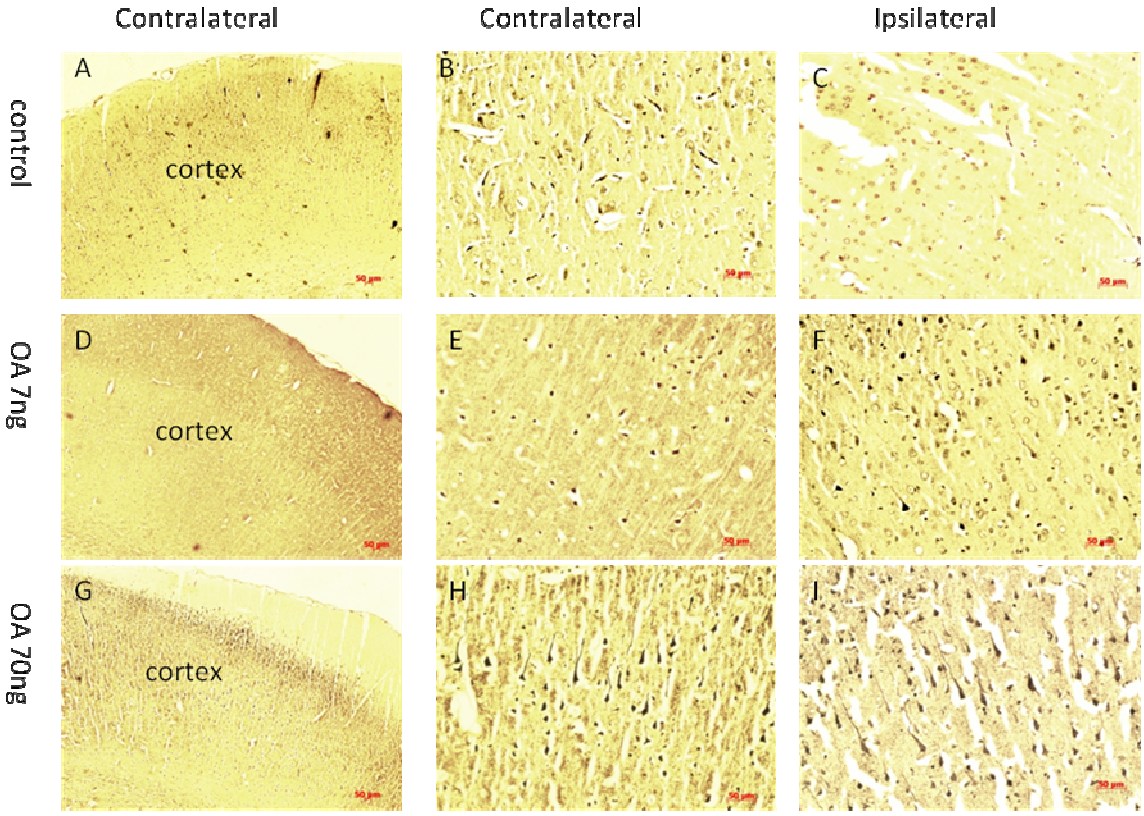

Contralateral
Contralateral
Ipsilateral
control
OA 7ng
OA 70ng
A
B
C
D
E
F
G
H
I
J
DG
D3V
cortex
CA1

Figure 6.


Contralateral
Contralateral
Ipsilateral
control
OA 7ng
OA 70ng
A
B
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G
H
I
cortex
cortex
cortex

Figure 7

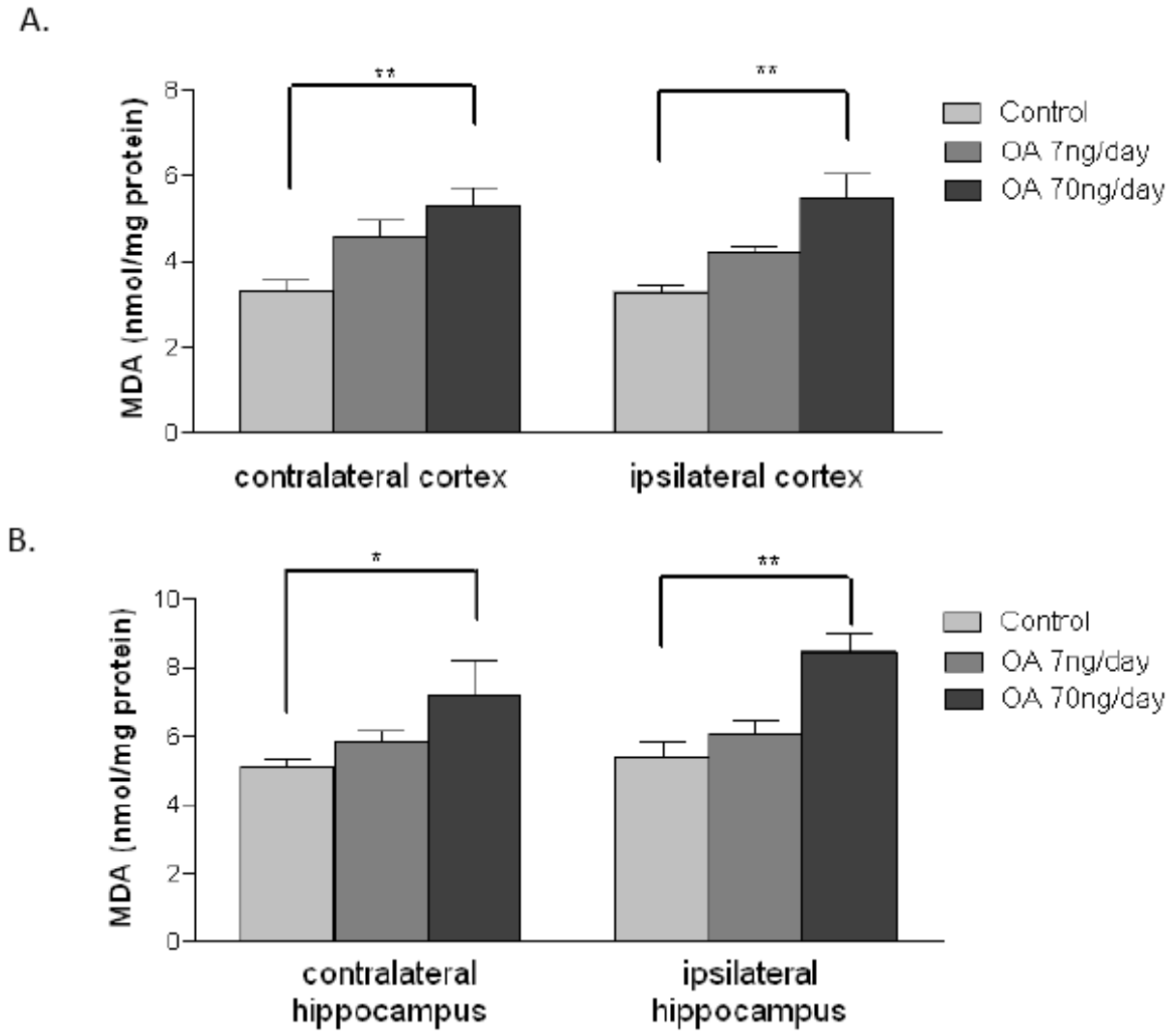
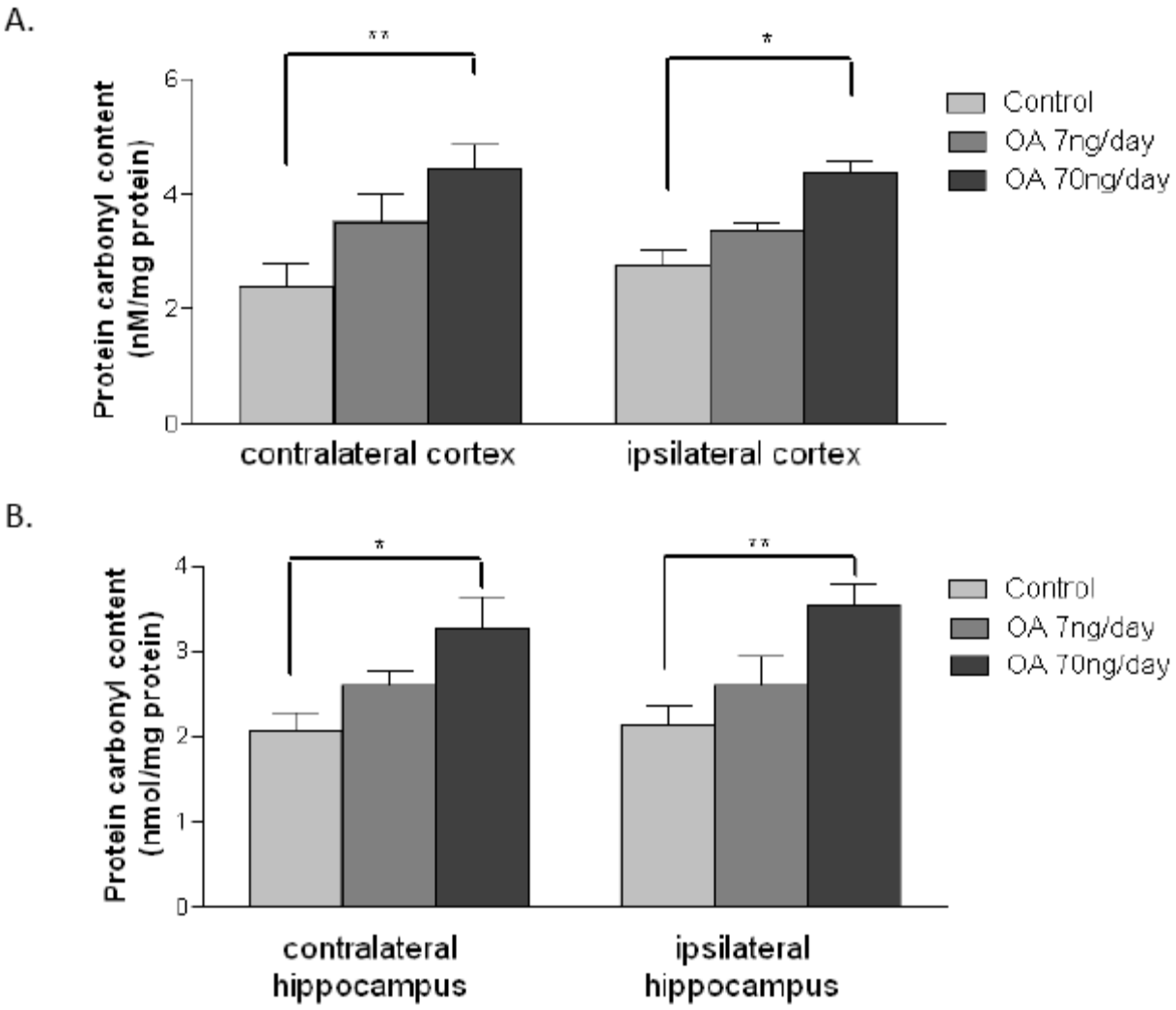

A.
MDA (nmol/mg protein)
0
2
4
6
8
**
**
contralateral cortex
ipsilateral cortex
Control
OA 7ng/day
OA 70ng/day
B.
MDA (nmol/mg protein)
0
2
4
6
8
10
*
**
contralateral hippocampus
ipsilateral hippocampus
Control
OA 7ng/day
OA 70ng/day

Figure 8


A.
**
*
Protein carbonyl content (nM/mg protein)
6
4
2
0
contralateral cortex
ipsilateral cortex
Control
OA 7ng/day
OA 70ng/day
B.
*
**
Protein carbonyl content (nmol/mg protein)
4
3
2
1
0
contralateral hippocampus
ipsilateral hippocampus
Control
OA 7ng/day
OA 70ng/day

Chapter 3

In vivo phosphorylation of tau induced by inhibition of protein phosphatases

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease and the most common form of dementia in the elderly. Although the cause and progression of AD is still unclear, affected brains of AD are characterized by the presence of senile plaques (SP), neurofibrillary tangles (NFTs) and loss of cholinergic neurons in the basal forebrain. Clinical studies have showed that dementia in AD patients is positively related to the numbers of NFTs rather than the degree of SP deposition. We have previously shown that okadaic acid (OA), a nonspecific serine/threonine phosphatase inhibitor, induces cognitive deficiency associated with oxidative stress. In the present study, we assessed the possible mechanisms of cognitive deficiency due to the inhibition of protein phosphatases by OA. The model was produced by the continuous microinfusion of OA into the dorsal hippocampus of adult female ovariectomized SD rats unilaterally for 14 days. In the brain of OA (70ng/day) treated rats, immunohistochemistry study showed positive staining of phospho-tau (Thr 205) in both cortex and hippocampus. Data from western blotting also showed that the unilateral dorsal hippocampal microinfusion of OA induced the increase of phospho-tau (Thr 205) levels on both sides of cortex and hippocampus in a dose-dependent manner, associated with the increase of cyclin-dependent kinase 5 (cdk5) and inactive glycogen synthase kinase 3 beta (p-GSK3β Ser 9) level. These results suggest that the microinfusion of OA into unilateral dorsal

hippocampus induce not only cognitive deficiency, but also AD-like NFTs changes resulted from significant increase of tau hyperphosphorylation, and cdk5 may be involved in OA induced tau hyperphosphorylation.

Introduction

Neurofibrillary tangles (NFTs), one of hallmarks of Alzheimer's disease (AD) besides senile plaques (SP), was identified high prevalence at death of tau pathology in the aging hippocampus, with very few individuals having no tau pathology (1). Tau proteins are microtubule associated proteins that are abundant in neurons at central nervous system (2). Under physiological condition, tau plays a key role in microtubules stabilization, axonal transportation and neurite outgrowth (2-5). Under pathological condition, deposits of abnormally hyperphosphorylated tau protein are found in many neurodegenerative disorders like AD (1, 3, 5-7). Meanwhile, tau pathology is also found in the aging brain (1, 8). However, the relationship between tau pathology in brain aging and development of tauopathy, neuronal disorders resulting from the pathological aggregation of tau protein in the brain, and cognitive deficiency is unclear.

Data from clinical studies have suggested that deposition of amyloid plaques does not correlate well with neuron loss and the severity of dementia in AD patients is positively related to the numbers of NFTs in cortex (9, 10). Tau hypothesis, the idea that tau protein abnormalities initiate the AD cascade, was also supported by the fact that NFTs are found in the neurons, aggregate to disintegrate the neuron's transport system and result in neuronal death (11-14). Studies from transgenic mice have showed that mice expressing a repressible human tau variant resulted in progressive age-related NFTs,

neuronal death and behavioral impairment which could be reversed by the suppression of transgenic tau except for the accumulation of NFTs (15, 16). Although tau hypothesis is supported by many studies, it remains unclear whether NFTs are the initiating factor or merely markers of the disease process and whether NFTs crosstalks with other AD hallmarks.

About 100 years since NFTs are first described by Dr. Alois Alzheimer in one of his patients suffering from AD, the breakthrough discovery of tauopathy was the finding that the main component of paired helical filaments (PHFs), which making up the NFTs, is aberrant phosphorylated tau (6, 7, 17-19). Within the last 20 years, a great deal of efforts had been put into the study of tau and tau phosphorylation in physiological and pathological settings. However, there is still a lot left to be learned. Several protein kinases, including glycogen synthase kinase-3 (GSK-3), PKC, MAP kinase and cyclin-dependent kinase 5 (cdk5) have been reported to phosphorylate tau protein at some but not all those sites that are found in AD tau (20-24), whereas its dephosphorylation is mainly catalyzed by protein phosphatase (PP) 1, 2A/B and 5, especially PP2A (25-29)(30, 31). It is proposed that an imbalance between tau phosphorylation and dephosphorylation is critical to AD (32, 33). This disturbance might be the result of either higher activities of tau kinases, lower activities of tau PPs, or both. Together, these findings have helped to establish the hypothesis that breaking the balance between tau protein phosphorylation

and dephosphorylation will lead to AD-like tauopathy.

We previously developed an experimental AD model via microinfusion of OA, a PP1/2A inhibitor, into the dorsal hippocampus of ovariectomized adult female SD rat unilaterally. Continuous administration of OA 70 ng/day into unilateral hippocampus for 14 days induced spatial cognitive impairment. To clarify the mechanism of cognitive impairment induced by OA in present study, pathological changes in these rats were observed by immunohistochemistry with specific anti-phospho-tau antibody. In order to investigate whether OA induced tau phosphorylation *in vivo* and the possible mechanisms, the model was used and the levels of phospho-tau, PP1/2A and certain proline-directed tau kinases including cdk5, GSK3β and ERK1/2 in both cortex and hippocampus were detected.

Materials and methods

Animals

Female Sprague Dawley (SD) rats were purchased from Charles Rivers (Wilmington, MA) and maintained in our animal facility in a temperature-controlled room (22-25 °C) with 12-hour dark-light cycles. All rats will have free access to laboratory chow and tap water. All animal procedures are reviewed and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee.

Materials

Okadaic acid (Cat #: 495604) was purchased from Calbiochem (Gibbstown, NJ) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 μM and diluted to appropriate concentration in artificial spinal-cerebral fluid. ALZET osmotic pumps (model 1002) and brain infusion kits were purchased from DURECT Corporation (Cupertino, CA). Anti-cdk5 (C-8), anti-PP1 (E-9), anti-PP2A (C-20), anti-tau (T1) and anti-p-ERK (E4) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK1/2 and anti-phospho-tau (T205) antibody came from Invitrogen (Carlsbad, CA). Anti-GSK3β antibody was purchased from Cell Signaling Technology (Danvers, MA). Immunohistochemistry ABC peroxidase staining kits and metal enhanced DAB substrate kit were purchased from Pierce (Rockford, IL).

Ovariectomy

To avoid the influence of estrogens on the model, all the rats used in this study were received ovariectomized bilaterally and were maintained on a phytoestrogen-free diet. Four week old female SD rats were anesthetized with ketamine (60mg/kg) and xylazine (10mg/kg). A small cut was made through skin and muscle. Ovaries were externalized and removed. Ovariectomies were performed at least two weeks before further procedure.

Implantation of brain infusion kit and osmotic pump

Adult ovariectomized female Sprague-Dawley rats (250-300g) were anesthetized by intraperitoneal ketamine (60mg/kg) and xylazine (10 mg/kg) and immobilized on a stereotaxic apparatus. A small cut was made through the scalp and a small hole was made through the exposed skull. A stainless steel brain infusion cannula of 0.36 mm outer diameter was embedded into the right dorsal hippocampus of animals by using stereotaxic surgery and fixed on the skull. The cannula terminal co-ordinates, with the incisor bar set at –3.3 mm, were in mm from bregma and dura: posterior 3.8, lateral ±2.5, depth –3. For microinfusion of OA, a brain infusion kit and ALZET Osmotic Pumps (Cupertino, CA) was used and a catheter tube was used to attach the cannula to the flow moderator of the ALZET pump, which was implanted subcutaneously. Before the placement of cannula and ALZET Osmotic Pump, the brain infusion assembly and

osmotic pump was prepared and filled with the solution to be delivered. Desired OA solution in artificial spinal-cerebral fluid (contain 1% DMSO for dissolving OA) was filled into the pump and allowed infusion at an appropriate rate into unilateral dorsal hippocampus. The control received the same surgery and microinfusion with the same volume of vehicle. This pump (model 1002, ALZET) had a 100 µl reservoir and two weeks duration. The cannula position was verified by injection of 10 µl of a solution of pontamine sky blue via the cannula. Ten minutes later, the rat was decapitated and its brain was removed for checking the validity of the dorsal hippocampus injection.

Immunohistochemistry

After 14 days OA dorsal hippocampal infusion, rats received transcardiac perfusion with 4% formaldehyde in PBS, followed by immersion fixation of removed brain, for at least another 24 hours before paraffin-embedding. For neuropathological diagnosis, tissue blocks were embedded in paraffin and sectioned at 10 µm. Brain slides were stained with HRP-conjugated polymer with DAB substrates. Afte deparaffinization and rehydration, the slides were placed in a boil in 10 mM sodium citrate buffer pH 6.0 and maintained at a sub-boiling temperature for 10 minutes. Cooled slides on bench top and then incubated sections in 3% hydrogen peroxide for 10 minutes. After three times of washing, the slides were blocked with 5% normal goat serum in 1% BSA for 1 hour at room temperature.

Removed the blocking solution and added appropriate primary antibody to each section and incubated overnight at 4 ºC. The biotinylated secondary antibody was applied after removing of the primary antibody and 3 times washing and incubated for 30 minutes at room temperature, followed by removing of secondary antibody and washing. The ABC (Avidin-Biotin Complex) reagent was applied to the tissue section with 30 minutes' incubation. The ABC reagent was washed off and the Metal enhanced DAB substrate working solution was incubated until the desired staining was achieved. As soon as the sections developed, the slides were immersed in distilled water followed by dehydration through 95% alcohol, absolute alcohol and xylene. Resinous medium was used to mount the coverslips.

Western blotting

For immunoblotting analysis with different antibodies, brain tissues were dissected into cortex and hippocampus, then homogenized in RIPA buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM sodium orthovanadate, 10mg/ml Aprotinin, 100 mg/ml Phenylmethyl Sulphonyl Fluoride (PMSF)). Samples were then centrifuged at 12,000g for 30 min, and supernatants were collected for analysis. Protein from the brain tissues was separated by SDS-PAGE and transferred to Immunobilon-P polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA) membrane. Membranes

were blocked with 5% try milk in PBS. Proteins were probed with specific antibodies at proper dilutions according to the manufacturer's instruction and incubated overnight at 4 ^{o}C. The blots were rinsed and applied with the appropriate secondary antibodies. After proper washing, the blots were developed with an enhanced chemiluminescent kit (Pierce, Rockford, IL). ECL results were digitized and quantified by using UVP (Upland, CA) Bioimaging System. All the blots were normalized and semi-qualified by beta actin, which was probed and detected on the same blots after stripping and re-blocking the membranes.

Statistics

The results were analyzed with one-way ANOVA with prism software (Graphpad Inc., San Diego, CA). The significance of differences among groups was determined by Tukey's multiple comparison tests. $p<0.05$ was considered significant for all the experiments. All results were expressed as mean ± SEM.

Results

OA induces NFT-like pathological formation by immunohistochemistry

In the previous study, we produced an experimental AD tauopathy model by microinfusion of OA into the dorsal hippocampus of adult ovariectomized female rats unilaterally and found that this model displayed a spatial learning and memory impairment without motor function deficit, and NFTs-like pathological changes were discovered in both hippocampus and cortex via Bielshowsky's silver staining. In order to further confirm the NFTs-like pathological changed induced by OA, the model was used and the brain samples were subject to immunohistochemistry probing with specific anti-phospho-tau (Thr205) antibody. To produce the experimental AD model induced by OA, OA (0, 7 and 70 ng/day) was continuously microinfused into the dorsal hippocampus of ovariectomized adult female rats unilaterally for 14 days. Then the rats were subject to transcardiac perfusion with formalin for further brain tissue paraffin sectioning. After deparaffinization, rehydration and blocking, the brain sections were probed with antibody raised against phosphorylated tau at Thr205 (p-Thr205). By using ABC reagent and DAB staining, we found that high dose OA infusion induced a big lesion in the ipsilateral dorsal hippocampal area, which was not seen in low dose OA and control group; and there were a few anti p-tau^{Thr205} immunoreactivity positive staining in the cortex and hippocampus of low OA dose group and much more positive staining were found in high

OA dose group, but no abnormal staining found in control (Figure 1 &2). These data are consistent with our previous study showing OA infusion can induce silver positive staining of NFTs-like formation in both hippocampus and cortex.

Tau phosphorylation in OA induced experimental AD tauopathy rats

14 days after the microinfusion of OA into the unilateral dorsal hippocampus, rats were subject to decapitation. The brains were removed, and the cortex and hippocampus of both ipsilateral and contralateral side were separated. After protein extraction in RIPA buffer and SDS-PAGE separation, the proteins were transferred onto a PVDF membrane that was probed with antibodies raised against total tau (T1) or phosphorylated tau (p-Thr205). Then, the ratio of phosphorylated tau over total tau was calculated to assess the tau phosphorylation in OA-induced tau model of AD. We found that there was a dose-dependent increase of phosphorylated tau in both cortex and hippocampus of OA treatment groups (Figure 3). In ipsilateral hippocampus, OA induced 4-fold increase of p-tau levels in low dose group ($p<0.05$) and about 6-fold increase in high dose group ($p<0.01$) (Figure 3A). There was also a slight increase of p-tau levels at contralateral hippocampus received high dose OA treatment ($p<0.05$) (Figure 3A). In ipsilateral cortex, p-tau levels were increased over 2-fold at low dose group ($p<0.01$) and over 3-fold in high dose group ($p<0.01$) (Figure 3B). Meanwhile, a slight increase of p-tau was

observed in the contralateral cortex of high OA dose group ($p<0.05$) (Figure 3B). The results suggested that OA could lead to tau hyperphosphorylation in vivo.

Effect of OA on tau protein phosphatases and kinases in OA induced experimental AD rats

It is proposed that the imbalance between tau kinases and phosphates is crucial to tau hyperphosphorylation. To further understand why tau was phosphorylated in vivo, we further detected the protein levels of PP1/2A and certain tau protein kinases, including cdk5, GSK3β and ERK1/2, in the cortex and hippocampus after 14 days microinfusion of OA into unilateral dorsal hippocampus of adult female OVX SD rats.

Effect of OA on PP1 and PP2A levels in OA induced experimental AD rats

As Figure 4 showed, although there were slight decreasing of PP1 levels in OA treatment groups, no significant changes of PP1 expression were found in both cortex and hippocampus of different treatment groups ($p>0.05$). The same results were observed about PP2A levels showing no significant decline of PP2A expression in both cortex and hippocampus after OA treatment ($p>0.05$) (Figure 5). These data suggested that OA infusion inhibits the activities other than the expression of PP1 and PP2A.

Effect of OA on GSK3 β levels in OA induced experimental AD rats

GSK3β is a one of the candidates for *in vivo* tau kinases. The activation of GSK3β is mediated by dephosphorylation at Ser 9 (34). In order to investigate whether GSK3β was involved in OA induced tau phosphorylation, we also detected the levels of inactive GSK3β over total GSK3β. As Figure 6 showed, there was an OA dose-dependent elevation of p-GSK3β (Ser 9) over total GSK3β in either side of cortex and hippocampus. In hippocampus, no significant increase of p-GSK3β (Ser 9) were found in OA treatment groups ($p>0.05$) (Figure 6A). In cortex, high dose OA (70ng) induced an increase of p-GSK3β (Ser 9) in both contraleteral side ($p<0.05$) and ipsilateral side ($p<0.01$) (Figure 6B). The results indicate that OA infusion inhibited the activation of GSK3β and GSK3β may not involve in OA infusion induced tau phosphorylation.

Effect of OA on cdk5 levels in OA induced experimental AD rats

In hippocampus as Figure 7A showed, there was an OA dose-dependent elevation of cdk5 levels in both sides. OA high dose induced 2-fold increase of cdk5 levels in contraleteral hippocampus ($p<0.05$) and about 3-fold increase in ipsilateral side ($p<0.01$) (Figure 7A). In the cortex, cdk5 levels were found to be increased in both sides of cortex of high dose group ($p<0.05$) (Figure 7B). The results suggested that OA infusion can

induce cdk5 upregulation which may be involved in OA induced tau phosphorylation *in vivo*.

Effect of OA on p-ERK1/2 levels in OA induced experimental AD rats

As Figure 8 showed, although there was an OA dose-dependent elevation of p-ERK1/2 over total ERK1/2 in either side of cortex and hippocampus, no significant increase of p-ERK1/2 were observed in both cortex and hippocampus of OA treated groups ($p>0.05$). The results suggest that OA infusion has no significant effect on ERK1/2 and ERK1/2 may be not involved in OA infusion induced tau phosphorylation.

Discussion

AD is the most common cause of dementia, comprising 50-70% of all cases. It is critical to develop useful animal models to study pathology of AD for preclinical testing of drugs, because very few species develop the behavioral, cognitive and neuropathological symptoms of AD spontaneously. Although much progress has been made in recent years with transgenic mice models of AD, these mice only present a small portion of AD patients via mimicking part of AD pathological events (35-37). To date, the complication of AD in human is beyond the current transgenic models can mimic and more efforts have to be made to explore the ideal AD models with different tools. A variety of animal models for AD have been introduced from invertebrates to mammals (37). Except for the transgenic technique, some conventional pharmacological techniques are still used to produce models for AD study, i.e. chemical lesion (38, 39). OA is one of the compounds widely used to produce both *in vivo* and *in vitro* AD model. In previous study, we produced an experimental AD model induced by microinfusion of OA into unilateral hippocampus, which mimicked the cognitive impairment and NFTs-like pathological changes. In the present study, the brain slides of this model were subject to immunohistochemistry. Progressive formation of anti p-tau^{Thr205} immunoreactivity was observed in both cortex and hippocampus, which is consistent with our previous data of silver staining and studies from other groups (12, 38). However, more profound positive

anti p-tau^{Thr205} immunoreactivity had been found in cortex than hippocampus. It may result from the massive neuronal death in hippocampus induced by OA.

The present study aimed at the tau pathology induced by OA, a PP1/2A inhibitor. Although tau pathology has received less attention than Aβ pathology in AD research, some studies from clinic had suggested that it is the numbers of NFTs, other than the degree of SP deposition, positively correlated with the degree of dementia in AD patients (9). Hyperphophorylated tau, the major component of NFTs, is dephosphorylated by PPs, including PP 1, 2A/B and 5, especially PP2A (25-29)(30, 31). We proposed that inhibition of phospho-tau protein dephosphorylation will lead to abnormal hyperphosphorylation of tau, which will further induce neurofibrillary degeneration of AD and other tauopathies. Our model was produced by continously microinfusion of OA into unilateral dorsal hippocampus of rats for 14 days and our western blotting data showed that the level of phosphorylated tau (p-tau^{Thr205}) was dramatically increased in the ipsilateral cortex and hippocampus, even at the contralateral cortex and hippocampus at a less degree, of both low dose (7ng/day) and high dose (70ng/day) groups. These data are in accordance with previous in vivo and in vitro studies (38, 40, 41).

Tau protein is a phospho-protein whose expression and phosphorylation is well regulated (7, 42, 43). On the longest central nervous system (CNS) tau isoform containing 441 residues, there are 79 putative serine or threonine residues and 5 tyrosine

residues located in two proline-rich regions whose phosphorylation affect the binding of tau to microtubules (5, 14, 44). These sites have been divided into proline-directed and non-proline-directed groups (45). Thr-205 is one of the proline-directed phosphorylation sites whose phosphorylation was associated with abnormally hyperphosphorylated tau in PHFs and tau induced neurodegeneration (46, 47). Our data suggested that OA could induce the phosphorylation of tau at Thr-205, one of proline-directed phosphorylation sites, which is consistent with the data from other groups (38, 48). Moreover, OA was also able to induce tau phosphorylation in a non-proline-directed epitope (Ser-262) (49). Based on the immunohistochemistry and immune blotting data, we found that microinfusion of OA into unilateral dorsal hippocampus induced tau phosphorylation and NFTs-like pathology, which are one of characteristic hallmarks of AD brain. It suggests that our model may be a useful tool to study the tauopathy of AD.

To clarify how the tau protein was hyperphosphorylated, we also detected the levels of PP1/2A and the levels of certain protein kinases, including cdk5, GSK3β and ERK1/2, in the cortex and hippocampus of the model. We found that there were no significant changes of PP1/2A levels in both cortex and hippocampus after OA infusion. So, the hyperphosphorylated tau observed in our model could be induced by the increase of tau protein kinases. Although many kinases have been considered as potential tau protein kinases, so far, only a few are thought to be good candidates in vivo. Three major

proline-directed protein kinase families have been characterized: MAPKs, GSK3 and CDC2-like kinases (cdk2 and cdk5) (50). GSK3β, a proline-directed serine/threonine kinase, is highly expressed in the brain (51) and is also recognized as tau protein kinase I (52), which could generate a PHF epitope on tau from microtubule proteins of bovine brain (53). In vitro studies showed that elevating GSK3 activity in the COS cells by transfection with GSK3β decreased the mobility of tau and facilitated the formation of PHF (54). Inhibition of GSK3 by lithium reduced the phosphorylation of tau, enhanced the binding of tau to microtubules and promoted microtubule assembly (55). Transgenic mice with GSK3β over expression showed tau hyperphosphorylation, neuronal death and spatial learning deficits (56). These findings provide strong evidence that GSK3β is one of the kinases phosphorylating tau in vivo, and GSK3β mediated tau phosphorylation could be one of contributing factor to AD pathology. The activation of GSK3β is mediated by dephosphorylation at Ser 9 which is regulated by Akt (34). PP2A can activate GSK3β directly by dephosphorylation at Ser 9, or indirectly by dephosphorylating Akt (57). Inhibition of PP2A by OA led to inactivation of GSK3β by increasing GSK3β (p-Ser 9), which is consistent with our data. It suggests that GSK3β may be not involved in OA induced tau phosphorylation in *vivo*.

Another in vivo candidate for tau kinase is cdk5, which is abundant in brain tissue and has been shown to associate with tau (58, 59). Cdk5 is one of the cdk family member and

is activated by interaction with the non-cyclins, p35 and p39, which are regulatory proteins exclusively expressed in postneurons (60). A subunit of tau protein kinase II, cdk5 is associated with its activator p25 (58). The p25 subunit, a proteolytic cleavage product of p35, accumulates in the brain and promotes the activation and mislocation of cdk5 in AD patients (61). The p25 is found in neurons containing NFT in AD brain and in vivo study showed that p25/cdk5 induces tau phosphorylation (61). Studies on transgenic mice (overexpressing human p25, which activates cdk5) showed that p25 transgenic mice display abnormally hyperphosphorylated tau and neurofilament, accompanied by cytoskeletal disruption (62). In vitro, tau is one of the first identified substrates of cdk5 (63) and many physiological relevant Ser/Thr sites on human tau have been identified as cdk5 sites, including Ser^{202}, Thr^{205}, Thr^{212}, Ser^{235}, Ser^{396} and Ser^{404} (64, 65). Due to the fact that in vitro cdk5 sites are also phosphorylated on PHF tau (46), it is proposed that cdk5 may involve in the tauopathy. It is supported by our data showing that, accompanied with the tau phosphorylation, the cdk5 levels were significantly elevated in both sides of the cortex in high dose (OA 70ng) group, and were also increased at ipsilateral hippocampus of both low dose (OA 7ng) and high dose groups; and our previous data showing that transient cerebral ischemia induced tau hyperphosphorylation and NFT-like conformational epetopes in adult female rat cortex could be reversed by inhibition of cdks (66, 67).

Besides GSK3β and cdk5, we also examined the p-ERK/ERK ratio in the model brain. Belong to the proline-directed protein kinase families, several members of MAP kinase family have been reported to phosphorylate tau, such as extracellular-signal-regulated kinase (ERK) (68, 69). However, we found that there were no significant changes of p-ERK/ERK ratio among groups.

Collectively, our data showed in vivo inhibition of PP1/2A by microinfusion of OA into unilateral hippocampus of adult ovariectomizd female SD rats could induce spatial learning and memory deficiency, tau phosphorylation and NFT-like tau pathology. Our data also suggested that cdk5 may involve in the tau hyperphosphorylation due to the inhibition of PP1/2A by OA. Although not fully mimicking the AD pathology, our model could be a useful tool to study the tauopathy and a valuable model for preclinical testing of drug targeting on PPs.

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Figure legends

Figure 1. The immunohistochemistry staining of the hippocampus in OA treated rats.

Fourteen days after microinfusion of OA into dorsal hippocampus unilaterally, rats received transcardiac perfusion with 4% formaldehyde in PBS, followed by immersion fixation of the removed brain. Then brain tissue was processed to paraffin sectioning and 10 µm slides were prepared for immunohistochemistry staining probed with antibody raised against phospho-tau (Thr 205). There was no positive staining found in either side of the hippocampus in the control group (A, B, C) or low OA dose (7ng) group (D, E, F). A few anti-p-Thr 205 immunoreactivity positive staining was found in the CA1 area of the contralateral hippocampus of rats rececived high dose infusion of OA (70ng) (G, H), and the ipsilateral hippocampus CA1 area was damaged (I).

Figure 2. The immunohistochemistry staining of the cortex in OA treated rats.

Fourteen days after microinfusion of OA into dorsal hippocampus unilaterally, rats were subjected to paraffin section preparation for immunohistochemistry staining. There was no positive staining found in either side of the cortex in the control group (A, B, C). A few anti-p-Thr 205 immunoreactivity positive staining was found in the both side of

the cortex in the rats rececived low OA dose infusion (D, E, F) and much more positive staining were found in high OA dose group (G, H, I).

Figure 3. Phospho-tau levels in OA induced experimental AD rats

Fourteen days after microinfusion of OA into dorsal hippocampus unilaterally, rats were decapitated and the brain were removed. Then cortex and hippocampus were separated, homogenized in RIPA buffer and centrifuged. The supernatants from different treatment groups were further subjected to western blot for assessing the ratio of phospho-tau (p-Thr 205) over total tau (T1) in both hippocampus (A) and cortex (B). Data were represented as mean ± S.E.M for n = 5. * < 0.05 and ** < 0.01.

Figure 4. PP1 levels in OA induced experimental AD rats

Supernatants from different treatment groups were further subject to western blot for assessing the levels of PP1 in both hippocampus (A) and cortex (B). Data were represented as mean ± S.E.M for n = 5.

Figure 5. PP2A levels in OA induced experimental AD rats

Supernatants from different treatment groups were further subject to western blot for assessing the levels of PP2A in both hippocampus (A) and cortex (B). Data were represented as mean ± S.E.M for n = 5.

Figure 6. GSk3β levels in OA induced experimental AD rats

Supernatants from different treatment groups were further subject to western blot for assessing the ratio of p-GSk3β (Ser 9) over total GSk3β in both hippocampus (A) and cortex (B). Data were represented as mean ± S.E.M for n = 5. * < 0.05 and ** < 0.01.

Figure 7. cdk5 levels in OA induced experimental AD rats

Supernatants from different treatment groups were further subject to western blot for assessing the levels of cdk5 in both hippocampus (A) and cortex (B). Data were represented as mean ± S.E.M for n = 5. * < 0.05 and ** < 0.01.

Figure 8. pERK1/2 levels in OA induced experimental AD rats

Supernatants from different treatment groups were further subject to western blot for assessing the ratio of p-ERK1/2 over total ERK1/2 in both hippocampus (A) and cortex (B). Data were represented as mean ± S.E.M for n = 5.

Figure 1.

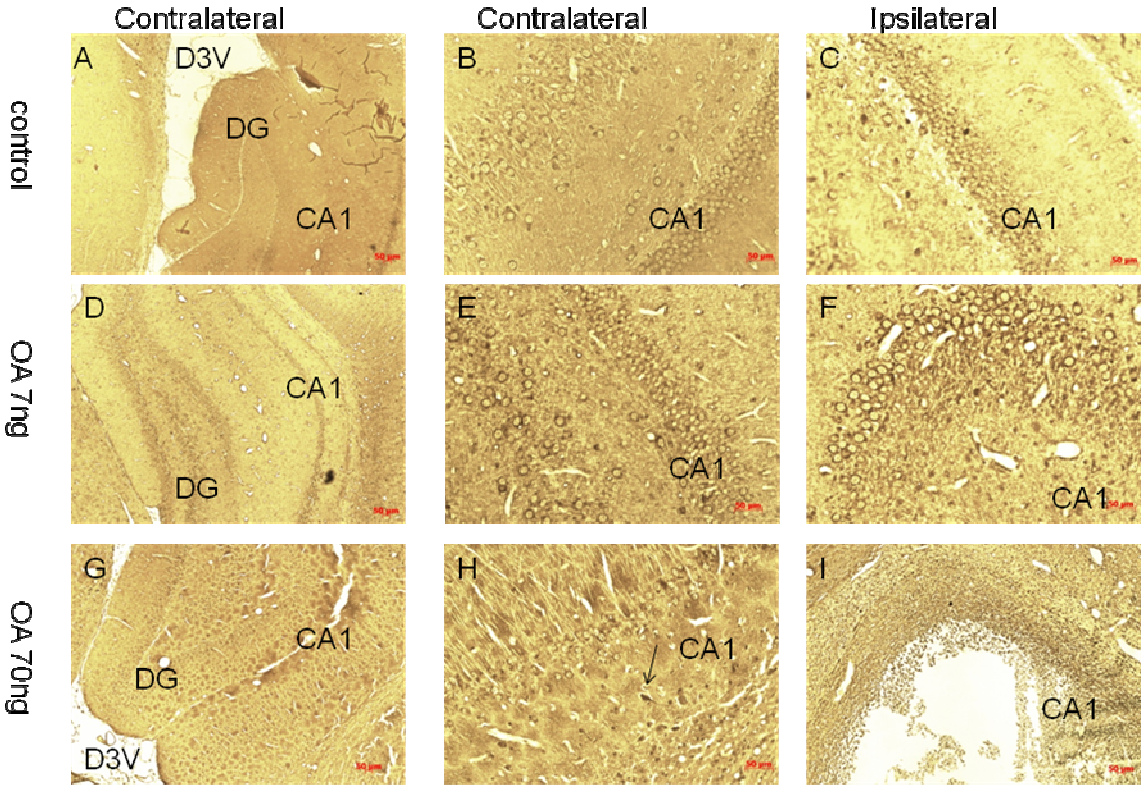
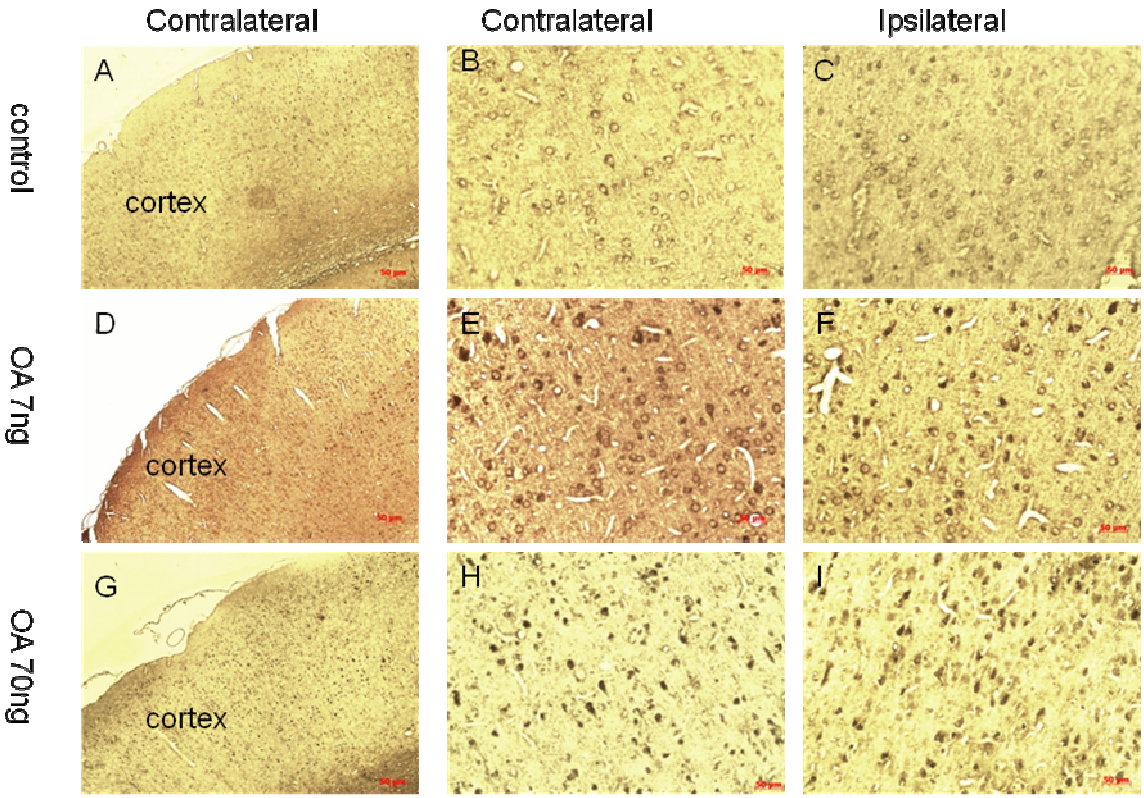

Contralateral
Contralateral
Ipsilateral
control
OA 7ng
OA 70ng
A
D3V
DG
CA1
B
CA1
C
CA1
D
CA1
DG
E
CA1
F
CA1
G
CA1
DG
D3V
H
CA1
I
CA1

Figure 2.


Contralateral
Contralateral
Ipsilateral
control
OA 7ng
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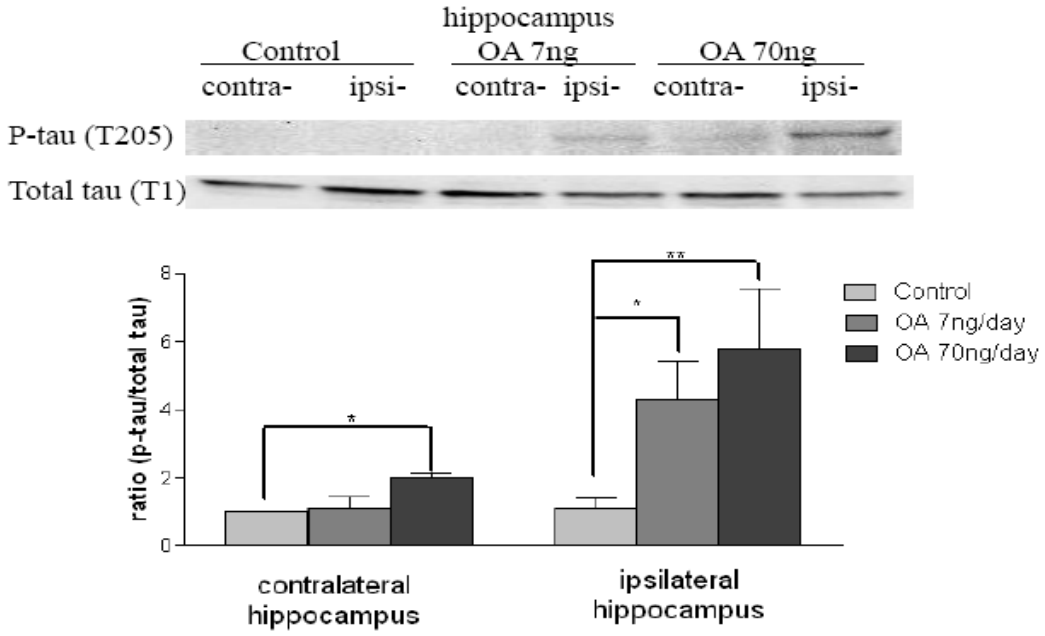
A
B
C
D
E
F
G
H
I
cortex
cortex
cortex

Figure 3.

A


hippocampus
Control
OA 7ng
OA 70ng
contra-
ipsi-
contra-
ipsi-
contra-
ipsi-
P-tau (T205)
Total tau (T1)
ratio (p-tau/total tau)
Control
OA 7ng/day
OA 70ng/day
contralateral hippocampus
ipsilateral hippocampus

B

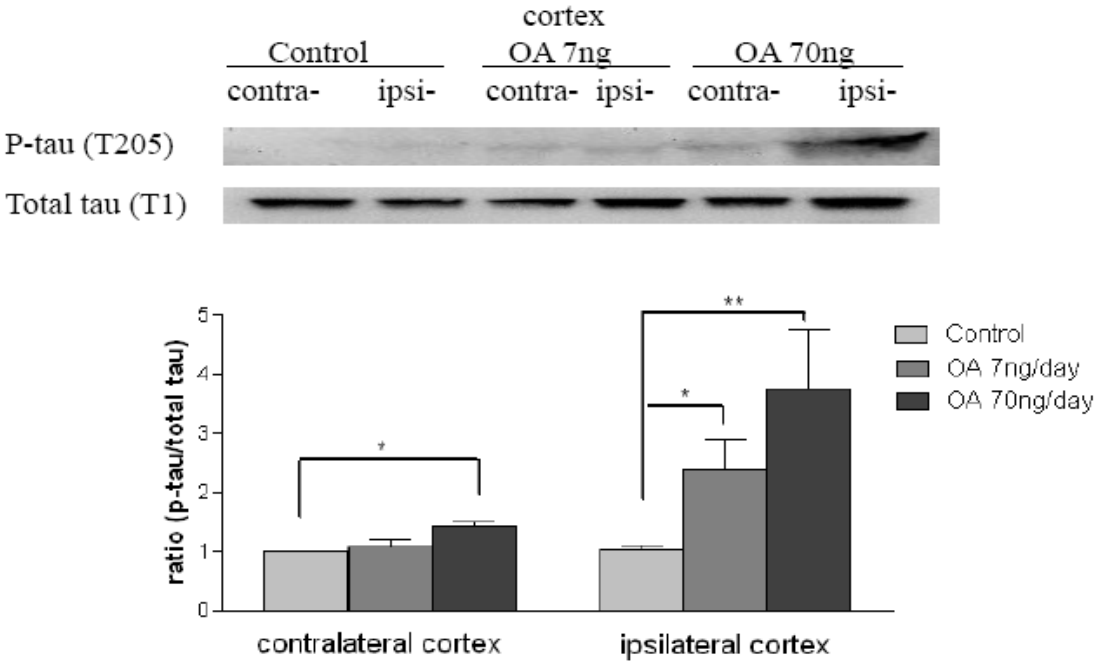
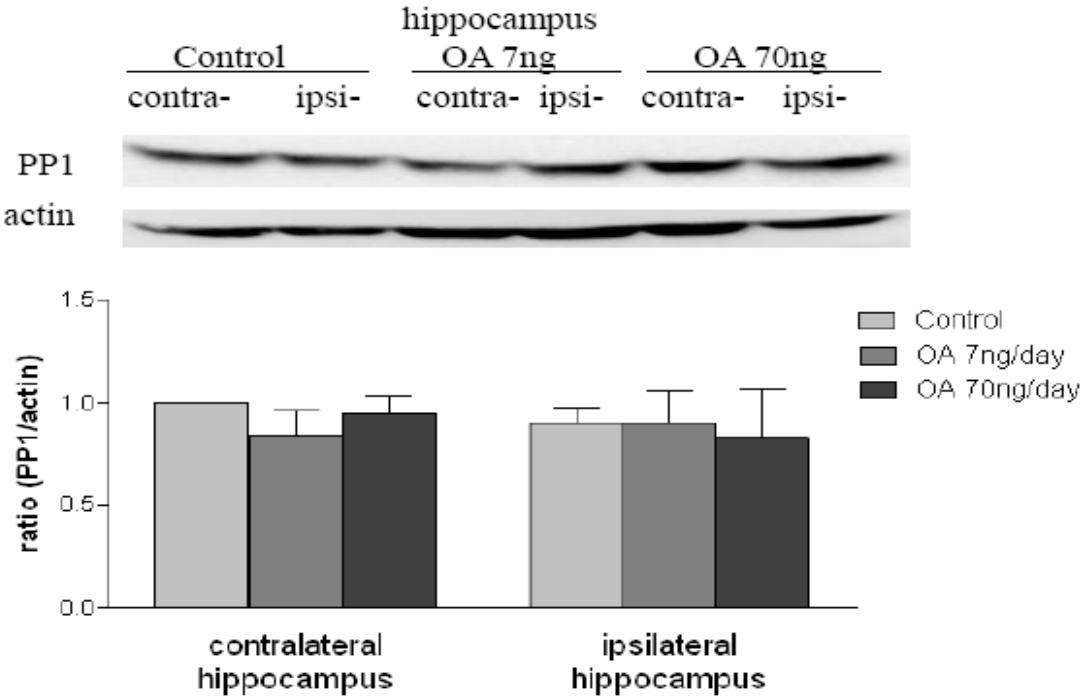

cortex
Control
OA 7ng
OA 70ng
contra-
ipsi-
contra-
ipsi-
contra-
ipsi-
P-tau (T205)
Total tau (T1)
ratio (p-tau/total tau)
Control
OA 7ng/day
OA 70ng/day
contralateral cortex
ipsilateral cortex

Figure 4.

A.


hippocampus
Control
OA 7ng
OA 70ng
contra-
ipsi-
contra-
ipsi-
contra-
ipsi-
PP1
actin
ratio (PP1/actin)
1.5
1.0
0.5
0.0
Control
OA 7ng/day
OA 70ng/day
contralateral hippocampus
ipsilateral hippocampus

B.

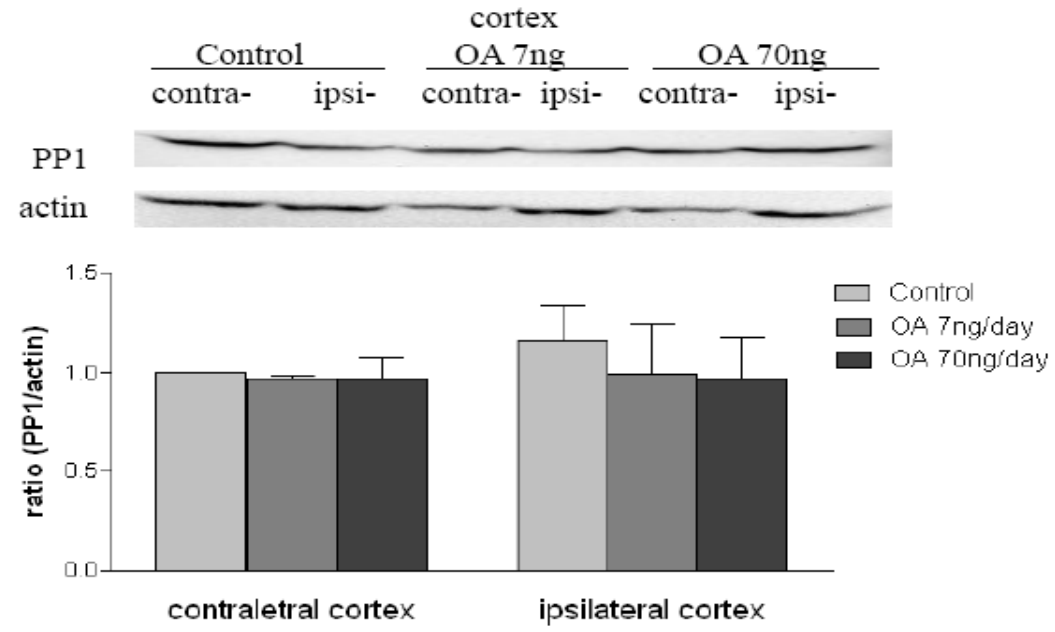
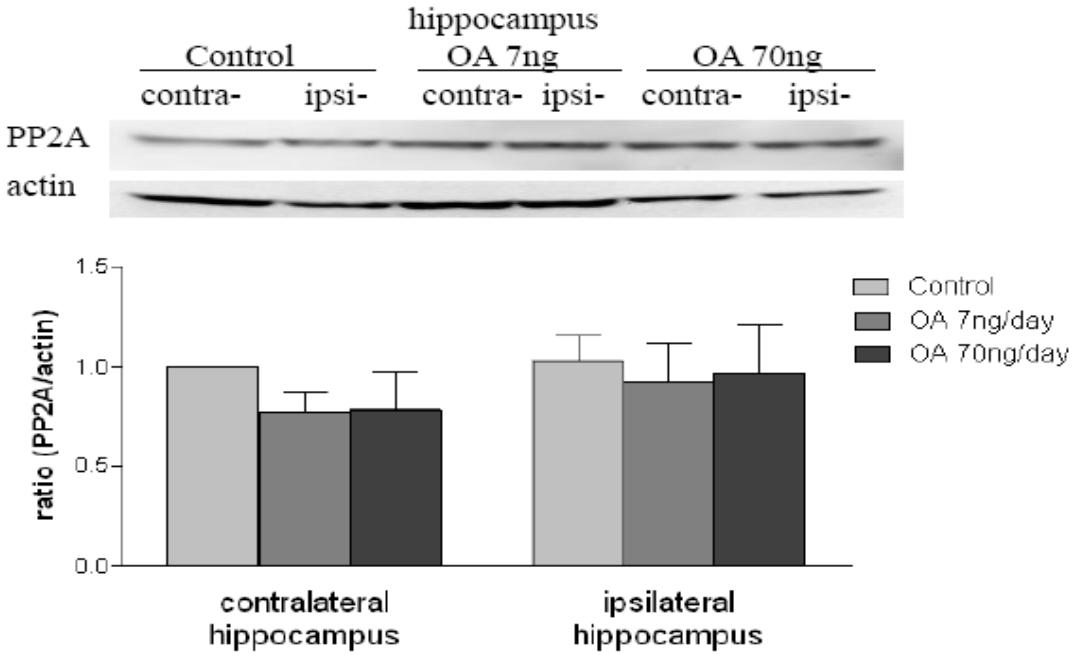

cortex
Control
OA 7ng
OA 70ng
contra-
ipsi-
contra-
ipsi-
contra-
ipsi-
PP1
actin
ratio (PP1/actin)
1.5
1.0
0.5
0.0
Control
OA 7ng/day
OA 70ng/day
contraletral cortex
ipsilateral cortex

Figure 5.

A.


hippocampus
Control
OA 7ng
OA 70ng
contra-
ipsi-
contra-
ipsi-
contra-
ipsi-
PP2A
actin
ratio (PP2A/actin)
1.5
1.0
0.5
0.0
Control
OA 7ng/day
OA 70ng/day
contralateral hippocampus
ipsilateral hippocampus

B.

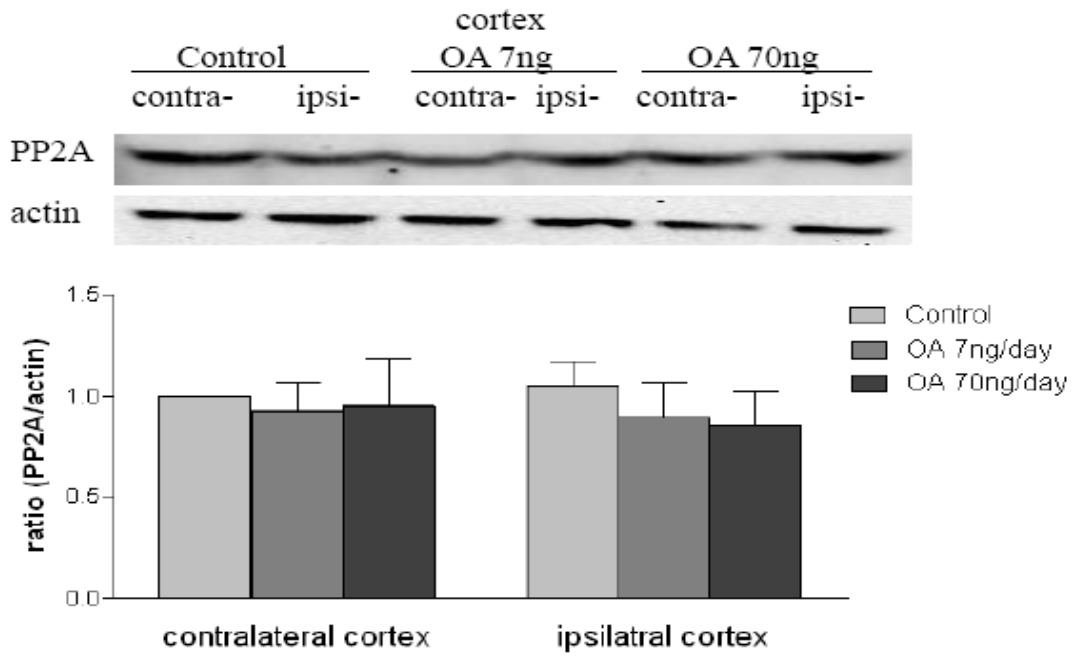
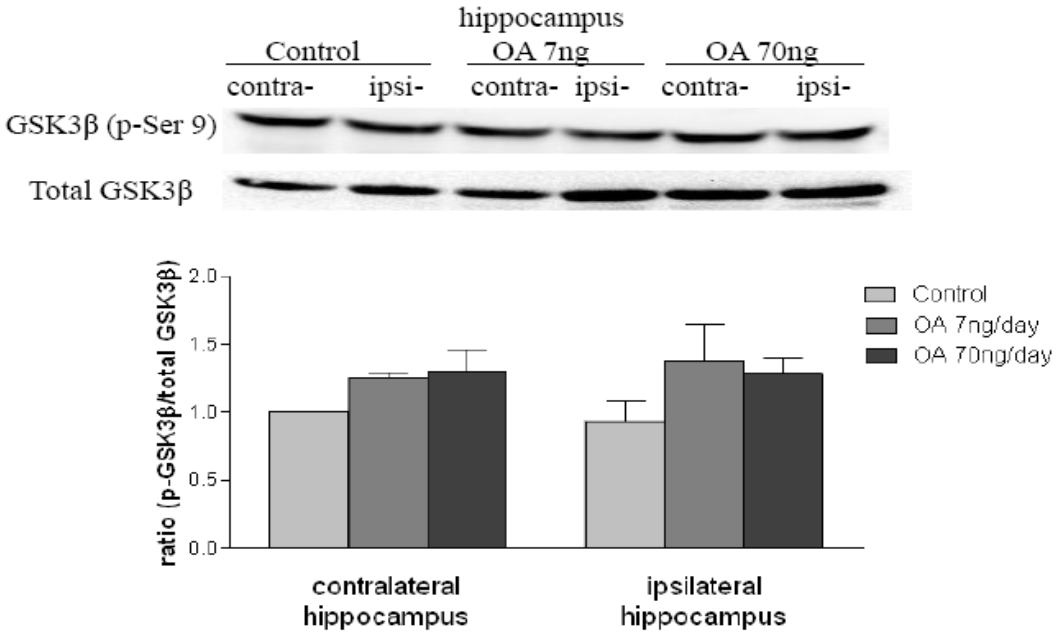

cortex
Control
OA 7ng
OA 70ng
contra-
ipsi-
contra-
ipsi-
contra-
ipsi-
PP2A
actin
ratio (PP2A/actin)
1.5
1.0
0.5
0.0
Control
OA 7ng/day
OA 70ng/day
contralateral cortex
ipsilatral cortex

Figure 6.

A.


hippocampus
Control
OA 7ng
OA 70ng
contra-
ipsi-
contra-
ipsi-
contra-
ipsi-
GSK3β (p-Ser 9)
Total GSK3β
ratio (p-GSK3β/total GSK3β)
2.0
1.5
1.0
0.5
0.0
Control
OA 7ng/day
OA 70ng/day
contralateral hippocampus
ipsilateral hippocampus

B.

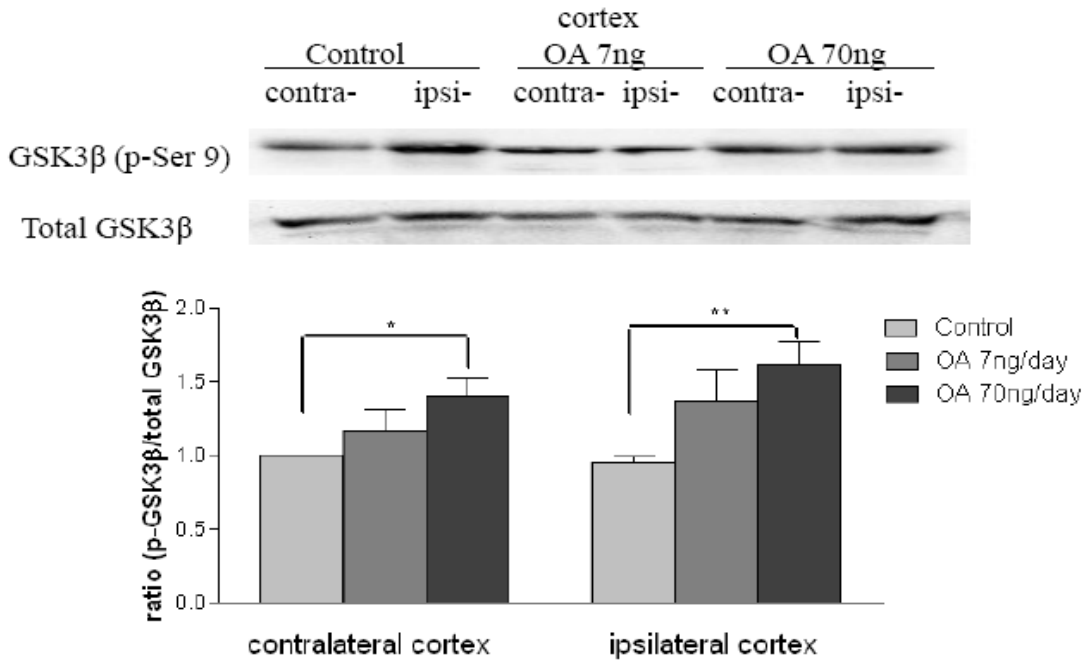
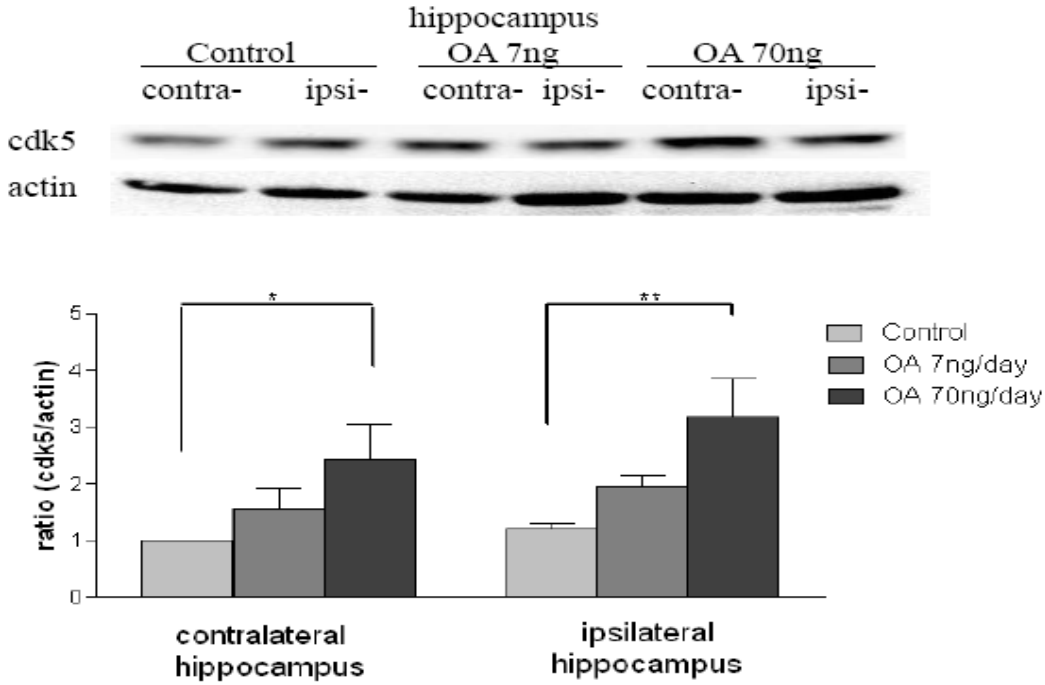

cortex
Control
OA 7ng
OA 70ng
contra-
ipsi-
contra-
ipsi-
contra-
ipsi-
GSK3β (p-Ser 9)
Total GSK3β
ratio (p-GSK3β/total GSK3β)
2.0
1.5
1.0
0.5
0.0
*
**
Control
OA 7ng/day
OA 70ng/day
contralateral cortex
ipsilateral cortex

Figure 7.

A.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Control
OA 7ng
OA 70ng
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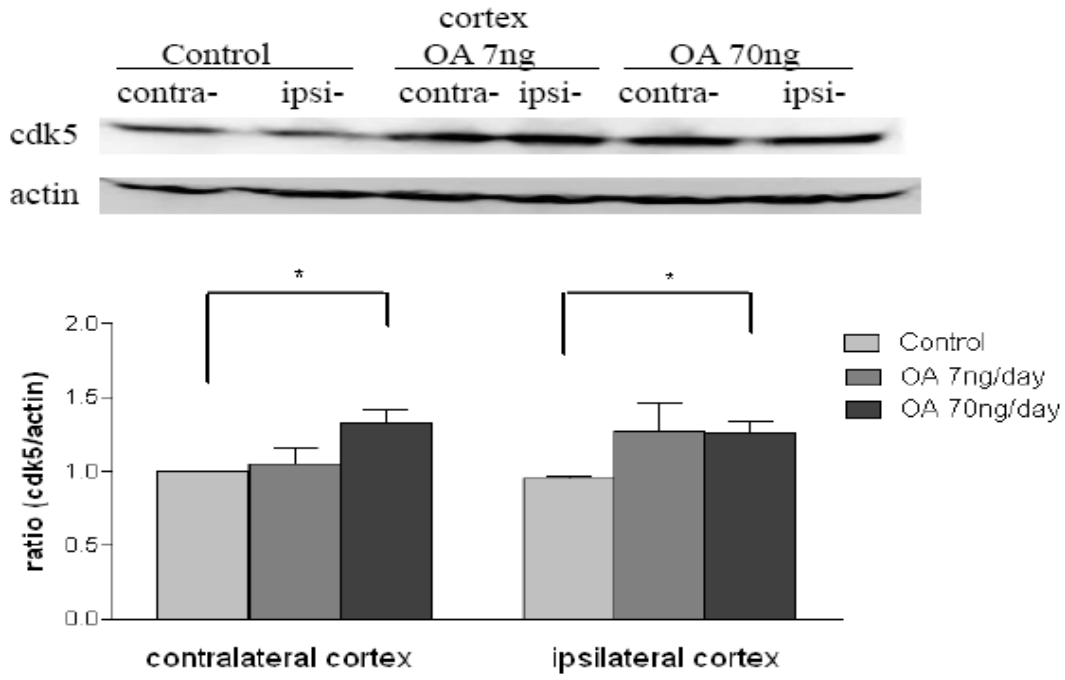
ipsi-
contra-
ipsi-
contra-
ipsi-
cdk5
actin
ratio (cdk5/actin)
0
1
2
3
4
5
*
**
Control
OA 7ng/day
OA 70ng/day
contralateral hippocampus
ipsilateral hippocampus

B.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OA 70ng
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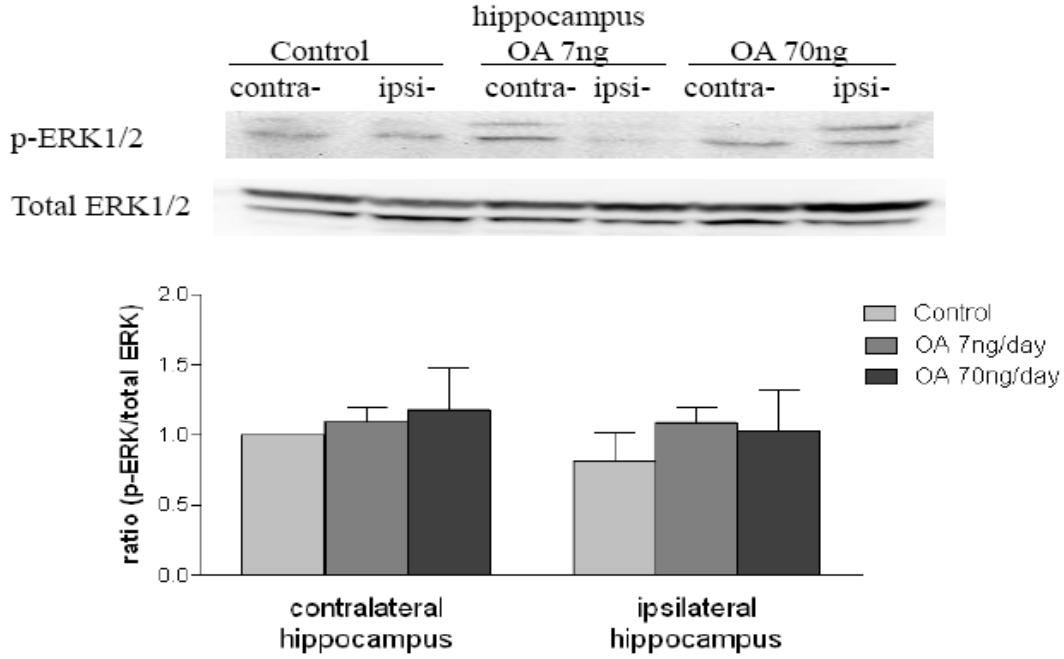
ipsi-
contra-
ipsi-
contra-
ipsi-
cdk5
actin
ratio (cdk5/actin)
0.0
0.5
1.0
1.5
2.0
*
*
Control
OA 7ng/day
OA 70ng/day
contralateral cortex
ipsilateral cortex

Figure 8.

A.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OA 70ng
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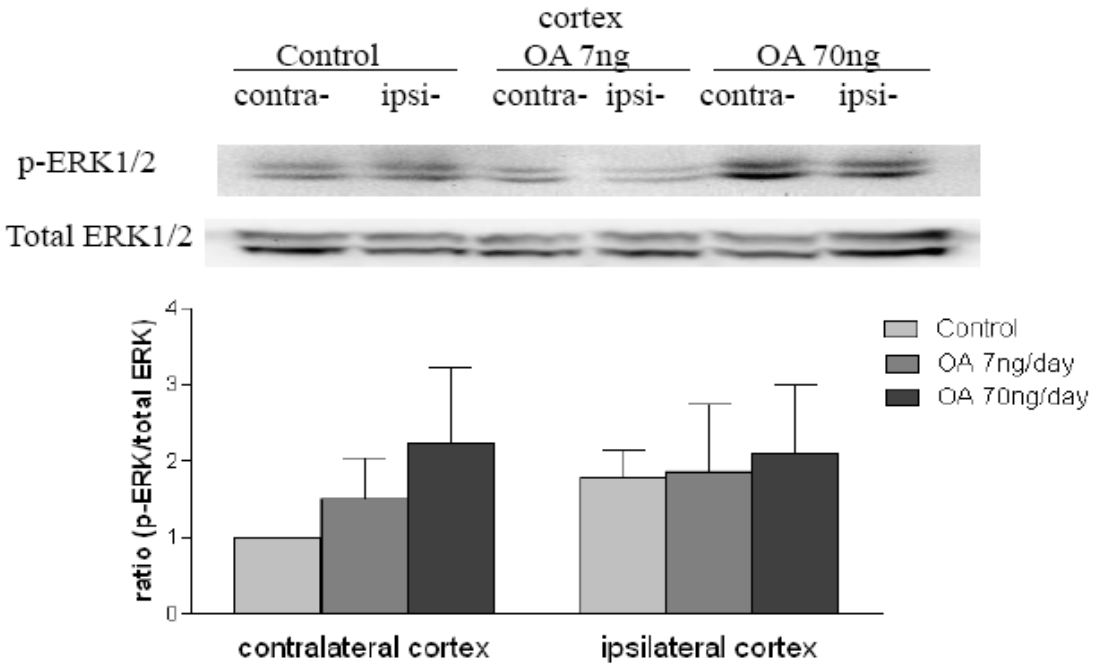
ipsi-
contra-
ipsi-
contra-
ipsi-
p-ERK1/2
Total ERK1/2
ratio (p-ERK/total ERK)
2.0
1.5
1.0
0.5
0.0
Control
OA 7ng/day
OA 70ng/day
contralateral hippocampus
ipsilateral hippocampus

B.


cortex
Control
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OA 70ng
contra-
ipsi-
contra-
ipsi-
contra-
ipsi-
p-ERK1/2
Total ERK1/2
ratio (p-ERK/total ERK)
4
3
2
1
0
Control
OA 7ng/day
OA 70ng/day
contralateral cortex
ipsilateral cortex

Chapter 4

Okadaic acid induced tau phosphorylation in SH-SY5Y cells in an estrogen preventable manner

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Key words : estrogen; tau phosphorylation; phosphatases; cdk5; GSK3β

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease and the most common form of dementia in the elderly. Epidemiological studies showed that AD is three times more prevalent than men and estrogen protects against AD. One of the pathological hallmarks of AD is neurofibrillary tangles (NFTs), which are composed of abnormally hyperphosphorylated tau. Clinical studies showed that the severity of dementia is more closely correlated with the numbers of NFTs in the AD cortex, but not the degree of senile plaque (SP) deposition, which is another hallmark of AD. The mechanism of tau hyperphosphorylation in AD is still unclear. To investigate the effect of estrogen on tau phosphorylation, SH-SY5Y cell line was treated with okadaic acid (OA), a protein phoserine/threonine phosphatase inhibitor, to induce tau phosphorylation and the neuroprotective effects of estrogen was observed by co-treatment with estrogen. Previously, we found that OA can induce tau hyperphosphorylation in vivo. In the present study, we found OA induced in vitro tau hyperphosphorylation, which was prevented by estrogen in dose-dependent manner. This preventing effect could be partially blocked by ICI 182,780, an estrogen receptor (ER) antagonist. Meanwhile, an OA induced ugregulation of cdk5 and inactive GSK3β (p-Ser 9) levels were also observed. 17β-estradiol was able to block this effect in a manner that was counteracted by ICI 182,780. Our results suggest that cdk5 may be involved in OA induced tau

hyperphosphorylation and estrogens can ameliorate the tau hyperphosphorylation via re-establishing the balance between tau kinases and phosphatases, which may be mediated by ER.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that causes progressive cognitive and behavior deterioration in the eldly (1, 2). One of the pathological hallmarks of AD is neurofibrillary tangles (NFTs) and its constituents, paired helical filaments (PHF), consisting mainly of hyperphosphorylated tau (3, 4). Clinical studies have shown that the severity of dementia in AD is closely correlated with the numbers of NFT. Although over 100 years' effort have been put into the study of AD, the pathology of AD still remain elusive. Several hypotheses have been proposed to explain the pathogenesis of AD. One of the hypotheses is tau hypothesis that tau pathology is the central event of AD pathogenesis (5).

Tau proteins are microtubule associated proteins that are abundant in neurons at central nervous system (6). Physiologically, tau plays a key role in microtubules stabilization, axonal transportation and neurite outgrowth (6-9). Pathologically, tau is abnormally heyperphosphorylated and its aggregation and deposition is found in many neurodegenerative disorders such as AD (8-12). Tau is a phosphoprotein whose expression and phosphorylation is well regulated (12-14). The longest human tau contains 441 residues, including 79 putative serine and threonine residues and 5 tyrosine residues located in two proline-rich regions (9). The phosphorylation of these residues affects the binding of tau to microtubules, leads to tau dysfunction and further result in

cell death (9, 15, 16). Although many protein kinases are able to phosphorylate tau in vitro (17-20), only a few are thought to be good candidates in vivo, such as GSK3β and cdk5, which are first isolated from bovine brain microtubules and named tau protein kinase I (TPK I) and TPK II (21-23). The dephosphorylation of phospho-tau is mainly mediated by protein phosphatases (PPs), among which PP2A is considered as the major phosphatase in vivo (24, 25). In selected areas of AD brain, both the expression and activity of PP2A have been reported to decline (26-29). It has been proposed that the imbalance between tau phosphorylation and dephosphorylation is critical to AD (30, 31). This disturbance could be either the increase of tau kinases activity, decrease of tau PPs activity, or both. Base on those studies, we propose that inhibithion of PP2A could lead to tau hyperphosphorylation.

Estrogen, a well known neuroprotectant, has been considered as a potential treatment for AD (32). AD is three times more prevalent in woman than man, and epidemiological studies have indicated that estrogen protects against AD (33). Clinical studies have shown that postmenopausal women with estrogen deficiency are at risk for neurodegenerative diseases (34) and postmenopausal estrogen therapy reduces the risk or delay the onset of AD (35). We previously found that estrogen may protect neuronal cells against oxidative stress and excitotoxicity by activating a combination of PPs, which play an important role in tau hyperphosphorylation (36-38). All the evidence suggests that

estrogen declining in the brain may be a contributor for AD and estrogen could be potential treatment for AD. However, the mechanisms of estrogen exerting neuroprotective effects still remain elusive.

In previous study, we found okadaic acid (OA), a PP1/2A inhibitor, induced tau hyperphosphorylation in vivo. In the present study, we investigated the effect of 17β-estradiol on OA induced tau phosphorylation in the (female) human neuroblastoma cell line (SH-SY5Y) and its possible mechanisms were also observed by using the ER antagonist, ICI 182,780.

Materials and methods

Materials

Okadaic acid (Cat #: 495604) was purchased from Calbiochem (Gibbstown, NJ) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 µM and diluted to appropriate concentration in artificial spinal-cerebral fluid. Anti-cdk5 (C-8), anti-tau (T1) and anti-p-ERK (E4) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK1/2 and anti-phospho-tau (Thr 205) antibody came from Invitrogen (Carlsbad, CA). Anti-GSK3β and GSK3β (p-Ser 9) antibody was purchased from Cell Signaling Technology (Danvers, MA). SH-SY5Y cell line was purchased from ATCC **(**Manassas, VA) and DME/F-12 media was obtained from Hyclone Laboratories, Inc. (Logan, Utah). Fulvestrant (ICI 182,780) and other reagents are from Sigma-Aldrich (St Louis, MO).

Cell culture and treatment

SH-SY5Y cells were grown in DME/F-12 medium supplemented with 10% charcoal-stripped FBS and penicillin/streptomycin (50 µg/ml) at 37°C in an atmosphere containing 5% CO_2 and 95% air. SH-SY5Y cells cultures were maintained at 50 and 100% confluency, respectively, in monolayers in plastic 75 cm^2 flasks.

OA was added into the media to a final concentration of 100 nM for three hours in the presence or absence of various concentrations of 17β-estradiol (10 nM, 100 nM, 1μM, 10 μM) to determine the dose-dependent effects of 17β-estradiol on the phospho-tau levels. For mechanism study, OA was added into the media to a final concentration of 100 nM for three hours in the presence or absence of 17β-estradiol (10 μM) and ICI 182,780 (1 μM), an ER antagonist.

Western blotting

For immunoblotting analysis with different antibodies, cells were harvested by scraping, washed in PBS, resuspended, then homogenized and sonicated in RIPA buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM sodium orthovanadate, 10mg/ml Aprotinin, 100 mg/ml Phenylmethyl Sulphonyl Fluoride (PMSF)). Lysates were then centrifuged at 12,000g for 10 min at 4 ºC, and supernatants were collected for analysis. The protein contents in the supernatants were determined by Bradford reagent assay. Protein from the treated cells was mixed with loading buffer, boiled for 5 minutes, separated by SDS-PAGE and then transferred to Immunobilon-P polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA) membrane. Membranes were blocked with 5% try milk in PBS. Proteins were probed with specific antibodies at proper dilutions according to the manufacturer's instruction and incubated overnight at 4

ºC. The blots were rinsed and applied with the appropriate secondary antibodies. After proper washing, the blots were developed with an enhanced chemiluminescent kit (Pierce, Rockford, IL). ECL results were digitized and quantified by using UVP (Upland, CA) Bioimaging System. All the blots were normalized and semi-qualified by beta actin, which was probed and detected on the same blots after stripping and re-blocking the membranes.

Statistics

The results were analyzed with one-way ANOVA with prism software (Graphpad Inc., San Diego, CA). The significance of differences among groups was determined by Tukey's multiple comparison tests. $p<0.05$ was considered significant for all the experiments. All results were expressed as mean ± SEM.

Results

Estrogen prevent OA induced tau phosphorylation in a dose-dependent manner

To examine the effect of OA on tau phosphorylation and the possible neuroprotective effects of estrogen, human neuroblastoma SH-SY5Y cells were treated with OA (100 nM) at the presence of various concentrations of 17β-estradiol (0, 10 nM, 100 nM, 1 μM, 10 μM). After three hours co-treatment, protein samples were collected for assessing tau phosphorylation by Western blot. As Figure 1 showed, OA (100 nM) induced a 3-fold increase in tau phosphorylation at a proline-directed site (Thr 205) ($p < 0.05$) in SH-SY5Y cells. And this OA induced tau phosphorylation was inhibited by 17β-estradiol in a dose-dependent manner and 10 μM of estradiol brought the phosphorylated tau down to control level ($p < 0.01$). Our data indicate that estrogen can prevent the tau phosphorylation induced by OA *in vitro*.

Estrogen prevent OA induced tau phosphorylation mediated by ER

According to the dose-dependent response of 17β-estradiol on tau phosphorylation, we treated SH-SY5Y cells with 10 μM of 17 β-estradiol at the presence or absence of OA (100 nM) and ICI 182,780 (1μM) for three hours. As Figure 2A showed, OA induced tau phosphorylation by over 5-folds. 17β-estradiol alone didn't affect the tau phosphorylation ($p > 0.05$), but it significantly prevented the OA induced tau

phosphorylation ($p < 0.05$) (Figure 2A). ICI 182,780 alone inhibited the OA induced tau phosphorylation. Co-treatment with estradiol, ICI 182,780 blocked part of the effect of estradiol ($p < 0.05$) (Figure 2A). Although there was slight increase of total tau (T1) induced by treatment either alone or combination of OA, 17β-estradiol, ICI 182,780, there were no significant changes among groups ($p > 0.05$) (Figure 2B). These data suggest that estrogen preventing tau phosphorylation may be ER-mediated effect.

Cdk5 involved in tau phosphoylation preventing effect of estrogen

In order to investigate the possible mechanisms of estrogen preventing OA induced tau phosphorylation, SH-SY5Y cells were treated with OA (100 nM) in the presence or absence of 17 β-estradiol (10 μM) and ICI 182,780 (1μM). After three hours exposure, protein samples were collected for assessing cdk5 levels by Western blot. As Figure 3 showed, OA alone increased cdk5 ($p < 0.05$) but not 17β-estradiol or ICI 182,780 alone. The OA induced cdk5 increasing was prevented by co-treatment with 17β-estradiol ($p < 0.01$), but not ICI 182,780 ($p > 0.05$), and this preventing effect of 17β-estradiol was partially blocked by ICI 182,780 ($p < 0.05$) (Figure 3). This suggested that cdk5 may be involved in both OA induced tau phosphorylation, and the tau phosphorylation preventing effect of estrogen.

Estrogen attenuated GSK3β inactivation induced by OA

We further investigated whether GSK3β was also involved in the effect of estrogen preventing OA induced tau phosphorylation, SH-SY5Y cells were treated with OA (100 nM) in the presence or absence of 17 β-estradiol (10 μM) and ICI 182,780 (1μM) for three hours and protein samples were collected for assessing GSK3β levels by Western blot. As Figure 4 showed, OA alone increased GSK3β (p-Ser 9), which is inactive status of the enzyme ($p < 0.05$) but not 17β-estradiol or ICI 182,780 alone. The OA induced GSK3β inactivation was prevented by co-treatment with 17β-estradiol ($p < 0.05$), but not ICI 182,780 ($p > 0.05$), and this preventing effect of 17β-estradiol was partially blocked by ICI 182,780 ($p < 0.01$) (Figure 4). The results showed that GSK3β was inhibited by OA and may be not involved in OA induced tau phosphorylation, and estrogen was able to inhibit the phosphorylation and inactivation of GSK3β which may be ER mediated effect.

ERK1/2 not involved in the effect of estrogen preventing tau phosphorylation induced by OA

To determine whether another proline-directed tau kinase, ERK1/2, was also involved in the effect of estrogen preventing OA induced tau phosphorylation, SH-SY5Y cells were treated with OA (100 nM) at the presence or absence of 17 β-estradiol (10 μM) and

ICI 182,780 (1μM) for three hours as above and the ratio of p-ERK/total ERK was detect via Western blot. As Figure 5 showed, OA induced a slight increasing of p-ERK, but no significance was found ($p > 0.05$), neither did 17β-estradiol or ICI 182,780 alone. The results showed that ERK1/2 may be not involved in OA induced tau phosphorylation.

Discussion

In the present study, we showed that OA induces tau phosphorylation at the proline-directed site (Thr 205) in SH-SY5Y neuroblastoma cells, which is consistent with our previous *in vivo* study that OA dorsal hippocampal infusion induces tau phosphorylation at the proline-directed site (Thr 205) in the hippocampus and cortex. Tau abnormally hyperphosphorylation has been associated with the decreased stability of microtubules and accumulation as tangles of PHFs in neurons undergoing degeneration (3, 39). The microtubules binding ability of tau is post-translationally regulated by serine/threonine-directed phosphorylation, which is considered to be the main mechanism that regulates the affinity of tau to the microtubules (39, 40). Responding to phosphorylation and dephosphorylation, the cycles of binding and detachment of tau from microtubules may affect axonal transportation (6-9). This equilibrium is decided by the phosphorylation state of tau, which is further controlled by the actions of kinases and phosphatases (41). Studies from Alvarez-de-la-Rosa *et al*. also showed that OA induced hyperphosphorylation of tau in non-proline-directed site (Ser 262) (42). However, we didn't find any effect of OA on the levels of non-phosphorylated tau, which is opposite to the study of Alvarez-de-la-Rosa (42). This could be explained as either induced expression of total tau, the action of other phosphatases (24) other than PP1/2A which are inhibited by OA (43), or both. *In vitro,* OA have been shown to induce phosphorylation

of tau at different sites, including Ser 396/404 (44), Ser 202/205 (45) and Ser 262 (42). We previously found that OA dorsal hippocampal infusion can induce tau phosphorylation and cognitive deficits similar to those seen in AD, which are also seen in other *in vivo* studies (3, 46).

We found that the OA induced tau phosphorylation at proline-directed site (Thr 205) in SH-SY5Y cells can be prevented by 17β-estradiol in a dose-dependent manner. Other study also showed that estradiol can prevent OA induced tau phosphorylation at non-proline-directed site (Ser 262) of the molecule (42). Clinical studies have shown that the decline of estrogen levels in postmenopausal women is a risk factor for neurodegenerative disease and this risk could be reduced by estrogen therapy (34, 35). Meanwhile, estrogen is also able to improve cognitive function during aging and estrogen therapy is reported to prevent the decline of cognitive function due to menopause (47-49). In addition, a growing body of evidence supports the neuroprotective effects of estrogens. For example, estrogen has shown protective effects against different insults such as serum deprivation (50-52), Aβ toxicity (53, 54), glutamate excitotoxicity (55, 56), hydrogen peroxide (H2O2) (57) and mitochondria toxins (58, 59) in both *in vitro* and *in vivo* studies. The neuroprotective effects of estrogens have also been described in a variety of cerebral ischemia models, including middle cerebral artery occlusion (MCAO), global forebrain ischemia models, photothrombotic focal ischemia models, and glutamate

induced focal cerebral ischemia models (60-63). Collectively, these results indicate that estrogens could be valuable candidates for brain protection.

Our study showed that the preventing effect of estrogen against OA induced tau phosphorylation at proline-directed site (Thr 205) can be partially blocked by ICI 182,780, an ER antagonist. Other study also showed that ER antagonist can block the effect of estrogen that prevents tau phosphorylation induced by OA at proline-directed site (Ser 262) (42). The partial blockade of 17β-estradiol's effect on tau phosphorylation by ER antagonist indicates that estrogen's effect on tau phosphorylation may be a receptor-mediated effect. It is reported that there are both ERα and ERβ expression in SH-SY5Y cell line (64). Except for the direct role as an antioxidant (65, 66), estrogens are known to exert their actions through members of the nuclear hormone receptor superfamily, ERα and ERβ (67-69). By binding to these receptors, estrogen promotes receptor dimerization and translocation to the nucleus, which will lead to the regulation of transcription of certain genes (32, 70). Although ICI 182,780 has been considered to bind to ER receptor as a pure ER antagonist, the actual mechanisms of ICI 182,780 remains poorly understood. It was reported that ICI 182,780, at very high concentration, is able to induce ER dimerization and ER-dependent transcription (71), which could probably explain why only partial blockade of estrogen effect on tau phosphorylation by ICI 182,780 was seen in our study and ICI 182,780 also prevented tau phosphorylation.

Another explanation could be due to the high concentration of estrogen which exerts its protective effect via a non-receptor pathway masking the receptor-mediated effect.

Our data showed that ER is at least partially involved in estrogen prevented OA induced tau phosphorylation. However, the post-receptor events and the mechanisms of estrogen on tau phosphorylation inhibition are not clear. Due to the fact that tau is phosphorylated by certain protein kinases, we investigated the effect of estrogen with or without the presence of OA on certain in vivo candidates of tau kinases, including GSK3β, cdk5 and MAPK. We found that OA increased GSK3β (p-Ser 9) and cdk5 levels which were prevented by estrogen and this effect of estrogen could be partially blocked by ICI 182,780. Our data indicates that the regulation of tau kinases could be one of the post-receptor events of estrogen preventing tau phosphorylation. The imbalance between tau phosphorylation mediated by tau kinases and dephosphorylation mediated by PPs is critical to AD (30, 31). Besides inhibiting PP1/2A, OA was also reported to activate calpain (72), which cleaves p35 to releases p25 (73). Conversion of p35 to p25 causes prolonged activation and mislocalization of cdk5, which leads to aberrant tau hyperphosphorylation (73). This is consistent with our data showing that OA increased cdk5 levels in SH-SY5Y cells. The activation of GSK3β is mediated by dephosphorylation at Ser 9 which is regulated by Akt (74). PP2A can activate GSK3β directly by dephosphorylation at Ser 9, or indirectly by dephosphorylating Akt (75).

Inhibition of PP2A by OA led to inactivation of GSK3β by increasing GSK3β (p-Ser 9), which is consistent with our data. However, no significant changes of p-ERK/ERK ratio were found in SH-SY5Y cells with the insult of OA. Moreover, all the changes of these proline-directed kinases induced by OA were found to be prevented via co-treatment with estrogen which effect was blocked by ICI 182,780. Our data indicates that OA can induce the imbalance between tau kinases and phosphatases and the balance could be reestablished by estrogen in a receptor-mediated manner.

Collectively, the balance between tau kinases and phosphatases is important for tau phosphorylation. Our data indicate that cdk5 may be involved in OA induced tau phosphorylation via inhibition of PP2A and the preventing effect of estrogen on tau phosphorylation induced by OA could be an ER-mediated neuroprotective effect.

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Figure legends

Figure 1. The dose-dependent effects of 17β-estradiol on tau phosphorylation in OA treated SH-SY5Y cells.

Human neuroblastoma SH-SY5Y cells were co-treated with OA (100 nM) and series of concentrations of 17β-estradiol (10 nM, 100 nM, 1 μM, 10 μM) for three hours. Lysates from different groups were subject to western blot for assessing phospho-tau (Thr 205). Control was treated with 0.1% DMSO. OA was dissolved in 0.1% DMSO. E2: 17β-estradiol. All data were normalized to percentage of control. Data were represented in mean ± S.E.M. for n=4. ## <0.01 compared with control and * <0.05, ** <0.01 compared with OA only.

Figure 2. The effect of 17β-estradiol on preventing tau phosphorylation in OA treated SH-SY5Y cells.

Human neuroblastoma SH-SY5Y cells were treated with OA (100 nM) at the presence of absence of 17β-estradiol (E2, 10 μM) and ICI 182,780 (1 μM) for three hours. Lysates from different groups were subject to western blot for assessing phospho-tau (Thr 205) (A) and non-phosphorylated tau (T1) (B). Control was treated with 0.1% DMSO as vehicle. OA was dissolved in 0.1% DMSO. All data were normalized to percentage of

control. Data were represented in mean ± S.E.M. for n=4. ## <0.01 compared with control and ** <0.01 compared with OA only.

Figure 3. cdk5 involvement in OA induced tau phosphorylation in SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells were treated with OA (100 nM) at the presence of absence of 17β-estradiol (E2, 10 μM) and ICI 182,780 (1 μM) for three hours and lysates from different groups were subject to western blot for assessing cdk5. Control was treated with 0.1% DMSO as vehicle. OA was dissolved in 0.1% DMSO. All data were normalized to percentage of control. Data were represented in mean ± S.E.M. for n=5. # means $p<0.05$ compared with control and * <0.05, ** <0.01 compared with OA only.

Figure 4. GSK3β is not involved in OA induced tau phosphorylation in SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells were treated with OA (100 nM) at the presence of absence of 17β-estradiol (E2, 10 μM) and ICI 182,780 (1 μM) for three hours and lysates from different groups were subject to western blot for assessing total GSK3β and p- GSK3β (Ser 9). Control was treated with 0.1% DMSO as vehicle. OA was dissolved in 0.1% DMSO. All data were normalized to percentage of control. Data were represented

in mean ± S.E.M. for n=4. # <0.05 compared with control and * <0.05 compared with OA only.

Figure 5. ERK1/2 is not involved in OA induced tau phosphorylation in SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells were treated with OA (100 nM) at the presence of absence of 17β-estradiol (E2, 10 μM) and ICI 182,780 (1 μM) for three hours and lysates from different groups were subject to western blot for assessing total ERK1/2 and p-ERK1/2. Control was treated with 0.1% DMSO as vehicle. OA was dissolved in 0.1% DMSO. All data were normalized to percentage of control. Data were represented in mean ± S.E.M. for n=4.

Figure 1.

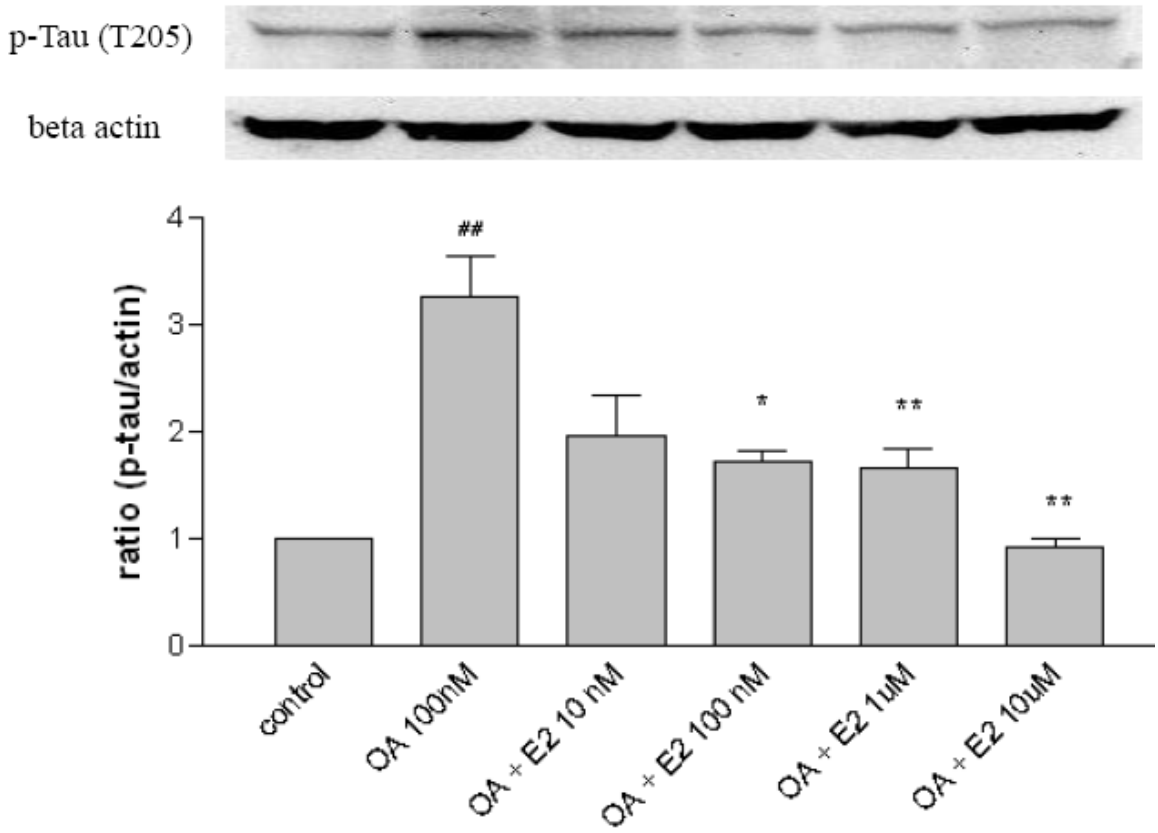

p-Tau (T205)
beta actin
ratio (p-tau/actin)
0
1
2
3
4
##
*
**
**
control
OA 100nM
OA + E2 10 nM
OA + E2 100 nM
OA + E2 1uM
OA + E2 10uM

Figure 2.

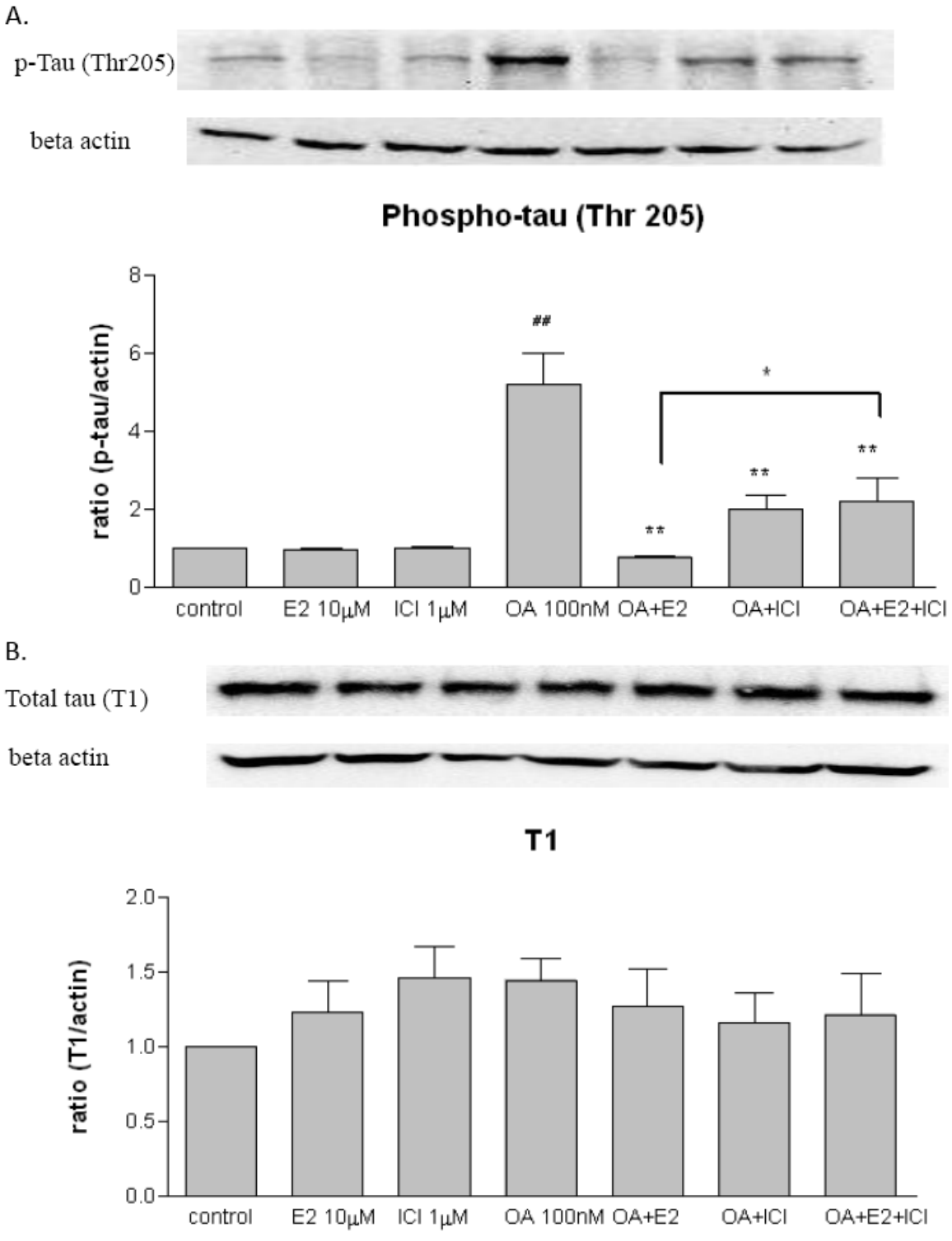

A.
p-Tau (Thr205)
beta actin
Phospho-tau (Thr 205)
ratio (p-tau/actin)
8
6
4
2
0
##
*
**
**
**
control
E2 10μM
ICI 1μM
OA 100nM
OA+E2
OA+ICI
OA+E2+ICI
B.
Total tau (T1)
beta actin
T1
ratio (T1/actin)
2.0
1.5
1.0
0.5
0.0
control
E2 10μM
ICI 1μM
OA 100nM
OA+E2
OA+ICI
OA+E2+ICI

Figure 3.

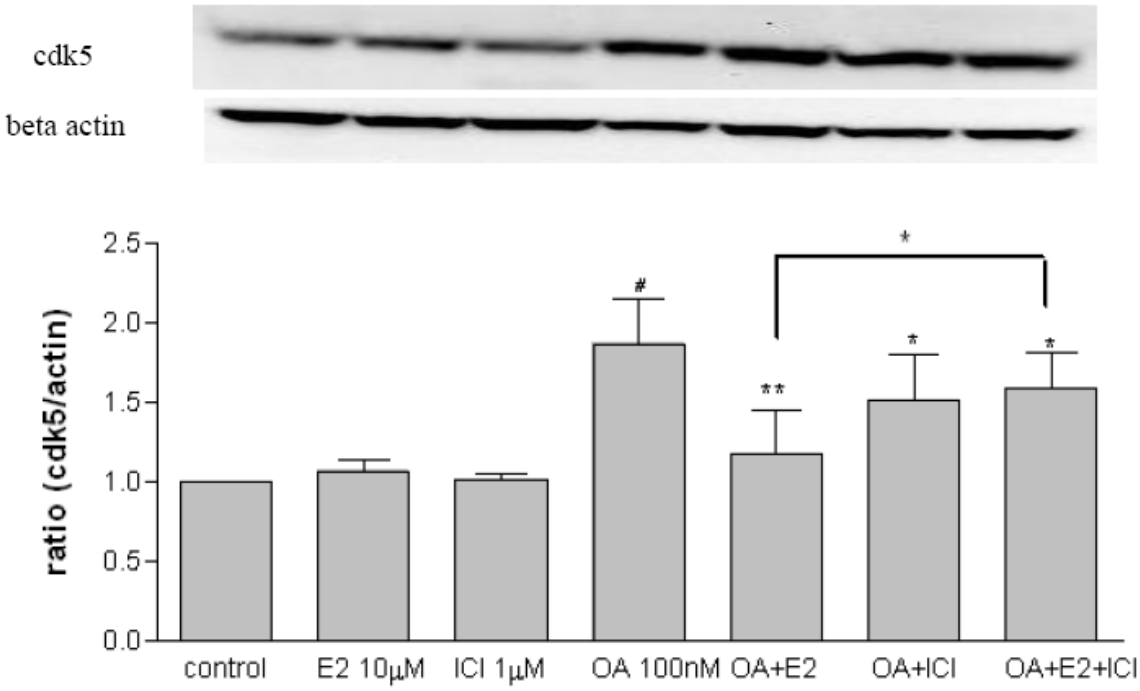

cdk5
beta actin
ratio (cdk5/actin)
2.5
2.0
1.5
1.0
0.5
0.0
#
*
**
*
*
control
E2 10μM
ICI 1μM
OA 100nM
OA+E2
OA+ICI
OA+E2+ICI

Figure 4.

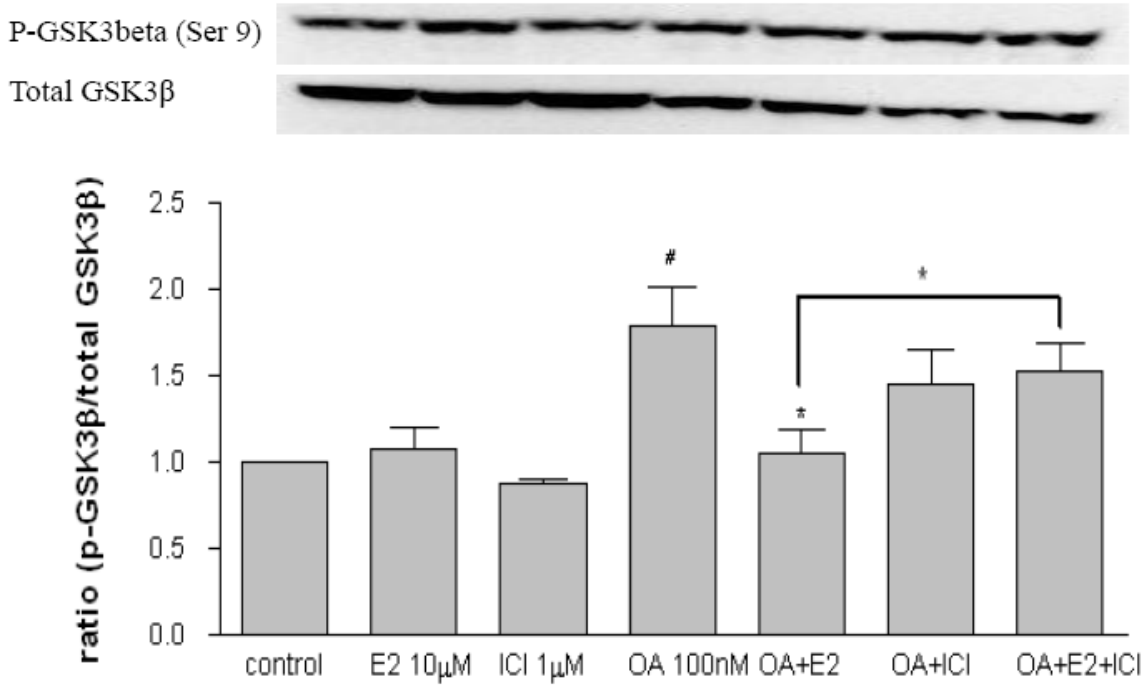
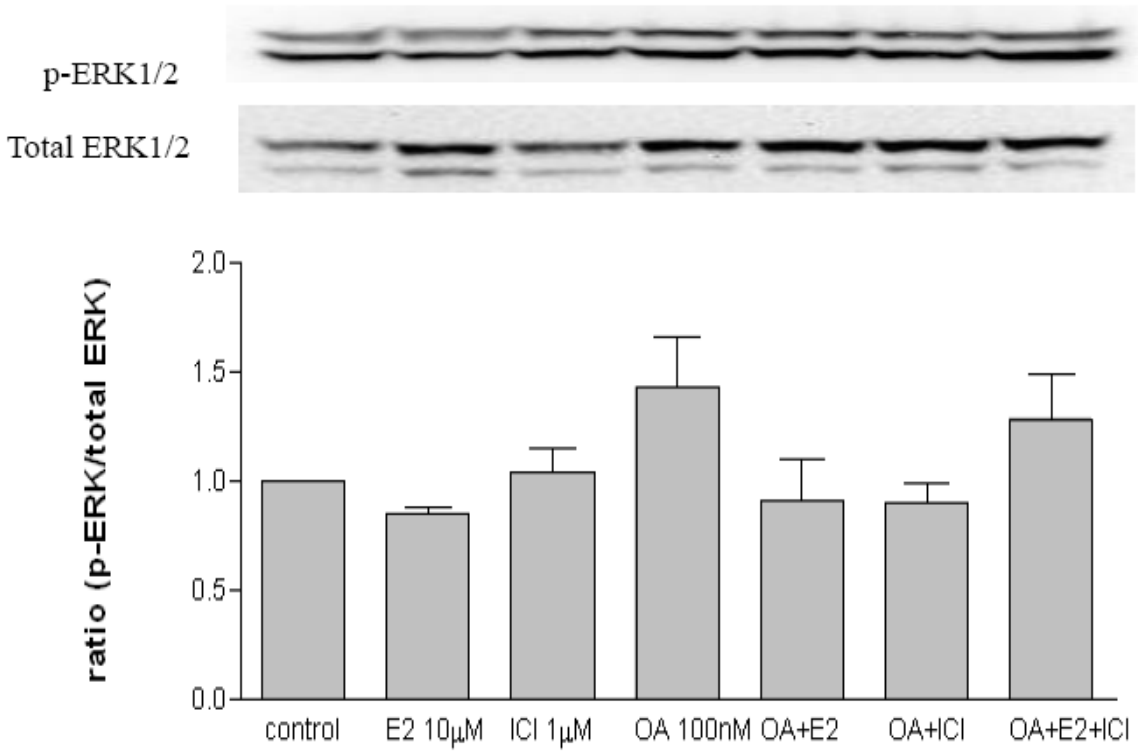

P-GSK3beta (Ser 9)
Total GSK3β
ratio (p-GSK3β/total GSK3β)
2.5
2.0
1.5
1.0
0.5
0.0
#
*
*
control
E2 10μM
ICI 1μM
OA 100nM
OA+E2
OA+ICI
OA+E2+ICI

Figure 5.


p-ERK1/2
Total ERK1/2
ratio (p-ERK/total ERK)
2.0
1.5
1.0
0.5
0.0
control
E2 10μM
ICI 1μM
OA 100nM
OA+E2
OA+ICI
OA+E2+ICI

Chapter 5: Summary and future directions

Summary

The current dissertation included two parts in the study of okadaic acid induced Alzheimer's model and the neuroprotective effects of estrogen. The first part was to characterize the experimental Alzheimer-like tauopathy model induced by okadaic acid *in vivo*; and the second part was to investigate the effect and potential mechanisms of estrogen on tauopathy induced by okadaic acid *in vitro*.

In the first part of this discussion, we observed no changes of the body weights in adult female ovariectomized SD rats that received microinfusion of okadaic acid into the dorsal hippocampus unilaterally; we found a spatial learning and memory deficits in okadaic acid infusion rats by testing the behavior performance (including Morris Water Maze, visible MWM and Rotarod tests); by using western blot, we also detected the tau phosphorylation in both cortex and hippocampus after okadaic acid infusion with a specific antibody, which recognizes a phosphorylation site at Thr 205 on tau protein; we also observed NFTs-like comformational changes in okadaic acid infusion brain by using Bielschowsky's silver staining and immunohistochemistry (probing with anti-p-tau Thr 205). Theses results may help to establish an *in vivo* model for tau phosphorylation and AD-like cognitive deficiency.

The pathology of AD tauopathy is still unclear. NFTs, composed of the intracellular aggregates of hyperphosphorylated tau protein, are another pathological hallmark of AD besides senile plaque. Based on the study of these AD hallmarks, two major competing hypotheses have been proposed: amyloid hypothesis and tau hypothesis. The latter has been supported by clinical studies showing the severity of dementia closely correlated with the numbers of NFTs rather than the degree of plaques. The present study supported the hypothesis that okadaic infusion can induce tau hyperphosphorylation as well as cognitive deficiency.

It is still a mystery how the tau protein is hyperphosphorylated and how NFTs is formed *in vivo*. In the present study, we proposed that an imbalance between tau protein phosphatases and kinases is essential to tau hyperphosphorylation. We investigated the levels of tau protein phosphatases PP1/2A and certain tau protein kinases, including cdk5, GSK3β and MAPK, after okadaic acid microinfusion into unilateral dorsal hippocampus of rat. We found that phosphorylated tau was significantly increased; and cdk5 was also increased in the brain after okadaic acid infusion. We also observed an increase of GSK3β (p-Ser 9), which is inactive status of GSK3β, and no significant changes of MAPK after okadaic acid infusion. It suggested that the inhibition of tau protein phosphatases by okadaic acid is sufficient to induce tau phosphorylation and cdk5 may be involved in this process. These data supported the hypothesis that imbalance between the

tau protein kinases and phosphatases is essential to tauopathy. Further study with Bielschowsky's silver staining and immunohistochemistry showed that NFTs-like pathological changes were formed in the okadaic acid infusion brain.

Based on the *in vivo* study, we identified aberrant tau phosphorylation, NFTs-like comformational changes and cognitive deficits from okadaic acid dorsal hippocampal infusion rats. There is still unsufficient evidence that okadaic acid induced NFTs are identical to those in AD patients. Although rat tau protein is known different from human tau protein, they share many identical characterizations, such as the specific phosphorylated sites. The okadaic acid infusion model may be a useful non-transgenic animal model for understanding the pathology of tauopathy and pre-clinical testing of drugs.

In the second part of dissertation, we observed the protective effects of estrogen on tau hyperphosphorylation induced by okadaic acid *in vitro*. We found that estrogen can block okadaic acid induced tau hyperphosphorylation in human neuroblastoma SH-SY5Y cells in a manner that was counteracted by estrogen receptor antagonist, ICI 182,780. We also observed that cdk5 and GSK3β (p-Ser 9) was induced by okadaic acid and estrogen can prevent this event in a manner of counteraction by ICI 182,780.

Estrogen is a well-known neuroprotectant and has been consider as a potential treatment for AD. We previous has found that estrogen can protect neuronal cells from a

variety of insults, such as serum deprivation, Aβ-induced toxicity, glutamate-induced excitotoxicity, mitochondria toxins and hydrogen peroxide. In the present study, we showed that estrogen prevents tau phosphorylation induced by okadaic acid in an estrogen-receptor mediated manner. Although the mechanisms of estrogen exerting neruoprotective effects still remain unclear, cdk5 may be a downstream target of estrogen in the prevention of tau hyperphosphorylation.

Collectively, our study have provided evidence that the inhibition of phosphatases can induce tau phosphorylation and NFTs-like comformational change which could be a useful animal model for tauopathy, estrogen can prevent the okadaic acid-induced tauopathy which are mediated by estrogen receptor and cdk5 may be one of the downstream target of estrogen.

Future direction

It is crucial to develop useful models to study the pathology of AD for pre-clinical testing of drugs. Our model has been able mimic some of symtoms of AD especially in tauopathy. However, much more studies need to be done to claim this model to be a good AD model. First of all, AD is an aging disease. Our model is induced by dorsal hippocampal microinfusion of okadaic acid, which inhibits the tau phosphatases and induces a hippocampal lesion as well. The next step should do is to lower the dose of okadaic acid and prolong the duration of infusion, which may rule out the influence of chemical lesion issue induced by okadaic acid.

The mechanisms of estrogen exerting neuroprotective effects remain elusive. Mounting evidence indicates that the deregulation of cdk5 is involved in many pathological events in neurodegenerative disease. But the role of cdk5 in okadaic acid induced tau phosphorylation is not clear. The cdk5 blocker should be tested if it can prevent the okadaic acid induced tau phosphorylation, and its effect should be compared with that of estrogen to rule out other existing pathways besides cdk5 pathway.