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Ovarian hormones ameliorate memory impairment, cholinergic deficit, neuronal apoptosis and astrogliosis in a rat model of Alzheimer's disease

ZHIYING HU[1], YANG YANG[2], KEQIANG GAO[2], JOHN A. RUDD[3] and MARONG FANG[2]

[1]Department of Obstetrics and Gynecology, Hangzhou Red Cross Hospital; [2]Institute of Neuroscience, Zhejiang University School of Medicine, Hangzhou, Zhejiang; [3]School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, SAR, P.R. China

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Abstract. Ovarian hormones, including progesterone (P4) and 17 β-estradiol (E2), have been shown to affect memory functions; however, the underlying mechanism whereby ovarian hormone replacement therapy may decrease the risk of Alzheimer's disease (AD) is currently unclear. The present study aimed to investigate the effects of P4 and E2 on spatial and learning memory in an ovariectomized rat model of AD. β-amyloid (Aβ) or saline were stereotaxically injected into the hippocampus of the rats and, after 1 day, ovariectomy or sham operations were performed. Subsequently, the rats were treated with P4 alone, E2 alone, or a combination of P4 and E2. Treatment with E2 and/or P4 was shown to improve the learning and memory functions of the rats, as demonstrated by the Morris water maze test. In addition, treatment with E2 and P4 was associated with increased expression levels of choline acetyltransferase and 5-hydroxytryptamine receptor 2A (5-HT2A), and decreased expression levels of the glial fibrillary acidic protein in the hippocampus of the rats. Furthermore, E2 and P4 treatment significantly attenuated neuronal cell apoptosis, as demonstrated by terminal deoxynucleotidyl transferase dUTP nick end labeling assays; thus suggesting that the ovarian hormones were able to protect against Aβ-induced neuronal cell toxicity. The results of the present study suggested that the neuroprotective effects of P4 and E2 were associated with amelioration of the cholinergic deficit, suppression of apoptotic signals and astrogliosis, and upregulation of 5-HT2A expression levels. Therefore, hormone replacement therapy may be considered an effective strategy for the treatment of patients with cognitive disorders and neurodegenerative diseases.

Correspondence to: Professor Marong Fang, Institute of Neuroscience, Zhejiang University School of Medicine, 866 Yuhangtang Road, Hangzhou, Zhejiang 310058, P.R. China
E-mail: fangmaro@zju.edu.cn

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Introduction

Alzheimer's disease (AD) is an age-associated, progressive neurodegenerative disorder, which is characterized by memory impairment and cognitive decline (1). Pathologically, AD is characterized by an abnormal deposition of β-amyloid (Aβ) protein, which initiates a series of events, including the formation of senile plaques, neurofibrillary tangles and neuropil threads, and glial cell activation and neuronal cell loss (2-4). In addition, the number of cholinergic neurons, and the levels of acetylcholine, has been shown to decrease with concurrent decreases in the levels of the enzyme choline acetyltransferase (ChAT) (5). These alterations have previously been suggested to occur due to dysfunctions in the serotonergic regulation of cholinergic neuronal cell activity (5).

Ovarian reproductive hormones are potent regulators of neuronal cell survival in the central nervous system (CNS) and of various biological processes, including development and neural injury (6-7). In particular, 17 β-estradiol (E2) and progesterone (P4) have been demonstrated to exert effects on cognitive functions (5,7-9), and P4 was reported to have promoted neuron survival by protecting neuronal cells against damage following brain injury (10). However, controversy exists regarding the beneficial effects of ovarian hormones on cognition during aging (11,12). Previous studies have suggested that hormone treatment may enhance memory in menopausal women; however, neither beneficial nor detrimental effects have been detected in other studies (12,13).

The present study aimed to investigate the mechanisms underlying the effects of ovarian hormones on learning and memory in a rat model of AD. The rat model was induced via central injection of aggregated Aβ1-40, which has been used in previous *in vivo* studies (1,2). An ovariectomized (OVX) rat model of AD was established in order to eliminate complications associated with endogenously produced E2 (14,15). The Morris water maze test (16) was used in order to assess the effects of E2 and P4 on spatial learning and memory functions in the rat model of AD. In addition, the apoptosis of neuronal cells and the protein expression levels of 5-hydroxytryptamine (5-H2, serotonin) receptor 2A (5-HT2A), glial fibrillary acidic protein (GFAP) and ChAT, were assessed

in the hippocampus of the rats, in order to establish whether improvements in memory were associated with neuroprotective mechanisms.

Materials and methods

Rats and agents. A total of 40 female Sprague-Dawley rats, aged 10 weeks and weighing, 250-300 g, were used (Zhejiang University Laboratory Animal Breeding and Research Center, Hangzhou, China). The rats were maintained under a 12 h light/dark cycle, with *ad libitum* access to food and water. Experiments were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (1), and with approval from the Animal Subjects Committee at Zhejiang University (Zhejiang, China). Aβ1-40 protein, which was purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in 0.9% (w/v) saline (final concentration, 5 mg/ml) and incubated at 37˚C for 1 week in order to induce aggregation (1,2).

Surgery and treatment with agents. Rats were anesthetized using an intraperitoneal injection of 40 mg/kg Nembutal (Merck Millipore, Darmstadt, Germany), and placed into a stereotaxic frame (Stoelting, Co., Wood Dale, IL, USA). A sham-operated group (n=8) received a bilateral injection (coordinates: -3.0 mm anteroposterior, -2.0 mm lateral to the bregma, and -3.3 mm ventral to the skull surface) of saline (2 µl, over 5 min), whereas ovariectomized rats (n=8) received 1 µl aggregated Aβ1-40 (10 µg/µl, over 5 min) into the hippocampus (17). After 1 day, the rats were anaesthetized using halothane (3% induction followed by 1% maintenance) (Sigma-Aldrich), an abdominal incision was made through the skin of the flank of the rats, and the left and right ovaries were isolated by ligation of the most proximal portion of the oviduct. Both ovaries and the ovarian fat were removed in ovariectomized rats. The abdominal incision was performed on the sham rats; however, the wound was closed without removal of the ovaries.

Four days following surgery, the Aβ1-40 aggregate-injected ovariectomized animals were randomly assigned into four groups (n=8/group): i) OVX + vehicle (vehicle-treated), ii) OVX + E2 (E2-treated), iii) OVX + P4 (P4-treated), and iv) OVX + E2 + P4 (P4 + E2-treated). The E2-treated group received daily subcutaneous injections of E2 (1 mg/kg) for 25 days (15). The P4-treated group received a total of four subcutaneous injections of P4 (5 mg/kg), every 7 days. The P4 + E2-treated group received both treatments, and the vehicle group received daily subcutaneous injections of sterile sesame oil for 25 days.

Morris water maze test. The effects of the ovarian hormones on the spatial cognitive performance of the AD rats was investigated using the Morris water maze test (16), in which the time taken for the rats to locate a submerged platform in a water maze (the escape latency) was used as an indication of spatial memory. In addition, alterations to the escape latency over consecutive days were used to assess learning with respect to long-term memory. Briefly, a circular polyester tank (diameter, 140 cm; height, 50 cm) was filled with water to a depth of 30 cm and maintained at 20±1˚C. The maze was divided geographically into four equal quadrants and release points in each quadrant were designated as North, East, South, or West. An escape platform measuring 15x15 cm was submerged 2 cm below the water surface in the center of one quadrant of the pool throughout the training and memory trials. Testing began 3 weeks after the rats were treated with Aβ1-40 or vehicle. All rats underwent initial training sessions consisting of 6 trials per day for 4 consecutive days. In each trial, the rats were placed at one of the four starting points and allowed to swim freely until they located the platform. The four starting points were ordered in a random manner between trials. Probe tasks were performed on the 5th day following training. The rats were allowed to swim for 120 sec to locate the platform, on which they were allowed to rest for 20 sec. Those rats unable to locate the platform were guided to it. Swim paths were recorded using a digital video camera (STC-TB33USB-AS; Sentech Co., Ltd., Japan) and analyzed using EthoVision 3.1 software (Noldus Information Technology, Leesburg, VA, USA).

Tissue preparation. Four rats per group were given a lethal dose of Nembutal (60 mg/kg body weight) 30 days post-Aβ1-40 injection. Following sacrifice, the rats were perfused intracardially with saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Subsequently, whole brains were carefully removed and dissected. Brain tissue was post-fixed at 4˚C in 4% paraformaldehyde overnight and then incubated with 30% (w/v) sucrose in phosphate-buffered saline (PBS) until the tissue dropped to the bottom of the container. Each brain was dissected into five sets of serial coronal sections through the hippocampal area using a cryostat at -20˚C. Each fifth section was collected for free floating immunohistochemical labeling and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Tissue sections (20 µm) were mounted onto gel-coated slides, upon which coverslips were mounted using Permount™ (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

An additional four rats per group were sacrificed by decapitation, after which the brains were carefully harvested, the hippocampus was dissected, and was immediately frozen in liquid nitrogen for western blotting.

Immunohistochemical labeling. All labeling experiments were performed in 24-well culture plates at room temperature. The sections were incubated in 3% H_2O_2/10% methanol/0.01 M PBS for 5 min in order to quench endogenous peroxidase, and were then blocked in 5% normal goat serum supplemented with 0.01 M PBS. Subsequently, the tissue sections were incubated with polyclonal rabbit primary anti-ChAT (1:250; AB144P; Chemicon International, Inc., Temecula, CA, USA), anti-5HT2A (1:500; sc-15073; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-GFAP (1:200; 18-0063; Thermo Fisher Scientific, Inc.) and anti-caspase-3 (1:500; 160745; Cayman Chemical, Ann Arbor, MI, USA) overnight at 4˚C. After a thorough wash in PBS, the sections were incubated with biotinylated goat anti-rabbit immunoglobulin (Ig) G (1:400; Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h at room temperature, followed by incubation with an avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; 1:300; Vector Laboratories, Inc.) for 1.5 h at room temperature. The reaction product

was developed with 0.025% 3,3-diaminobenzidine (DAB) and 0.0033% H_2O_2 in 0.2 M Tris-HCl (pH 7.6), after which the reaction was quenched by rinsing the slides with 0.2 M Tris-HCl.

The ChAT, 5HT2A and caspase-3 labeled sections were counterstained with hematoxylin, and then mounted onto 0.02% poly-L-lysine-coated slides and allowed to dry at room temperature. Subsequently, the tissue sections were dehydrated using a graded series of alcohol, cleared in xylene and cover-slipped. Primary antibody omission controls were used in order to confirm the specificity of the immunohistochemical labeling. Five sections from each animal were selected at random and images were captured under 200x using a Nikon E600 microscope (Nikon Corporation) magnification in three visual fields/per section (Olympus Corporation, Tokyo, Japan). The ChAT, 5HT2A, GFAP and caspase-3 immunoreactive cells in each hippocampal region were counted.

Western blot analysis. Prior to total protein extraction, the brain tissue was homogenized in homogenization buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 1 ml ice-cold radioimmunoprecipitation assay buffer (EDTA-free; both Thermo Fisher Scientific, Inc.) 1 mg/ml pepstatin A, 1 mg/ml aprotinin and 1 mg/ml leupeptin (Roche Diagnostics, Basel, Switzerland), using a Polytron® PTA 10S homogenizer (Kinematica, Inc., CA, USA). Protein concentrations were determined using the Bradford Protein assay (Beyotime Institute of Biotechnology, Haimen, China). Proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) at 70 V for 1.5 h at 4˚C using Bio-Rad TransBlot® Turbo™ apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, the membranes were blocked for 1 h at room temperature with 5% (w/v) nonfat milk in Tris-buffered saline (TBS) supplemented with 0.05% (v/v) Tween 20 (50 mM Tris base, 200 mM NaCl, 0.05% Tween 20), and then incubated for 12 h with polyclonal rabbit anti-ChAT (1:500; Chemicon International, Inc.), anti-5HT2A (1:800; Santa Cruz Biotechnology, Inc.), and anti-GFAP (1:500; Thermo Fisher Scientific, Inc.) primary antibodies. The membranes were then washed three times for 10 min using TBS supplemented with 0.05% Tween 20, and subsequently incubated for 1 h with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology, Inc.), prior to further washing. Proteins were detected using enhanced chemiluminescence (ECL reagents; GE Healthcare Life Sciences, Chalfont, UK) and exposed to radiographic film (Hyperfilm ECL; GE Healthcare Life Sciences), according to the manufacturer's protocol.

The densitometry of the immunoreactive bands of interest was determined via digital images of the radiographic film using Quantity One 1-D software (Bio-Rad Laboratories, Inc.). In order to normalize protein bands to a gel loading control, the membranes were washed in TBS-Tween 20 and probed with rabbit anti-β-actin (1:5,000; Abcam, Cambridge, MA, USA), followed by incubation with HRP-conjugated goat anti-rabbit (1:5,000; Santa Cruz Biotechnology, Inc.) and ECL detection. For the negative control, the primary antibody was omitted.

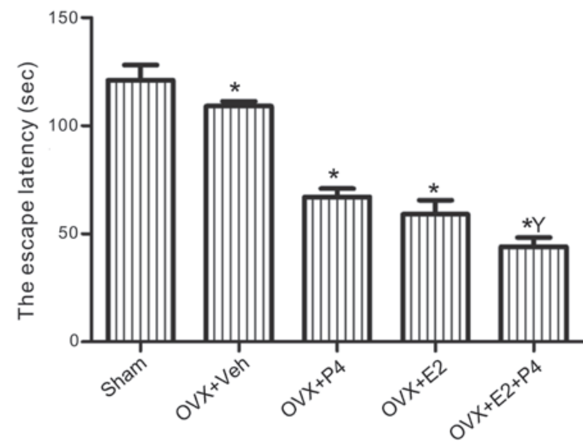

Figure 1. Effects of E2 and/or P4 on the escape latencies of rats in the Morris water maze test. Data are presented as the mean ± standard deviation. *P<0.01 vs. the vehicle-treated rats; YP<0.01 vs. the P4-treated rats. E2, 17-β-estradiol; P4, progesterone; OVX, ovariectomized; veh, vehicle.

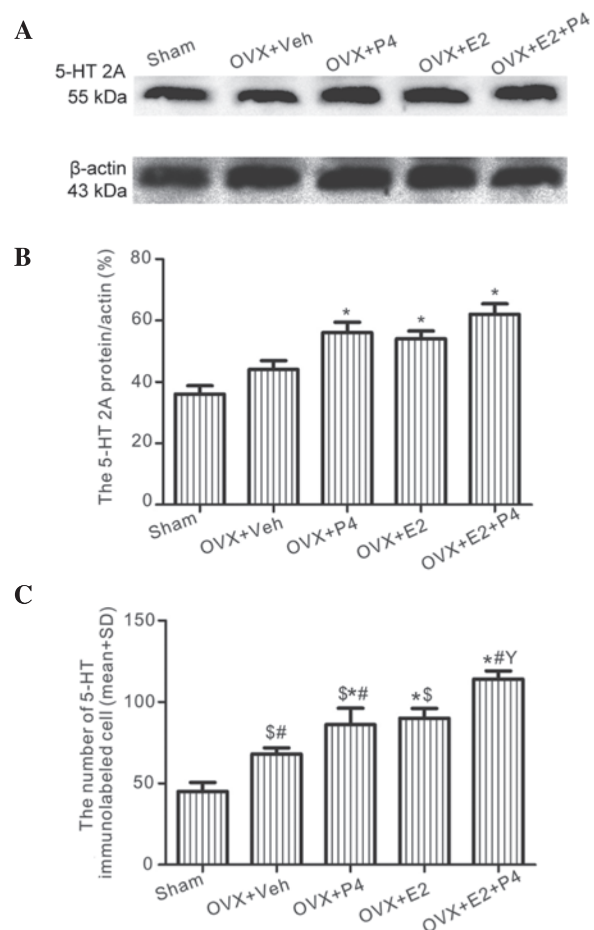

Figure 2. E2 and P4 treatment increased the protein expression levels of 5-HT2A and its immunoreactivity in the hippocampus of a rat model of AD. (A) The expression levels of 5-HT2A significantly increased following E2 and P4 treatment, as compared with the sham rats, as demonstrated by western blotting. (B) The expression levels of 5-HT2A were quantified by densitometric analysis of the western blot. Mean ratios were calculated by normalizing to β-actin. *P<0.05 vs. the sham and vehicle-treated rats. (C) The number of 5-HT2A immunoreactive cells in each group were calculated. Data are presented as the mean ± standard deviation. *P<0.01 vs. the sham and vehicle-treated groups; #P<0.05 vs. the E2-treated group; YP<0.05 vs. the P4-treated group; $P<0.05 vs. the E2 + P4-treated group. E2, 17 β-estradiol; P4, progesterone; 5-HT2A, 5-hydroxytryptamine (serotonin) receptor 2A; AD, Alzheimer's disease; OVX, ovariectomized; veh, vehicle.

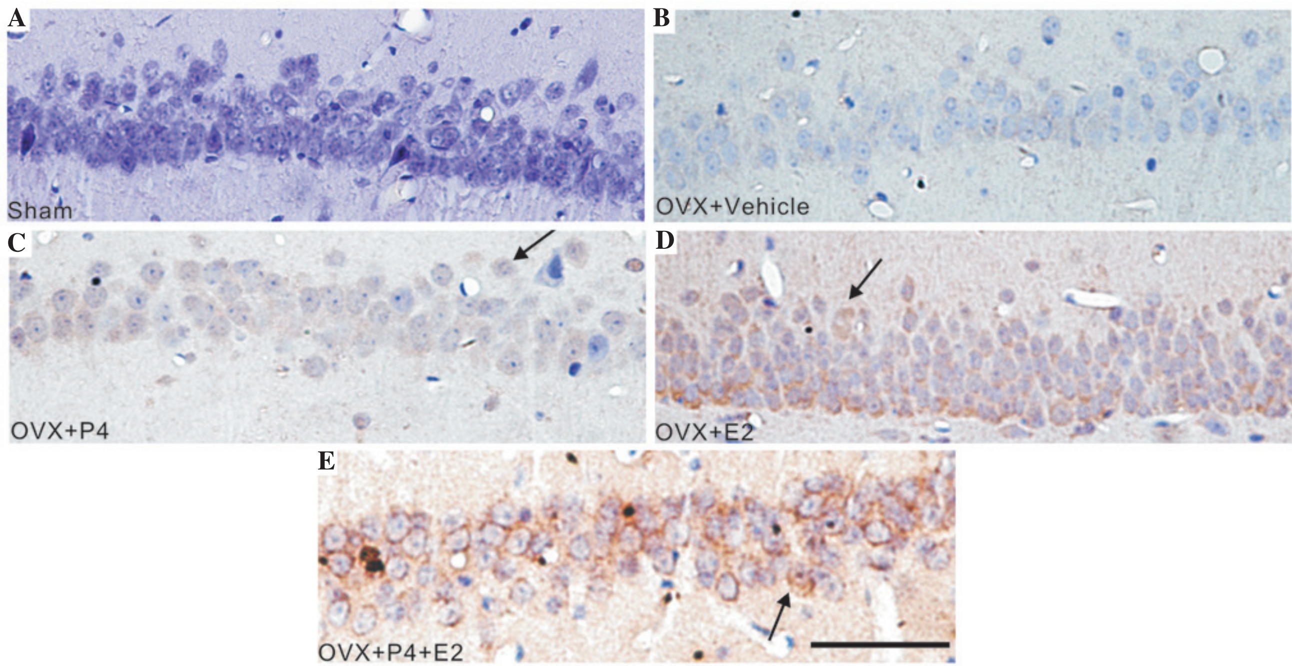

Figure 3. E2 and P4 treatment increased the 5-HT2A immunoreactivity of astrocytes in the hippocampus of a rat model of AD. The tissue sections were developed using 3,3-diaminobenzidine (brown) chromogen and counterstained with hematoxylin. Arrows indicate 5-HT2A-positive cells. (A) Sham group, (B) vehicle-treated group, (C) P4-treated group, (D) E2-treated group and (E) P4+ E2-treated group (scale bar=100 μm). E2, 17 β-estradiol; P4, progesterone; OVX, ovariectomized; veh, vehicle; 5-HT2A, 5-hydroxytryptamine (serotonin) receptor 2A; AD, Alzheimer's disease.

Apoptosis assay. TUNEL assays were performed according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA). Briefly, tissue sections from each group were fixed with 1% paraformaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and then rinsed with PBS and incubated with TUNEL reaction mixture (enzyme, nucleotides) in a humidified atmosphere at 37˚C for 1 h. Staining was then performed using DAB/H_2O_2 at 37˚C for 5 min. Finally, the sections were counterstained with methyl green (Santa Cruz Biotechnology, Inc.) and analyzed under 400x magnification using a Nikon E600 microscope (Nikon Corporation) in 5 vision fields/per section. Labeled cells in the hippocampus were counted.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical significance was analyzed using one-way analysis of variance with post-hoc Tukey t-tests. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses and graphs were performed or generated using GraphPad Prism Version 4.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

E2 and P4 improve spatial memory in a rat model of AD. Morris water maze tests were conducted in order to investigate the effects of ovarian hormones on the spatial and learning memory functions of a rat model of AD. Sham rats, which had received bilateral injections of Aβ into the hippocampus but had not undergone an ovariectomy, exhibited escape latencies of ~120 sec, whereas the escape latencies of the ovariectomized vehicle-treated rats were ~108 sec. Treatment with E2 and/or P4 significantly decreased the escape latencies of the ovariectomized rats (Fig. 1), as compared with the sham-operated and vehicle-treated rats, and this effect was most significant for the E2 + P4-treated group ($P<0.01$; Fig. 1).

E2 and P4 increase the protein expression levels of 5-HT2A and ChAT in the hippocampus. Western blot analysis demonstrated that the protein expression levels of 5-HT2A in the hippocampus of the P4 and/or E2-treated groups were significantly increased, as compared with those in the sham and vehicle-treated rats ($P<0.01$; Fig. 2A and B). These results were consistent with immunostaining (Fig. 2C), which detected that, as compared with the sham and vehicle-treated groups (Fig. 3A and B), 5-HT2A was upregulated in the P4-, E2-, and P4 + E2-treated groups (Fig.3C-E). In addition, the P4 + E2-treated group demonstrated significantly increased 5-HT2A expression levels, as compared with the rats treated with E2 or P4 alone ($P<0.05$; Fig. 2C; Fig. 3C and D).

The protein expression levels of ChAT were increased in the ovarian hormone-treated groups, particularly in the E2- and E2 + P4-treated groups, as compared with the sham and vehicle-treated rats ($P<0.01$; Figs. 4 and 5A), as demonstrated using immunolabeling. Western blotting detected a significant increase in the expression levels of ChAT in the P4 and E2-treated groups ($P<0.05$; Fig. 5B and C).

E2 and P4 decrease the protein expression levels of GFAP in the hippocampus. Immunolabeling detected significantly decreased levels of GFAP in the astrocytes of the ovarian hormone-treated rats, as compared with the vehicle-treated rats ($P<0.01$; Figs. 6 and 7A), and this effect was most significant for the E2-treated group, as compared with the P4- and E2 + P4-treated groups ($P<0.05$; Figs. 6 and 7A). In addition, western blotting detected significantly decreased

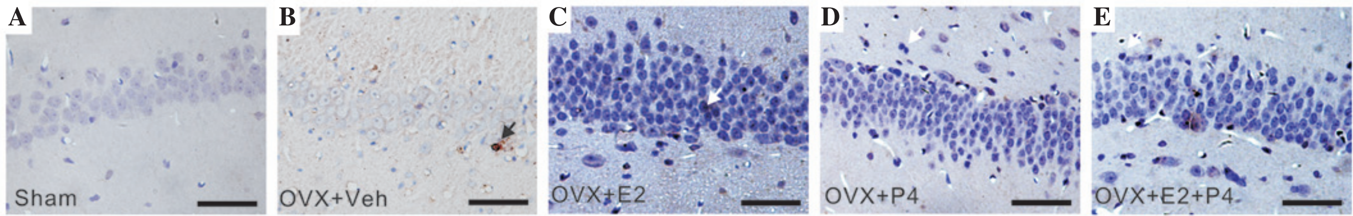

Figure 4. E2 and P4 treatment increased the ChAT immunoreactivity of astrocytes in the hippocampus of a rat model of AD. The tissue sections were developed using 3,3-diaminobenzidine (brown) chromogen and counterstained with hematoxylin. Arrows indicate ChAT-positive cells. (A) Sham group, (B) vehicle-treated group, (C) P4-treated group. (D) E2-treated group and (E) P4 + E2-treated group (scale bar=100 μm). E2, 17 β-estradiol; P4, progesterone; OVX, ovariectomized; veh, vehicle; ChAt, choline acetyltransferase; AD, Alzheimer's disease.

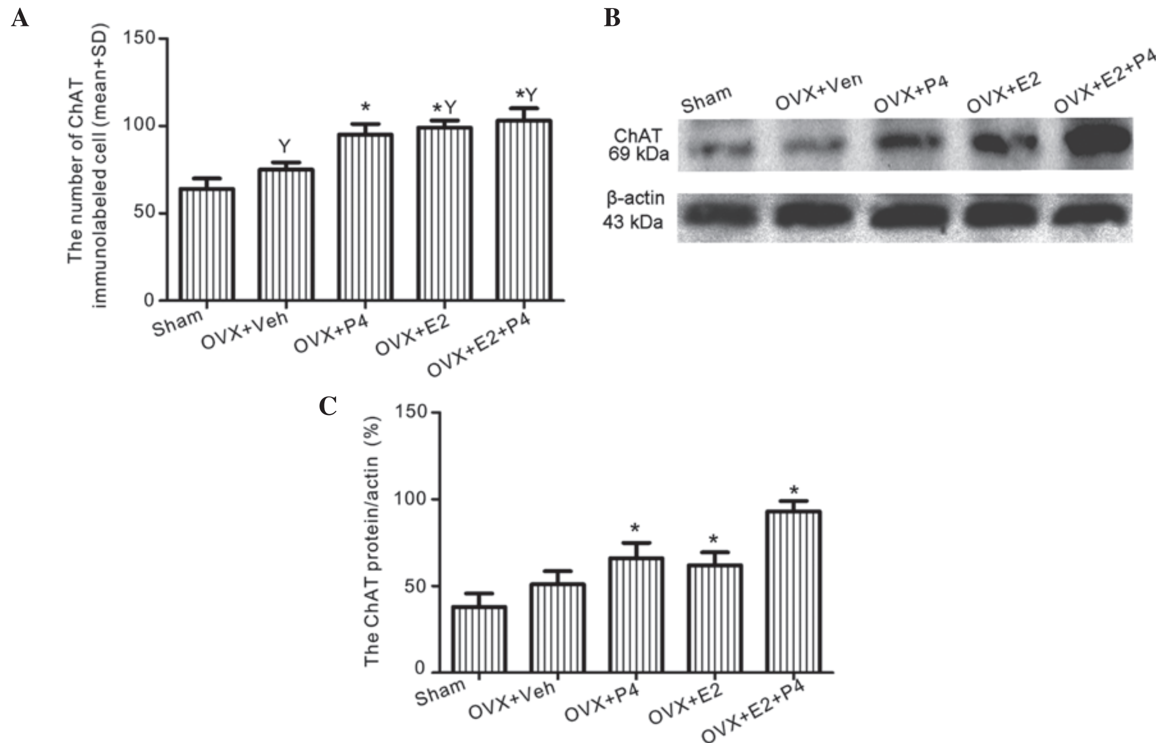

Figure 5. E2 and P4 treatment upregulated the protein expression levels of ChAT in the hippocampus of a rat model of AD. (A) The number of ChAT immunoreactive cells in each group was calculated. (B) The protein expression levels of ChAT significantly increased following E2 and P4 treatment, as compared with the sham and vehicle-treated rats, as demonstrated by western blotting. (C) The expression levels of ChAT were quantified by densitometric analysis of the separated protein bands. Mean ratios were calculated by normalizing to β-actin. Data are presented as the mean ± standard deviation. *P<0.05 vs. the sham and vehicle-treated rats. *P<0.01 vs. the sham and vehicle-treated group; YP<0.01 vs. the P4-treated group. E2, 17 β-estradiol; P4, progesterone; OVX, ovariectomized; veh, vehicle; ChAt, choline acetyltransferase; AD, Alzheimer's disease.

GFAP protein expression levels in the hippocampus of the E2-, P4-, and E2 + P4-treated groups, as compared with the vehicle-treated rats (P<0.01; Fig. 7B and C).

E2 and P4 suppress apoptotic signals and inhibit cell apoptosis. A significant decrease in caspase-3 immunoreactivity of the neuronal cells was detected in the hippocampus of the P4- and/or E2-treated group, and this was associated with a visible decline in the levels of apoptosis, as demonstrated by the TUNEL assay, as compared with the sham rats (P<0.01; Fig. 8 and 9). This effect was most significant for the P4 + E2-treated group, as compared with the rats treated with P4 or E2 alone (P<0.05; Fig. 8 and 9); thus suggesting that the combined treatment is more effective than either ovarian hormone alone.

Discussion

Various steroid hormones have been shown to have a role in regulating the production of Aβ protein; thus suggesting that selective lowering of Aβ levels using steroids may be considered a potential strategy in the prevention and treatment of AD (18). E2 and P4, which are ovarian hormones involved in reproduction, have previously been demonstrated to exert additional effects on the CNS (7,19). Previous studies have suggested that there may be an increased risk of neuronal dysfunction following menopause, or an ovariectomy; thus suggesting that reduced levels of ovarian hormones may increase the risk of AD (19,20). In addition, E2 and P4 have previously been associated with anxiety and mood (21-23), and E2 replacement therapy has been suggested as a potential

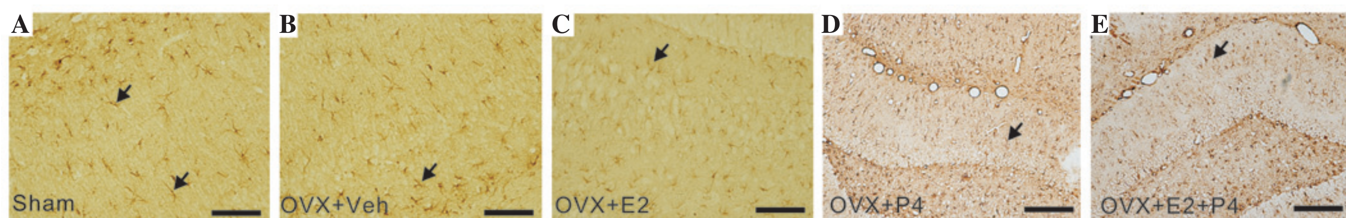

Figure 6. E2 and P4 treatment decreased the protein expression levels of GFAP in the hippocampus of a rat model of AD, as detected by GFAP immunoreactivity staining. Antibody-GFAP complexes were visualized via 3,3-diaminobenzidine staining and hematoxylin counterstaining. Arrows denote GFAP-positive cells. (A) Sham group, (B) vehicle-treated group, (C) E2-treated group, (D) P4-treated group and (E) P4 + E2-treated group (scale bar=100 μm). E2, 17 β-estradiol; P4, progesterone; OVX, ovariectomized; veh, vehicle; GFAP, glial fibrillary acidic protein; AD, Alzheimer's disease.

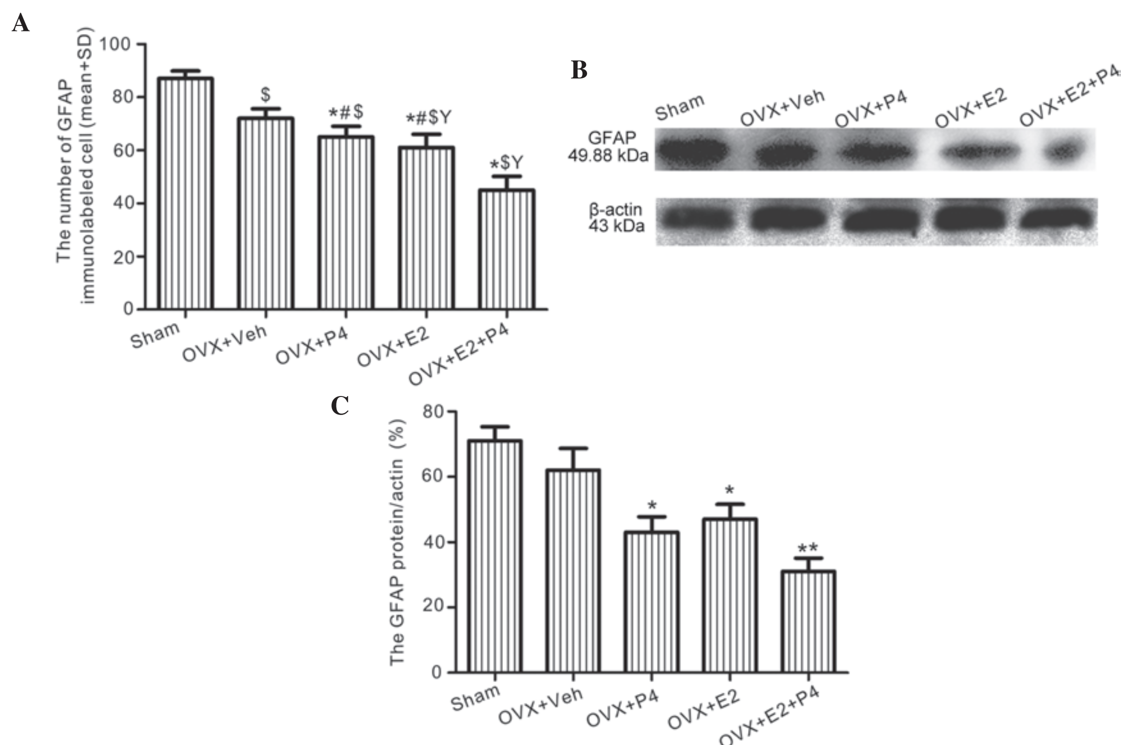

Figure 7. E2 and P4 treatment decreased the protein expression levels of GFAP in the hippocampus of a rat model of AD. (A) The number of GFAP immunoreactive cells in each group was calculated. [*]P<0.01 vs. the sham and vehicle-treated groups; [Y]P<0.01 vs. the P4-treated group; [#]P<0.01 vs. the E2 + P4-treated group; [$]P<0.01 vs. the shame group. (B) The GFAP expression levels significantly increased following E2 and P4 treatment, as compared with the sham and vehicle-treated rats, as demonstrated by western blotting. (C) The expression levels of GFAP were quantified by densitometric analysis of the separated protein bands. Mean ratios were calculated by normalizing to β-actin. Histograms depict the mean densitometric value of GFAP:β-actin. [*]P<0.05 vs. the sham and vehicle-treated groups; [**]P<0.01 vs. the sham and vehicle-treated groups. E2, 17 β-estradiol; P4, progesterone; OVX, ovariectomized; veh, vehicle; GFAP, glial fibrillary acidic protein; AD, Alzheimer's disease.

strategy for restoring the deficits that occur following an ovariectomy, in order to improve mood, cognitive function and the overall quality of life (5,8,11,12). The effects of hormonal treatment on cognition during aging are controversial (12). Previous studies have suggested that P4 replacement therapy may reduce the susceptibility of neuronal cells to toxic insult in a manner that is relevant to aging and AD (7,19). Whilst the results from older animal models have provided a strong rationale for the use of hormone replacement therapy to prevent dementia and AD, the results from clinical trials have been conflicting (12,24). For example, numerous clinical trials have reported an increased risk of dementia and cognitive decline in women treated with E2 alone or in combination with a progestin (13,25-27).

In the present study, Aβ1-40 was injected into the hippocampus of OVX rats in order to generate a model of AD, and the therapeutic effects of E2 and P4 were investigated. In particular, the spatial and learning memory functions of all rats were assessed using the Morris water maze test. The spatial memory of the Aβ1-40- and vehicle-treated rats declined, as compared with the sham-operated rats, whereas treatment with E2 and P4 was shown to exert protective effects. These results suggested that E2 and P4 treatment may have improved learning and memory functions in a rat model of AD. The increased ChAT and 5-HT2A immunoreactivity of the neurons in the ovarian hormone-treated rats, as well as the observed amelioration of astrogliosis (indicated by upregulation of GFAP expression) in the hippocampus of

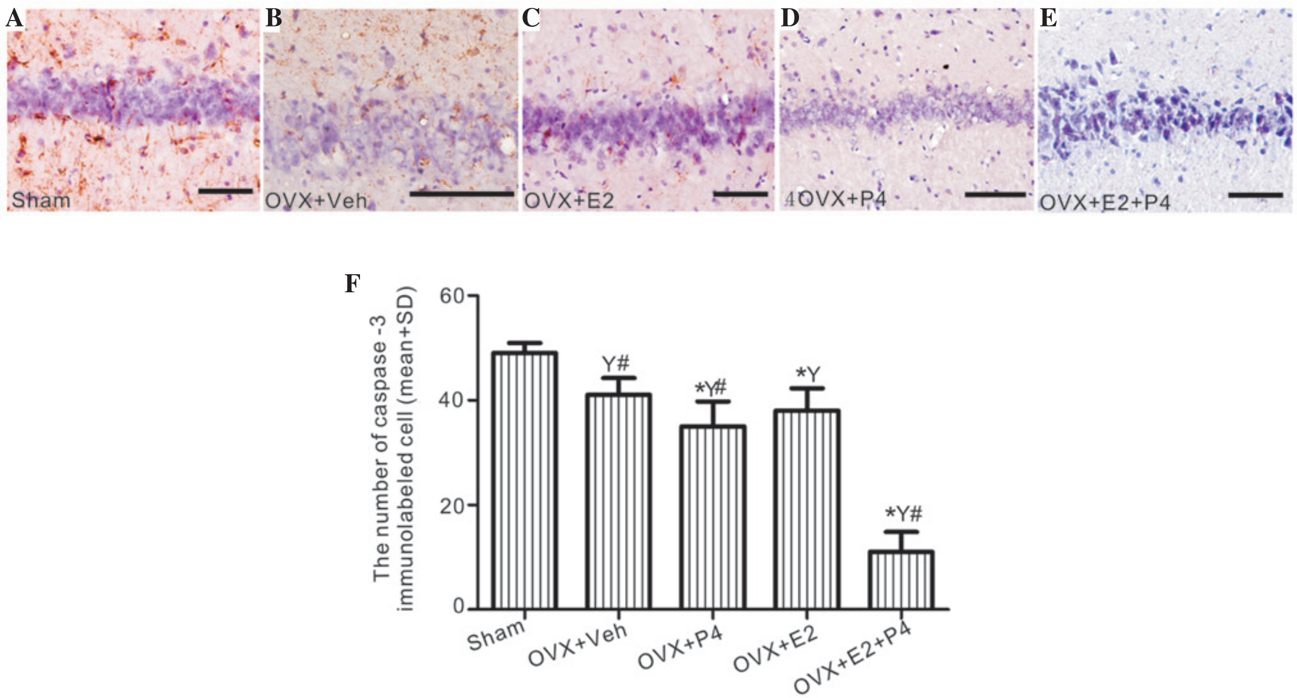

Figure 8. The effects of E2 and P4 treatment on the expression levels of caspase-3 in the hippocampus of a rat model of AD. (A-E) Caspase-3 immunoreactivity counterstaining via hematoxylin. (A) Sham group, (B) vehicle-treated group, (C) E2-treated group, (D) P4-treated group and (E) P4 + E2-treated group (scale bar=100 μm). (F) The number of GFAP immunoreactive cells in each group was calculated. *P<0.01 vs. the vehicle-treated group; $^\#$P<0.05 vs. the E2-treated group; ^{Y}P<0.01 vs. the sham group. E2, 17 β-estradiol; P4, progesterone; OVX, ovariectomized; veh, vehicle; AD, Alzheimer's disease.

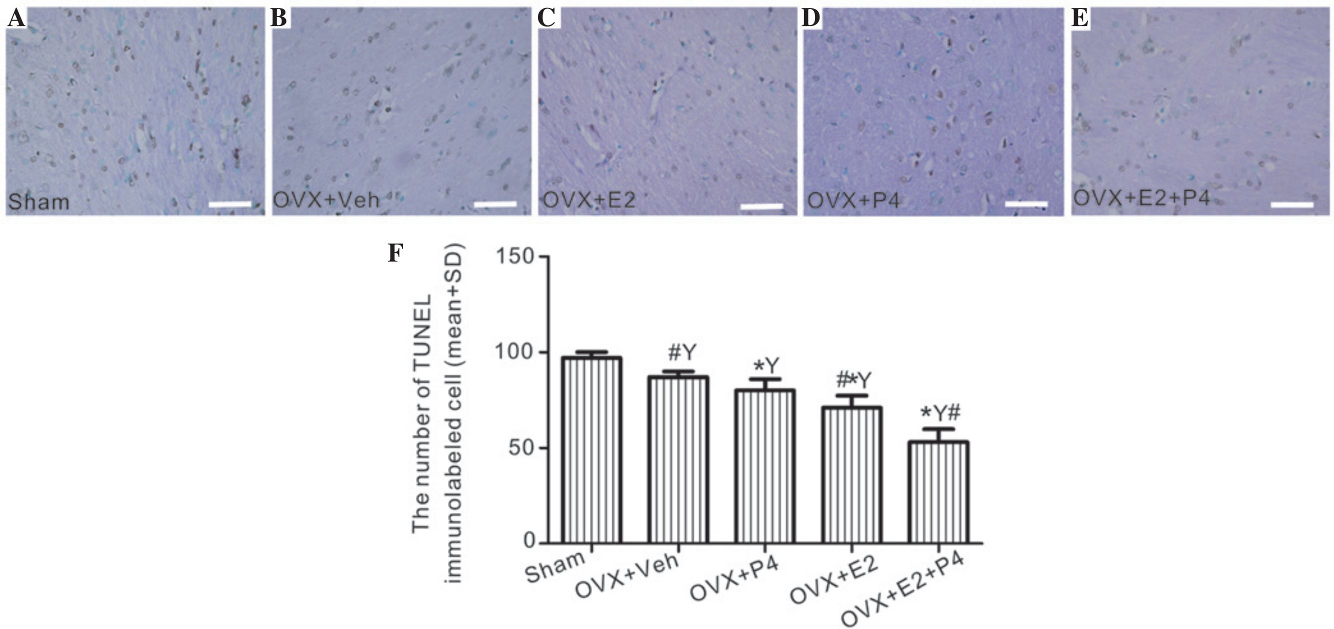

Figure 9. The effects of E2 and P4 treatment on apoptotic cells in the hippocampus of a rat model of AD. (A-E) Apoptotic cells in the tissue sections were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis, and were counterstained using methyl green. (A) Sham group, (B) vehicle-treated group, (C) E2-treated group, (D) P4-treated group and (E) P4 + E2-treated group (scale bar=100 μm). (F) The number of TUNEL-labeled cells in each group was calculated. *P<0.05 vs. the sham and vehicle-treated rats; $^\#$P<0.05 vs. the P4-treated group; ^{Y}P<0.01 vs. the sham group. E2, 17 β-estradiol; P4, progesterone; OVX, ovariectomized; veh, vehicle; GFAP, glial fibrillary acidic protein; AD, Alzheimer's disease.

the rats, provided further evidence for potential therapeutic effects of E2 and P4. However, there were various limitations associated with the rat model of AD used in the present study, including that the experimental rats were of a young age.

In a previous study, ChAT activity in the frontal cortex and hippocampus decreased in an amyloid precursor protein 695 transgenic mouse model, which also had learning and memory deficits (28). The hippocampus is necessary for various types of learning and memory formation in rats and other mammals (29),

and the loss of cholinergic neurons is considered a key event in the pathogenesis of dementia (30). Therefore, the decline of ChAT-positive neurons detected in the present study may have contributed to the age-associated learning and memory deficits observed. However, the reduction in the number of ChAT-positive neurons in the hippocampus of the rat model of AD was attenuated by treatment with E2 and/or P4, and this may have ensured that normal levels of acetylcholine were available for memory formation and consolidation.

5-HT is another neurotransmitter, which has previously been associated with behavior and cognition (31). 5-HT2C receptors are distributed throughout the human CNS, including within the prefrontal cortex and hippocampus (32), and drugs with a high affinity for 5-HT2A receptors have been shown to modulate memory formation in rats (33). In addition, previous studies have suggested that 5-HT dysfunction may have important functional consequences in patients with AD (32,34). In a study analyzing retrospective data, low densities of 5-HT2A binding sites were associated with aggressive progression of AD (35).

In the present study, 5-HT2A expression levels were decreased in the rat model of AD, and this was attenuated by exogenous ovarian hormone replacement therapy. In particular, P4 was shown to have the greatest protective effects. As well as the direct effects of steroidal hormones on cholinergic neurons, previous studies have reported indirect effects for E2 on serotonergic neurons, which facilitated acetylcholine release (5). Notably, the present study demonstrated an association between ChAT and 5-HT2A immunoreactivity in the hippocampus following E2 and P4 treatment, which was consistent with the hypothesis that serotonin is able to modulate cholinergic systems involved in cognition.

Astrogliosis, which involves an augmentation and increase in size of GFAP-immunoreactive astrocytes, is frequently detected in scenarios of brain damage (36,37). Upregulation of GFAP in astrocytes has been reported for various neurodegenerative disorders and in the autogenic senescence-accelerated mouse model (36-38). Increased astrocyte-neuron contact may restrict or reduce synaptic plasticity by decreasing potential interneuron contacts (39). The results of the present study demonstrated that GFAP-positive astrocytes increased with age in the hippocampus of an Aβ-induced rat model of AD; thus suggesting that there were age-associated pathological changes occurring in the CNS, and that an increase in astrocyte volume may lead to a corresponding decrease in neuronal cell volume (36).

In the present study, less severe astrogliosis was detected in the brains of the E2- and P4-treated rats, as compared with the sham and vehicle-treated rats; this was particularly evident in the E2-treated group. Furthermore, the marked decrease in the expression levels of GFAP in the E2- and/or P4-treated rats was associated with increased 5-HT2A immunoreactivity and ChAT expression levels in these rats. The results of the present study suggested that the increased astrogliosis in the rat model of AD may have contributed to specific functional deficits, including impairment of learning ability and memory, and alterations in cholinergic and serotonergic function, whereas E2 and P4 treatment was able to alleviate the astrogliosis.

In the present study, the effects of E2 and P4 on apoptosis in the rat model of AD were also investigated. Increased caspase-3 expression levels and a higher number of TUNEL-positive cells were detected in the vehicle-treated rats, as compared with the sham rats. Treatment with E2 and P4 significantly downregulated caspase-3 expression levels and decreased the number of TUNEL-positive cells, thus suggesting that they were able to suppress apoptosis. In addition, P4 treatment was associated with the most significant neuroprotective effects, which is consistent with previous studies in which P4 was shown to protect neurons against glutamate toxicity (40,41) and to inhibit caspase-3 activation (42). In the present study, it is possible that the anti-apoptotic effects of P4 and E2 resulted in a better performance in the spatial memory task.

The ability of E2 and P4 to reduce cholinergic deficits, apoptotic signaling, astrogliosis and 5-HT2A immunoreactivity, may have protected against memory impairment in the rat model of AD. In general, E2 was better able to protect the cholinergic neurons suppressing reactive gliosis, as compared with P4, whereas P4 was more able to protect the 5-HT2A system and to prevent apoptotic mechanisms. Notably, when the two drugs were combined, the effects were not additive, thus suggesting that E2 and P4 exerted neuroprotective effects which had partially overlapping mechanisms, or that they antagonized each others actions using unidentified mechanisms. Therefore, in order to fully elucidate the mechanisms involved, further studies are required, which is particularly important when considering hormone replacement therapy for treatment of the menopause or of patients with AD.

In conclusion, the present study investigated the effects of ovarian hormones on spatial and learning memory in an ovariectomized rat model of AD, which was generated by stereotaxically injecting Aβ(1-40) bilaterally into the hippocampus of ovariectomized rats. The results of the present study demonstrated that treatment with ovarian hormones significantly reduced the expression levels of GFAP, and increased the expression levels of ChAT and 5-HT2A in the hippocampus, alleviated neuronal apoptosis and improved learning and memory functions in the rats. These results suggested that the ovarian hormones, E2 and P4, may confer protection against Aβ-induced toxicity *in vivo*.

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