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Research Paper

ERα and/or ERβ activation ameliorates cognitive impairment, neurogenesis and apoptosis in type 2 diabetes mellitus mice

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ABSTRACT

Estrogen receptors (ERs) are thought to be associated with the onset and progression of neurodegenerative injuries and diseases, but the relationship and mechanisms underlying between ERs and cognition in type 2 diabetes remain elusive. In the current study, we investigated the effects of ERα and ERβ on the cognition, neurogenesis and apoptosis in high-fat diet and streptozocin-induced diabetic mice. We found that ERα and/or ERβ activation using their agonists (0.5 mg/kg E2, PPT or DPN) ameliorate memory impairment in the Morris water maze and Y-maze tests, increase hippocampal neurogenesis and prevent hippocampal apoptotic responses. Importantly, treatment with the pharmacologic ERs agonists caused significant increases in the membrane ERα and ERβ expression and subsequent PI3K/Akt, CREB and BDNF activation in the hippocampus of type 2 diabetes mellitus mice. Our data indicate that ERα and ERβ are involved in the cognitive impairment in type 2 diabetes, and that activated ERs, such as application of ERs agonists, could be a novel and promising strategy for the treatment of diabetic cognitive impairment.

1. Introduction

Type 2 diabetes mellitus (T2DM) is associated with cognitive decrements and an increased risk to develop dementia (Pasquier et al., 2006; Spauwen et al., 2013); these will become a major worldwide clinical problem in the future. In several recent clinical and animal experimental studies, older women or female mice with T2DM and obesity have a higher frequency of cognitive decline compared with the men or male mice of the same age (Gregg et al., 2000; Sakata et al., 2010; Yaffe et al., 2004). Especially, epidemiological studies have shown increased risk of cognitive impairment with the age-related loss of sex steroid hormones, while cognitive impairment is more prevalent in postmenopausal women than in age-matched men (Vina and Lloret, 2010). The sharp decline of estrogens after menopause has been presumed to account for the increased female susceptibility to cognitive impairment in T2DM.

Estrogens are the primary female sex hormones and involved in female sexual development and maintenance of normal reproductive functions. They also play very important roles in the immune system as well as in the central nervous system (CNS) in human body (Warner and Gustafsson, 2015). A lot of evidence has documented profound effects of estrogens on learning, memory and neurodevelopmental processes (Brann et al., 2007; Craig et al., 2008; Craig and Murphy, 2007a, 2007b). Animal studies have shown that endogenous estrogen levels changed by reproductive experience in females are associated with enhanced hippocampus-dependent memory (Li et al., 2013). Furthermore, women who underwent surgical menopause or had menopause before 47 years old without hormone treatments had an increased risk for global cognitive impairment and dementia in later life, suggesting that earlier menopause is associated with a higher risk for cognitive impairment (Hogervorst, 2013). Moreover, it has reported that 17β-estradiol the most potent estrogen increased neurogenesis in various brain regions such as dentate gyrus of hippocampus, and decrease both brain inflammation and the activation of apoptosis, these effects in the brain contribute to region-specific learning and memory (Gatson et al., 2009; McClure et al., 2013). Usually, the beneficial effects of estrogens on memory are likely mediated through classical estrogen receptors (ERs), designated as ERα and ERβ (Gronemeyer et al., 2004). In more recent years, the focuses on ERs have intensified, because its novel pathophysiological role has emerged in the CNS disorders including spinal cord injury, multiple sclerosis, Parkinson's disease, and Alzheimer's disease (Chakrabarti et al., 2014). It has been shown that ER agonists possess neuroprotective effects in enhancing memory and cognition and ameliorating neurodegenerative diseases, it not only provide neuroprotection by inhibition of microglia activation, but also modulation of cell survival mechanisms, synaptic reorganization,

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regenerative responses to axonal injury, and neurogenesis process. These effects of ER agonists might be a useful therapeutic option for delaying the onset or progression of neurodegenerative injuries and diseases. However, very few reports on the association of ERs with cognition in type 2 diabetes are found yet. In this study, we investigated the relationship between ERs and cognition in type 2 diabetes, and then explored the possible mechanisms of ERs in memory impairment, neurogenesis and apoptosis.

2. Materials and methods

2.1. Animals and reagents

Female ICR mice (Yangzhou University Medical Center, China), weighing 18–22 g (6–8 weeks old) were used for the experiments. Mice were housed in a constant room with maintained temperature (22 ± 2 °C), humidity (55 ± 5%), and lighting (12-h light/dark cycle) and allowed access to water and food freely. All experiments were approved by the Institutional Review Committee for the use of Animal Subjects of China Pharmaceutical University and experimental procedures are subjected to the guidelines of the institutional animal care and use committee of China.

17β-Estradiol (E2, Cat.No.E2758) and streptozocin (STZ, Cat.No.S0130) were purchased from Sigma Aldrich (St. Louis, MO, USA). ERα selective agonist 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT, Cat.No.1426) and ERβ selective agonist 2,3-Bis (4-hydroxyphenyl)-propionitrile (DPN, Cat.No.1494) were purchased from Tocris Bioscience (Ellisville, MO, USA). High-fat diet (HFD) was purchased from Medical Center of Yangzhou University (Yangzhou, China), consisted of 13% lard, 2% sesame, 20% sugar, 3% cholesterol, 0.1% sodium cholate, 5% peanut. Other reagents have been described in the methods.

2.2. Animal model and treatment

The mice were randomly divided into seven groups: (1) sham operated control mice (Veh + Veh), (2) STZ and high-fat diet induced type 2 diabetes mellitus mice (DM + Veh), (3) ovariectomized mice (OVX + Veh), (4) DM mice with OVX operation (DM&OVX + Veh), (5) DM mice with OVX operation and E2 treatment (DM&OVX + E2), (6) DM mice with OVX operation and PPT treatment (DM&OVX + PPT), (7) DM mice with OVX operation and DPN treatment (DM&OVX + DPN). The mice were anesthetized and then carried out bilateral ovariectomy surgery except the group (1) and (2) mice with sham operation. One week later, the mice except the group (1) and (3) were fed with HFD for 4 weeks and then injected once with low dose of STZ (in the tail vein at 100 mg/kg body weight) to induce partial insulin deficiency, followed by continued HFD feeding for an additional 4 weeks, and then the mice presence of hyperglycemia (> 11.0 mmol/L) were used for the next experiments (Jiang et al., 2012).

Mice in group (5) (6) and (7) were injected subcutaneously with E2 (0.5 mg/kg), PPT (0.5 mg/kg) and DPN (0.5 mg/kg) respectively, and other groups were injected vehicle (bean oil) every other day for 4 weeks. The dose used was as previously reports (Bliedtner et al., 2010; Mancuso et al., 2011; Sakata et al., 2010). After treatment, one part of mice was submitted to the behavior tests, a further part was tested for hippocampal neurogenesis and neural differentiation, a third part was used to evaluate the fasting blood glucose and insulin serum levels, and the fourth part was used for assays of ERα, ERβ, caspase-3, Bcl-2, Bax, PI3K, p-PI3K, Akt, p-Akt, CREB, p-CREB and BDNF.

2.3. Morris water maze (MWM) task

Spatial learning and memory was assessed by the MWM test (Tang et al., 2013). The test consisted of 5 d training (visible and invisible platform training sessions) and a probe trial on day 6. Mice were individually trained in a circular pool (diameter 120 cm, height 50 cm) containing 30 cm-high water maintained at 25 °C. A platform (9 cm diameter) was placed in the centre of one quadrant of the pool and its position was fixed throughout the training sessions. Each mouse was individually trained in both visible-platform (days 1–2) and hidden-platform (days 3–5) versions. Visible-platform training was performed for baseline differences in vision and motivation; the platform was placed 1 cm below the surface of the water and was indicated by a small flag (5 cm in height). The hidden-platform version (the flag was removed) was used to evaluate spatial learning and determine the retention of memory to find the platform. On each day, the mice were subjected to four trials with a 2 h interval between trials. Each trial lasted for 90 s unless the mice reached the platform first. The time (escape latency) that elapsed until the mouse reaches the platform was noted. If an animal failed to find the platform within 90 s, the test was ended and the animal was gently navigated to the platform by hand for 30 s. In the probe trial (day 6), the platform was removed and the mice had 90 s to search for the previous platform. The time spent in the target quadrant (i.e. the quadrant where the platform was previously located) and the number of platform location crossings was recorded. Data of the escape latency, the time spent in the target quadrant, the number of platform location crossings and swim speed were collected by the video tracking equipment and processed by a computer equipped with an analysis-management system (Viewer 2 Tracking Software; Ji Liang Instruments, China).

2.4. Y-maze test

This was performed as descried previously (Tang et al., 2013). The Y-maze was constructed of black plastic walls (height 10 cm), consisting of three compartments (10 × 10 cm) connected with passages (4 × 5 cm), with the floor of 3.175 mm stainless steel rods (8 mm apart). The test was conducted for two consecutive days. On day 1 (learning trial), each mouse was placed in one of the compartments and allowed to move freely for 5 min (habituation) before moving to the next session with electric power on. During the training, electric shocks (2 Hz, 125 ms, 10 V) were available through the stainless steel grid floor in two of the compartments and the light was on in the shock-free compartment. Each mouse was trained 10 times. The training was stopped once the mouse entered the shock-free compartment and stayed for 30 s, which was recorded as a correct choice. If the mouse did not enter this compartment, it was gently navigated to the compartment and allowed to stay for 30 s. On day 2 (testing trial), each mouse was also tested 10 times following the same procedures as on day 1. The numbers of correct choices during the 10 trials and the latency to enter the shock-free compartment on day 2 were recorded manually.

2.5. Open-field test

The test was used to evaluate the general locomotor activity of the mice associated with each treatment and performed as descried previously (Chen et al., 2016). The open-field chamber was made of Plexiglas, with a black-painted floor (50 × 50 cm) and transparent walls (height 40 cm). The experiments were carried out in a sound-attenuated room under low-intensity light (7 lx). At the beginning, the mice were gently placed at the center of the open field and allowed to explore for 5 min. The total distance traveled (m) was recorded in the 5 min period by the ANY Maze® video tracking. After each trial the chamber was cleaned with ethanol solution (10% v/v) and dried with paper towels in order to avoid odor impregnation.

2.6. BrdU administration and immunofluorescence

For analyzing hippocampal neurogenesis and neural differentiation, mice were injected intraperitoneally with 50 mg/kg of bromodeoxyuridine (BrdU, Cat.No.B5002, Sigma-Aldrich) thrice, at 2 h interval. BrdU

intercalates into the nascent DNA strain during cell division and therefore labels dividing cells. 14 days later, the mice were transcardially perfused with 0.1 M PBS followed by 4% ice-cold paraformaldehyde. The brains were postfixed in 4% paraformaldehyde overnight, and dehydrated with 30% sucrose over 2 days. Coronal sections (7 μm) were cut using an oscillating tissue slicer and fixed on the glass slides. Then the sections blocked with PBS containing 0.3% Triton X-100 and 3% normal goat serum and incubated with the first antibody of rat anti-BrdU (1:50, Cat.No.SC-56258, Santa Cruz.), rabbit anti-NeuN antibody (1:200, Cat.No.ABN78, Millipore Bioscience) and rabbit anti-GFAP antibody (1:100, Cat.No.12389S, Cell Signaling) under 4 °C overnight. After rinsing with PBS, the sections were incubated with Cy3-conjugated goat anti-rat IgG (1:100; Cat.No.A0507, Beyotime Biotechnology) for BrdU labeling and FITC conjugated goat anti-rabbit IgG (1:100; Cat.No.A0562, Beyotime Biotechnology) for NeuN or GFAP labeling for 1 h. After staining DAPI for 10 min, the sections were added 50% glycerol and covered coverslips. Fluorescent signals were detected and captured using the Laser Scanning Confocal Microscope (LSM700, Carl Zeiss, Germany). The quantification was carried out using Image-Pro Plus software. BrdU-positive cells from single labeled sections were counted along all SGZ of the dentate gyrus from three animals per group, six sections per animal (18 different sections per group) through a 20 × objective; the values were averaged and expressed as mean number of $BrdU^+$ cells in SGZ of the DG. The percentage of differentiated cells was calculated as the number of $NeuN^+$ or $GFAP^+$ cells divided by the $BrdU^+$ cells through a 20 × objective.

2.7. Blood glucose and serum insulin determination

For the fasting blood glucose determination, blood samples (10 μl) were collected from the tail vein and measured with a blood glucose monitoring system (Roche).

For the serum insulin determination, blood samples (approximately 800 μl) were collected from the orbit and centrifuged (3000 rpm, 15 min, 4 °C) to obtain supernatants for the assay. Serum insulin was determined using mouse insulin ELISA kit (Cat.No.CSB-E05071m, Cusabio Biotech Co., Ltd., Wuhan, china). The ELISA kit procedures were performed following the manufacturer's instructions. In brief, the sample (10 μl) and the Sample Diluent (40 μl) were added into the precoated plate and added 100 μl HRP-conjugate reagent to each well, then incubated for 60 min at 37 °C in the dark. After washing each well of the precoated plate with washing buffer, chromogen was then added and the mixture was incubated at 37 °C in the dark for 15 min. After the addition of the stop solution, the resulting color was recorded at 450 nm using a microplate absorbance reader.

2.8. Western blot analysis

Mouse hippocampus were chopped into small pieces and homogenized in ice-cold RIPA (50 mM Tris–HCl (pH 7.4), 150 nM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). The dissolved proteins were collected from the supernatant after centrifugation at 4 °C, 12000 rpm for 15 min. Protein concentration was determined by a BCA protein assay kit according to the manual (Cat. No. P0010, Beyotime Biotechnology, Jiangsu, China) and then assessed for expression of BDNF, pro- or cleaved caspase-3, Bax, and Bcl-2 proteins. Protein extracts were separated by a SDS-polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane. The membrane was blocked with 5% BSA in Tris buffer saline and then incubated at 4 °C overnight with respective primary antibodies for anti-BDNF antibody (1:1000, Cat.No. BS6533, Bioworld Technology), anti-pro or cleaved caspase-3 antibody (1:500, Cat.No.9665S, Cell Signaling Technology), anti-Bcl-2 antibody (1:500, Cat.No.BS1031, Boster Technology), anti-Bax antibody (1:500, Cat.No.BS1030, Boster Technology), or β-actin (1:10000, Cat.No.AP0060, Bioworld Technology). After washing with tris buffered saline–tween 20 (TBST), the membranes were incubated with a horseradish peroxidase conjugated secondary antibody (1:10000, Cat.No.#GGHL-15P, Bioworld Technology) for 2 h at room temperature. The antibody-reactive bands were visualized by using enhanced chemiluminescence detection reagents and a gel imaging system (Tanon Science & Technology Co., Ltd., China).

Membrane, nuclear and cytoplasmic protein extracts were prepared using Membrane, Nuclear and Cytoplasmic Protein Extraction kit (Cat.No.C510002, Sangon Biotech, China). Briefly, mouse hippocampus were chopped into small pieces and homogenized in ice-cold buffer A containing 0.5% phosphatase inhibitor, 0.1% protease inhibitor, 1% phenylmethylsulfonyl fluoride and 0.1% DL-dithiothreitol, then centrifuged at 4 °C, 12000 rpm for 20 min. The supernatant was cytoplasmic protein extracts, and stored at −80 °C. The precipitate was added buffer B containing 0.5% phosphatase inhibitor, 0.1% protease inhibitor, 1% phenylmethylsulfonyl fluoride and 0.1% DL-dithiothreitol, and centrifuged at 4 °C, 12000 rpm for 20 min. Then, the supernatant as nuclear protein extracts were stored at −80 °C. Finally, the precipitate was added buffer C containing 0.5% phosphatase inhibitor, 0.1% protease inhibitor, 1% phenylmethylsulfonyl fluoride and 0.1% DL-dithiothreitol, centrifuged at 4 °C, 12000 rpm for 10 min, and the supernatant as membrane protein extracts were stored at −80 °C. The cytoplasmic protein extract was subjected to Western blot for assay of PI3K (1:1000, Cat.No.BS3006, Bioworld Technology), p-PI3K (1:1000, Cat.No.AP0152, Bioworld Technology), Akt (1:1000, Cat.No.BS1810, Bioworld Technology), and β-actin (1:10000, Cat.No.AP0060, Bioworld Technology) were used as a loading control. The nuclear protein extract was subjected to Western blot for assay of ERα (1:1000, Cat.No.SC-787, Santa Cruz Biotechnology), ERβ (1:1000, Cat.No.SC-8974, Santa Cruz Biotechnology), p-Akt (1:1000, Cat.No.BS4007, Bioworld Technology), CREB (1:1000, Cat.No.BS1077, Bioworld Technology), p-CREB (1:1000, Cat.No.BS4053, Bioworld Technology), and laminB1 (1:1000, Cat.No.BS3547, Bioworld Technology) was used as a loading control. The membrane protein extract was subjected to Western blot for assay of ERα (1:1000, Cat.No.SC-787, Santa Cruz Biotechnology), ERβ (1:1000, Cat.No.sc-8974, Santa Cruz Biotechnology), and Na,K-ATPase (1:1000, Cat.No.#3010, Cell Signaling Technology) was used as a loading control.

2.9. Statistical analysis

Quantitative data are reported as mean ± SEM. In the water maze, the escape latencies, swimming speed and total distance traveled were analyzed using repeated two-way ANOVA with “days” as the within-subject factor and “group” as the between-subject factor. All other data were analyzed using one-way ANOVA followed by Tukey or Dunnett's tests for post hoc multiple comparisons. The level of statistical significance was $P < 0.05$. All analysis was carried out using SPSS 18.0.

3. Results

3.1. Estrogen receptors activation ameliorates cognitive deficits in type 2 diabetes mellitus mice

To investigate whether ERs activation enhances memory and cognition in type 2 diabetes, we treated the mice using ER nonselective agonist E2, ERα selective agonist PPT and ERβ selective agonist DPN respectively and then performed different behavior tests. Firstly, the performance of mice in a non-spatial visible-platform variant of the MWM to test for baseline differences in vision and motivation among treatment groups were assessed. Mice in each group exhibited similar escape latency in the visible-platform test, suggesting no influence of E2, PPT or DPN on vision and basal motivation of mice (4 trials/mouse/day for 2 days; effect of day, $F(6,439) = 6.347$, $p < 0.05$; effect of group, $F(6,439) = 0.166$, $p > 0.05$; effect of group-by-day interaction,

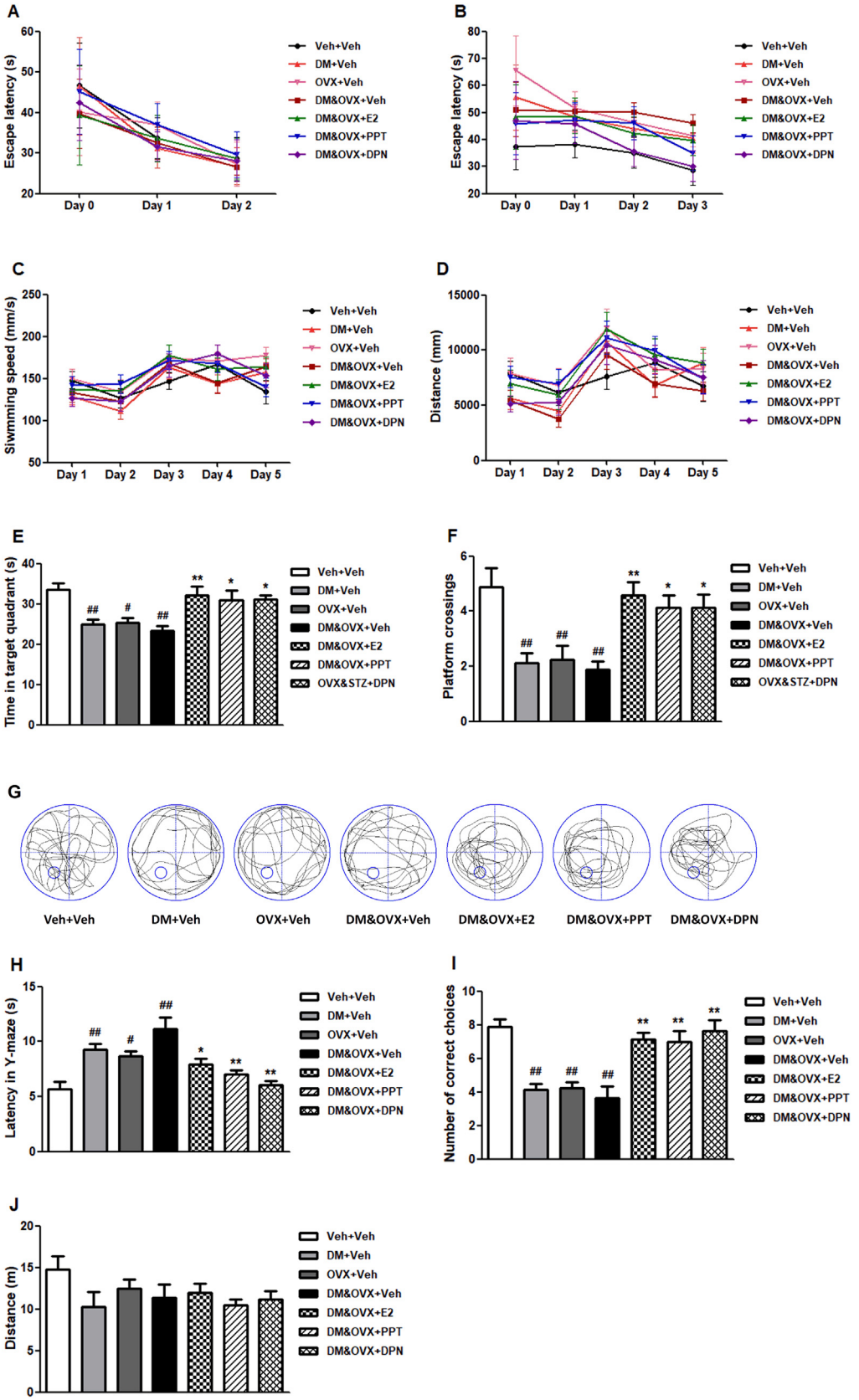

Fig. 1. Estrogen receptors activation ameliorated memory impairment in type 2 diabetes mellitus mice. Day 0 indicates performance on the first trail and subsequent points represent an average of all daily trails in the Morris water maze task. (A) No differences were found in the escape latency among all groups during the visible platform test (days 1–2). (B) Changes in escape latency to reach the hidden platform during acquisition trials (days 3–5). The swimming speed (C) and total distance traveled (D) on day 1–5 are shown. The time spent in the target quadrant (E), the number of platform location crossings (F), and the representative swim paths of individual mice in each group during the probe trial test on day 6 (G) are presented. The latency to enter the shock-free compartment (H) and the number of correct choices (I) on day 2 were measured in the Y-maze test. (J) Total distance of each group mice traveled in 5 min were measured in the open field test. Results are expressed as means ± SEM (n = 8–10). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Veh + Veh group, $^{*}P < 0.05$, $^{**}P < 0.01$ versus DM&OVX + Veh group. Abbreviation: Veh, vehicle; DM, diabetes mellitus; OVX, ovariectomized.

F(6,439) = 0.088, p > 0.05; Fig. 1A). We then tested the mice in the spatial hidden-platform variant, and data showed that mice in DM + Veh, OVX + Veh and DM&OVX + Veh group displayed increased escape latencies compared to the Veh + Veh group on day4 and day5, but treatment with ER agonists including E2, PPT and DPN for 4 weeks decreased the escape latency (4 trials/mouse/day for 3 days; effect of day, F(6,647) = 6.179, p < 0.01; effect of group, F (6,647) = 2.410, p < 0.05; effect of group-by-day interaction, F

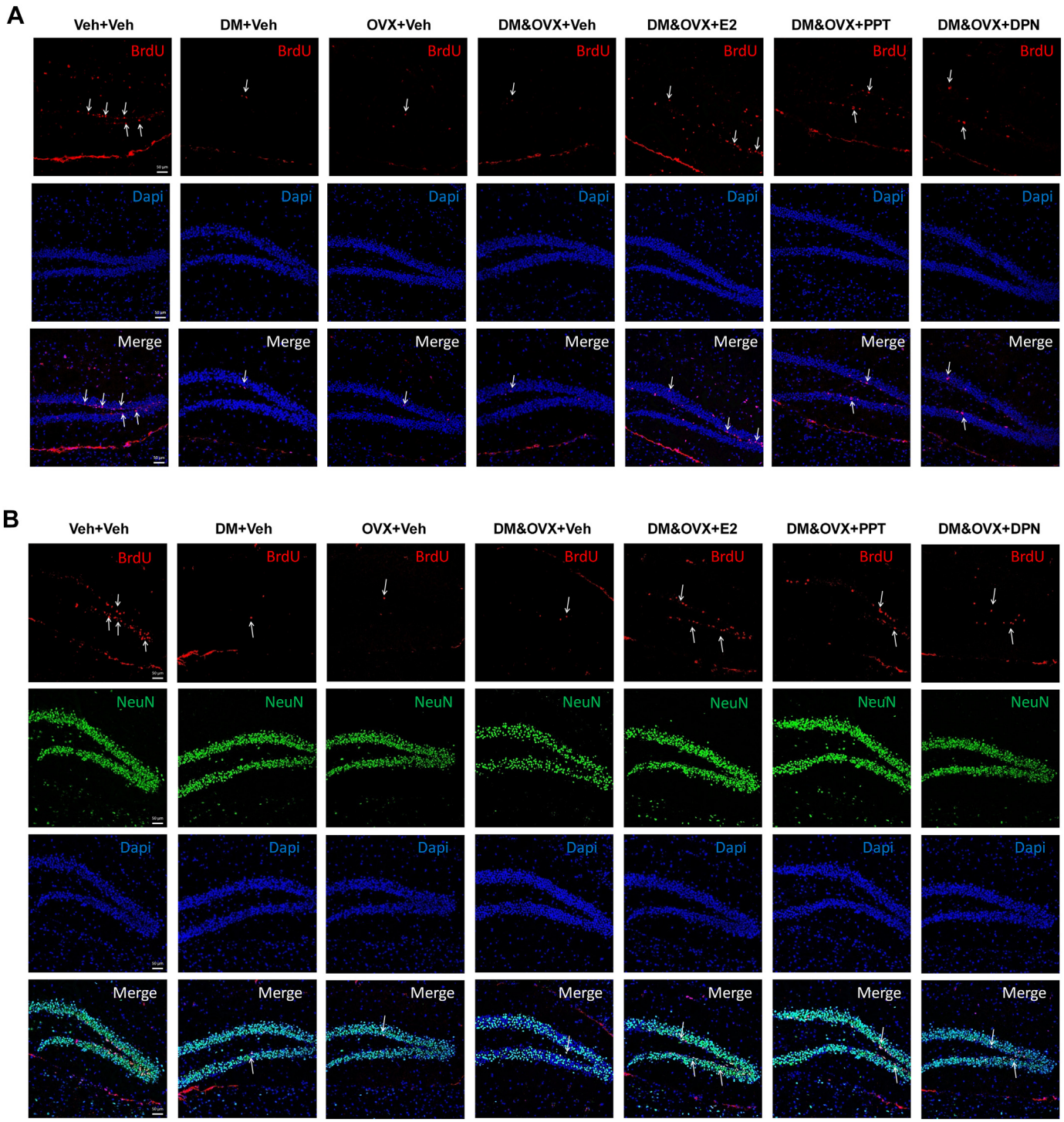

Fig. 2. Estrogen receptors activation enhanced hippocampal neurogenesis and did not change the differentiation types of new born cells in type 2 diabetes mellitus mice. (A) Confocal micrographs of the dentate gyrus stained for BrdU (red) and DAPI (blue). (B, C) Representative confocal micrographs of BrdU-positive cells (red) and NeuN (green) or GFAP (green) in the dentate gyrus from all groups. (D) Histogram showing the number of BrdU-immunostained cells per slide. (E) Percentages of neuronal and glial cells labeled by BrdU in the dentate gyrus. Scale bars = 50 μm. Data are shown as means ± SEM (n = 3–4). $^{\#\#}P < 0.01$ versus Veh + Veh group, $^{*}P < 0.05$, $^{**}P < 0.01$ versus DM&OVX + Veh group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(6,647) = 0.189, $p > 0.05$; Fig. 1B). Moreover, mice in each group showed similar swim speed and total distance traveled during the visible-platform and hidden-platform variant (Fig. 1C and D). In the probe trial, a putative measure of spatial learning and memory retention, mice in DM + Veh, OVX + Veh and DM&OVX + Veh group displayed a significant decrease in the time spent in the target quadrant ($p < 0.05$ or $p < 0.01$; Fig. 1E) and the number of platform location crossings ($p < 0.01$; Fig. 1F) compared to the Veh + Veh group. It is noteworthy that, the DM mice with OVX appeared to have more serious cognitive impairment than only DM or OVX, but this was not statistically significant. In contrast, mice treated with E2, PPT and DPN showed significant increases in both indices compared to the DM mice

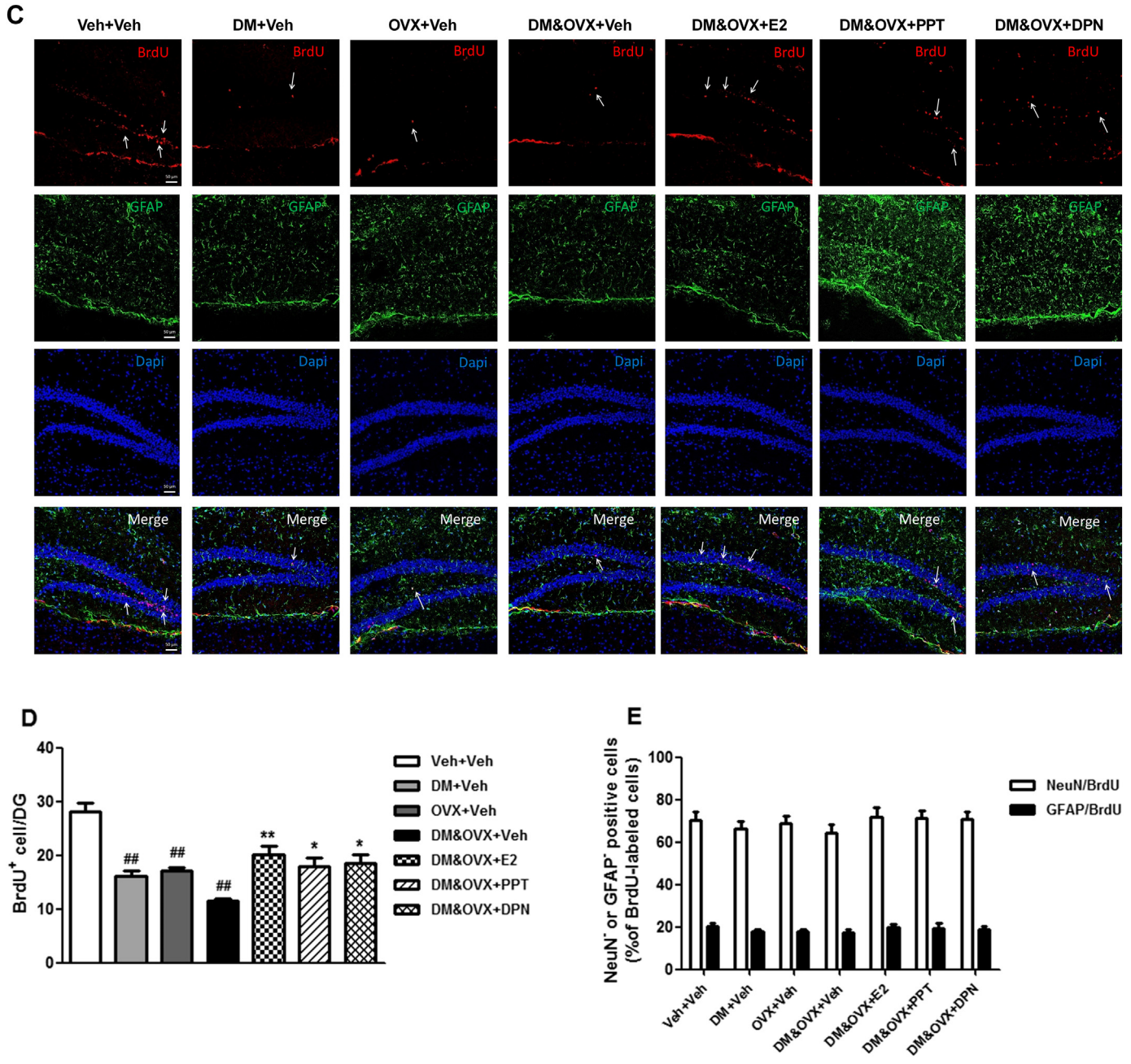

Fig. 2. (*continued*)

with OVX ($p < 0.05$ or $p < 0.01$; Fig. 1E and F) (1 trial/mouse for last day; $F(6,53) = 6.738$, $p < 0.001$ for the time; $F(6,53) = 7.419$, $p < 0.001$ for the number). These data suggest that ERα and/or ERβ activation improve spatial learning and memory in the estrogen-deficient type 2 diabetic mice.

To confirm the results observed in the MWM task, we also carried out the Y-maze test. The data showed that mice in DM + Veh, OVX + Veh and DM&OVX + Veh group significantly increased the latency to enter the shock-free compartment and decreased the number of correct choices compared to the Veh + Veh group ($p < 0.05$ or $p < 0.01$; Fig. 1H and I), but treatment with E2, PPT and DPN significantly reversed these changes ($p < 0.05$ or $p < 0.01$; Fig. 1H and I) ($F(6,55) = 9.697$, $p < 0.001$ for the latency to enter the shock-free compartment; $F(6,55) = 10.216$, $p < 0.001$ for the number of correct choices). These data further prove that ERα and/or ERβ activation improve spatial learning and memory in the estrogen-deficient type 2 diabetic mice.

Moreover, to determine whether general locomotor activity interferes with cognitive behavior test, we conducted open field test. Results showed that there was no significant difference among the groups in the total distance traveled in the open field test ($F(6,55) = 1.285$, $p = 0.282$; Fig. 1J). These behavior data indicate that the ER agonists do not affect the spontaneous activity of mice. Thus, the effect of spontaneous activity on cognitive function in mice was ruled out. All the behavior results demonstrate that ERα and/or ERβ activation rescues cognitive deficits in the estrogen-deficient diabetic mice.

3.2. Estrogen receptors activation enhances hippocampal neurogenesis in type 2 diabetes mellitus mice

Most preclinical models have shown that diabetes results in reduced hippocampal neurogenesis that may contribute to cognitive decline, a complication commonly observed in humans with diabetes (Ho et al., 2013). We investigated whether ERs is involved in hippocampal

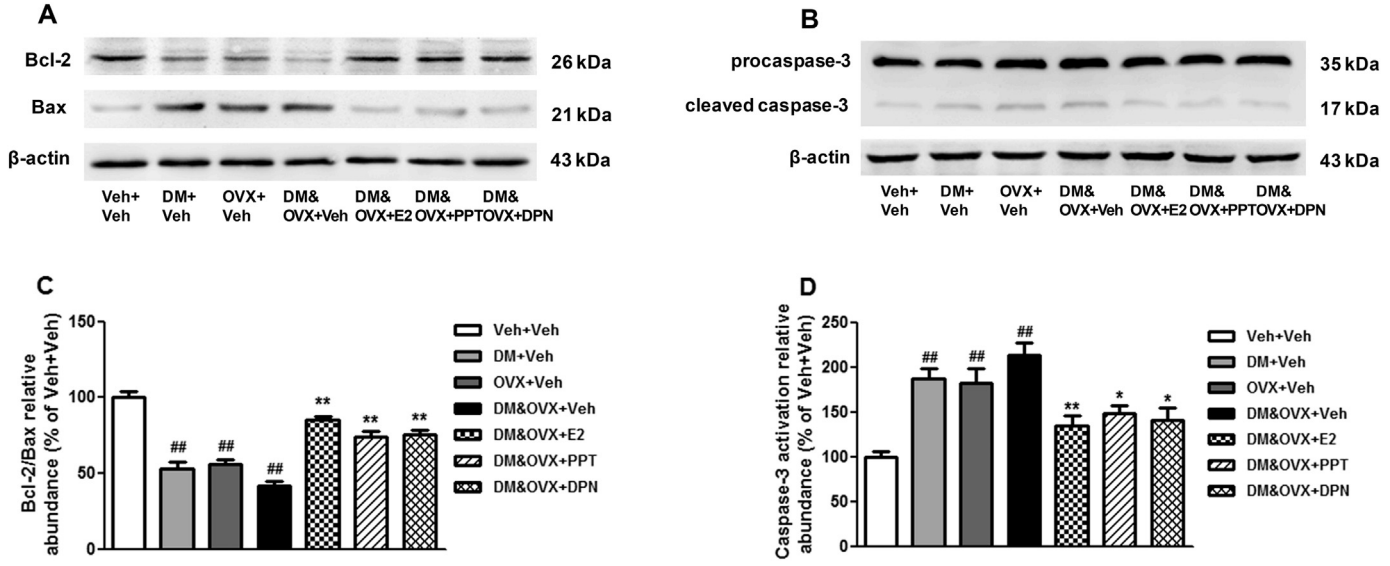

Fig. 3. Estrogen receptors activation prevents hippocampal apoptotic responses in type 2 diabetes mellitus mice. (A, B) Bcl-2, Bax, Procaspase-3 and cleaved caspase-3 were determined in hippocampus by Western blot using respective antibodies. (C) Ratio of Bcl-2/Bax is expressed as the percentage of the Veh + Veh. (D) Caspase-3 activation is expressed as the ratio of caspase-3 fragment to procaspase-3. Data are shown as means ± SEM (n = 3–4). $^{\#\#}P < 0.01$ versus Veh + Veh group, $^{*}P < 0.05$, $^{**}P < 0.01$ versus DM&OVX + Veh group.

Table 1
Effects of estrogen receptor agonists on blood glucose and serum insulin in type 2 diabetes mellitus mice

Group	Blood glucouse (mmol/L)	Serum insulin (mIU/L)
Veh + Veh	5.13 ± 0.09**	67.54 ± 1.67##
DM + Veh	19.89 ± 0.95	47.25 ± 2.36
OVX + Veh	5.23 ± 0.18**	69.04 ± 1.73##
DM&OVX + Veh	23.45 ± 1.13	46.09 ± 1.34
DM&OVX + E2	18.18 ± 1.17	49.99 ± 2.87
DM&OVX + PPT	19.70 ± 1.48	49.79 ± 3.58
DM&OVX + DPN	20.30 ± 1.39	49.31 ± 2.14

Data are expressed as mean ± SEM (n = 4–10).
** P < 0.01.
P < 0.01 versus DM&OVX + Veh group.

neurogenesis in type 2 diabetes mellitus mice. BrdU staining was carried out 16 d after the first BrdU injection in mice, by which time newborn cells develop differentiated phenotypes (Kempermann et al., 2003). To examine the phenotype of BrdU-positive cells in the dentate gyrus (DG), double labeling for BrdU and NeuN, a neuronal marker, or GFAP, a glial marker, was performed. Analysis by confocal microscopy showed that the number of $BrdU^+$ cells in the DG in the DM + Veh, OVX + Veh and DM&OVX + Veh group significantly decreased relative to the Veh + Veh mice ($p < 0.001$; Fig. 2A and D), and the number in the DM&OVX + Veh group was less than only DM or OVX, but this was not statistically significant. However, the number of $BrdU^+$ cells in the DG in the mice treated with E2, PPT and DPN was significantly increased compared to the DM mice with OVX ($p < 0.05$ or $p < 0.01$; Fig. 2A and D) ($F(6,20) = 15.034$, $p < 0.001$). Furthermore, no significant differences in the percentage of $NeuN^+/BrdU^+$ or $GFAP^+/BrdU^+$ were observed among the various groups ($F(6,20) = 0.414$, $p = 0.858$ for $NeuN^+/BrdU^+$; $F(6,20) = 0.255$, $p = 0.949$ for $GFAP^+/BrdU^+$; Fig. 2B–C and E–F). These data suggest that ERα and/or ERβ activation enhances hippocampal neurogenesis in type 2 diabetes mellitus mice.

3.3. Estrogen receptors activation prevents hippocampal apoptotic responses in type 2 diabetes mellitus mice

Mounting evidence suggests that neuronal apoptosis occurs in the diabetic animals (Sadeghi et al., 2016). To evaluate whether the activation of ERs plays an antiapoptotic role in the diabetic mice, we examined hippocampal pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2, as well as caspase-3, being a crucial mediator of apoptosis through its protease activity by Western blot analysis. As shown in Fig. 3, compared to the Veh + Veh group, the ratio of Bcl-2/Bax in the hippocampus was significantly decreased and the caspase-3 activation, as indicated by increases in the ratio of caspase-3 fragment to procaspase-3, was significantly increased in the DM + Veh, OVX + Veh and DM&OVX + Veh group ($p < 0.01$, Fig. 3), and the DM&OVX + Veh group changed the most among the three groups. However, ER non-selective agonist E2, ERα selective agonist PPT and ERβ selective agonist DPN treatment reversed these changes induced by DM and OVX ($p < 0.01$, Fig. 3). ($F(6,20) = 34.084$, $p < 0.001$ for Bcl-2/Bax; $F(6,20) = 9.919$, $p < 0.001$ for caspase-3 activation) The results showed that ERα and/or ERβ activation prevents hippocampal apoptotic responses in type 2 diabetes mellitus mice.

3.4. Effects of estrogen receptor agonists on blood glucose and serum insulin in type 2 diabetes mellitus mice

Next, we detected the levels of fasting blood glucose and serum insulin in each group. As shown in Table 1, compared with the DM mice with OVX, ER agonists including E2, PPT and DPN treatment did not affect the levels of fasting blood glucose and serum insulin ($F(6,69) = 40.099$, $p < 0.001$ for serum glucose; $F(6,27) = 17.240$, $p < 0.001$ for serum insulin). This suggests that the ERα and ERβ may be involved in diabetic cognitive impairment through other signals.

3.5. Effects of estrogen receptor agonists on the hippocampal estrogen receptors expression in type 2 diabetes mellitus mice

To determine the effects of subcutaneous injection of ER agonists on the expression of ERs in hippocampus, we detected nuclear and membrane ERs protein levels in the hippocampus using Western blot. As shown in Fig. 4, compared to the Veh + Veh group, the membrane and nuclear ERα and ERβ protein levels in the hippocampus were significantly decreased in the DM + Veh, OVX + Veh and DM&OVX + Veh group ($p < 0.01$), and the DM&OVX + Veh group reduced more than the other two groups, but with no statistical difference. In

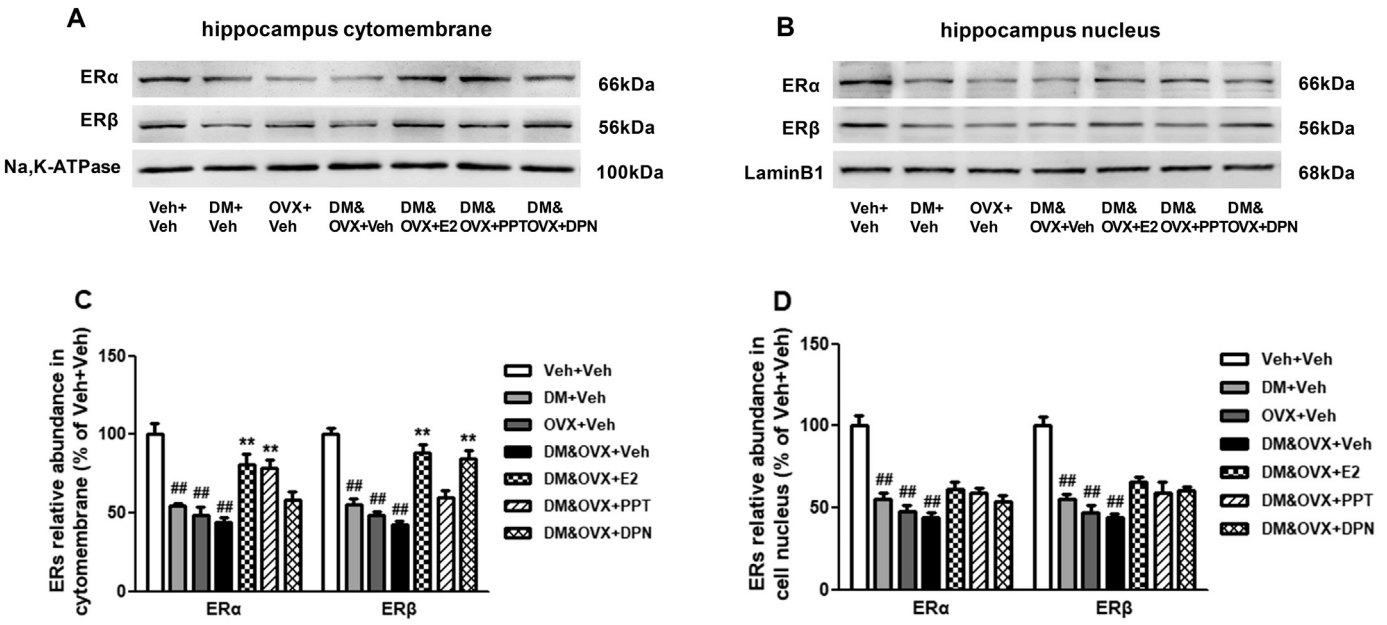

Fig. 4. The effects of estrogen receptors agonists on the hippocampal estrogen receptors expression in type 2 diabetes mellitus mice. (A, B) The levels of ERα and ERβ protein expression in hippocampus cytomembrane and nucleus of mice were measured using Western blot. (C, D) Quantification of the protein levels of ERα and ERβ was expressed as the ratio (in percentage) of Veh + Veh group Data are shown as means ± SEM (n = 5). $^{\#\#}P < 0.01$ versus Veh + Veh group, $^{**}P < 0.01$ versus DM&OVX + Veh group.

addition, compared to the DM&OVX + Veh group, ER nonselective agonist E2 treatment significantly increased the membrane ERα and ERβ protein levels ($p < 0.01$, Fig. 4A, C), and ERα selective agonist PPT and ERβ selective agonist DPN treatment significantly increased the membrane ERα or ERβ protein levels in the hippocampus respectively ($p < 0.01$, Fig. 4A, C). However, although these ER agonists increased the nuclear ERα and ERβ protein levels in the hippocampus, there was no statistical difference ($p > 0.05$, Fig. 4B, D). ($F(6,34) = 40.005$, $p < 0.001$ for membrane ERα; $F(6,34) = 29.417$, $p < 0.001$ for membrane ERβ; $F(6,34) = 21.292$, $p < 0.001$ for nuclear ERα; $F(6,34) = 14.232$, $p < 0.001$ for nuclear ERβ).

3.6. Hippocampal estrogen receptors upregulation activate PI3K/Akt, CREB and BDNF signaling in type 2 diabetes mellitus mice

In the previous reports, the ER action is present and defined as a rapid effect (within minutes even seconds) initiated by interaction with plasma membrane-associated ERs, leading to activation of signaling pathways such as PI3K/Akt (Marino et al., 2006). Once Akt is activated, CREB undergoes phosphorylation and thereby up-regulating one of its downstream genes, brain-derived neurotrophic factor (BDNF), to provide neuroprotection (Du and Montminy, 1998). Using Western blot analyses, compared to the Veh + Veh group, the levels of p-PI3K/PI3K, p-Akt/Akt, p-CREB/CREB and the expression of BDNF protein in the hippocampus were obviously decreased in DM + Veh, OVX + Veh and DM&OVX + Veh group ($p < 0.05$ or $p < 0.01$, Fig. 5), and the DM& OVX + Veh group reduced more than the other two groups, but with no statistical difference. However, compared to the DM&OVX + Veh group, ER nonselective agonist E2, ERα selective agonist PPT and ERβ selective agonist DPN treatment significantly increased the expression of these proteins ($p < 0.05$ or $p < 0.01$, Fig. 5). ($F(6,20) = 11.292$, $p < 0.001$ for p-PI3K/PI3K; $F(6,20) = 12.654$, $p < 0.001$ for p-Akt/Akt; $F(6,20) = 10.333$, $p < 0.001$ for p-CREB/CREB; $F(6,20) = 12.512$, $p < 0.001$ for BDNF) These data suggest that the membrane-associated ERs may activate the PI3K/Akt, CREB and BDNF signaling in type 2 diabetes mellitus mice.

4. Discussion

Most preclinical models have shown that diabetes results in reduced hippocampal neurogenesis and neuroplasticity that may contribute to cognitive decline, a complication commonly observed in humans with diabetes. Currently, there is no diabetes-specific treatment for cognitive decline in humans. Although the goal of clinical treatments for diabetes includes the maintenance of euglycemia to prevent the onset and progression of cognitive symptoms (Biessels et al., 2007), long-term studies with different treatments have not validated the approach. The level of estrogen synthesis during aging is critical biochemical change for females and sharp reduction of estrogen during menopausal period is commonly associated with the decline of cognitive function observed in post-menopausal women (Gregg et al., 2000; Yaffe et al., 2004). Rodent studies have confirmed that cognitive impairment and hippocampal neuropathology of diabetes mellitus is relieved by estrogen treatment (Garris, 1999; Lannert et al., 1998; Sakata et al., 2010; Saravia et al., 2006; Toung et al., 2000). In this study,consistent with the previous reports, DM or OVX could induce cognitive impairment, and the DM mice with OVX appeared to have more serious cognitive impairment than only DM or OVX, but this was not statistically significant. Therefore, we used the combination of hyperglycemia and estrogen deficiency model to investigate the role of ERs in the type 2 diabetes mellitus mice. The mice carried out bilateral ovariectomy surgery (OVX), which widely employed to mimic postmenopausal metabolism syndrome, and treated with HFD/STZ inducing T2DM. The majority of HFD/STZ-treated mice displayed hyperglycemia, insulin resistance, and glucose intolerance as previously reported (Luo et al., 1998). And we found that ERα and/or ERβ activation using their agonists ameliorate memory impairment in the T2DM mice, which was confirmed by behavioral tests such as MWM and Y-maze tasks.

Pronounced pathological changes characterize the brain of diabetic animals, particularly the hippocampus. The hippocampus has repeatedly been implicated in learning and memory, and numerous studies have shown that experimental diabetes has negative impacts and induce apoptosis in hippocampal neurons (Sadeghi et al., 2016; Sun et al., 2014). In addition, an important function of the dentate gyrus of the hippocampus is in neurogenesis. Adult neurogenesis in the dentate

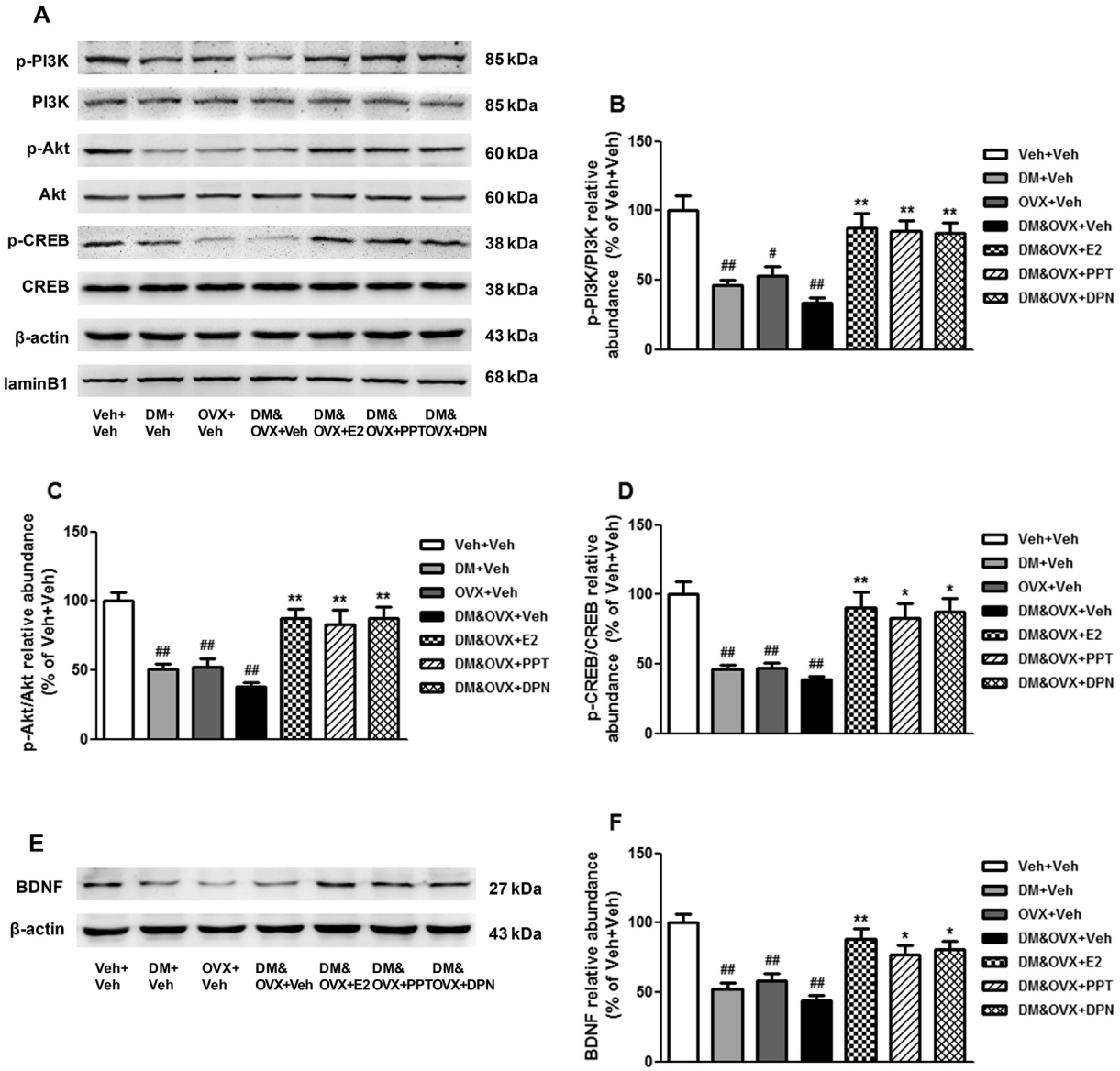

Fig. 5. Hippocampal estrogen receptors upregulation activate PI3K/Akt, CREB and BDNF in type 2 diabetes mellitus mice. (A) The protein levels of p-PI3K, PI3K, p-Akt, Akt, p-CREB, and CREB in the hippocampus of mice were detected by Western blot with respective antibodies. (B-D) Quantification of p-PI3K/PI3K (B), p-Akt/Akt (C), and p-CREB/CREB (D) were expressed as the ratio (in percentage) of Veh + Veh group. (E) The protein level of BDNF was also determined by western blot. (F) Quantification of BDNF was expressed as the ratio (in percentage) of Veh + Veh group. Results are expressed as means ± SEM (n = 3–4). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Veh + Veh group, $^{*}P < 0.05$, $^{**}P < 0.01$ versus DM&OVX + Veh group.

gyrus has been proposed to regulate information processing in the hippocampus, and young neurons may contribute to the circuitry both by integrating new information and by inhibiting the activity of the dense network of mature granule cells (Duarte-Guterman et al., 2015). Hippocampal function and morphology are sensitive to changes in estrogens that occur across the reproductive cycle, pregnancy, motherhood, and aging in females (Daniel, 2013; Pawluski et al., 2009). Estrogens not only increase spine and synapse density in CA1 of the hippocampus and to impact hippocampal synaptic plasticity, but also stimulate proliferation and differentiation of progenitors into mature granule neurons in the DG (Daniel, 2013; Ormerod et al., 2003; Saravia et al., 2006). Moreover, estrogen protects neuronal cells from apoptosis in the hippocampus (Nilsen et al., 2006; Nilsen and Diaz Brinton, 2003; Sharma and Mehra, 2008). These effects of estrogen in the hippocampus may be mediated by ERα and ERβ, which widely distributed and expressed in the hippocampus, cerebral cortex, amygdale and etc., these regions helps to regulate the autonomic nervous system, memory and cognition, and emotional reactions (Brannvall et al., 2002; Cui et al., 2013; McEwen et al., 2001; Ormerod et al., 2003). In our investigations, we used ER nonselective agonist E2, ERα selective agonist PPT and ERβ selective agonist DPN respectively, and found that hippocampal ERα and/or ERβ activation promote the hippocampal cells survival, induce both neuonal and glial differentiation and prevent hippocampal apoptotic responses as evidenced by decreased caspase-3 activity and increased ratio of Bcl-2/Bax. Furthermore, both ERα and ERβ activation appeared to have better effects of improving memory, increasing neurogenesis and suppressing apoptosis than only ERα or ERβ activation, but this was not statistically significant. These demonstrate that both ERα and ERβ play the critical role in cognition in T2DM. However, we found the effects of ERα and ERβ on cognition, neurogenesis and apoptosis cannot be due to amelioration of the diabetic state, since blood glucose and serum insulin levels values did not significantly alter compared to controls, suggesting the ERα and ERβ may be involved in diabetic cognitive impairment through other

signals.

ERα and ERβ localize in nuclear and extranuclear sites, including the membrane (McEwen et al., 2012; McEwen and Milner, 2007; Vasudevan and Pfaff, 2008). In this study, we found the mice treated with ERs agonists significantly increased the membrane ERα and ERβ protein levels in the hippocampus, but there was no obvious change in the nuclear ERα and ERβ. Therefore, we firstly focused the ER-dependent mechanism named “membrane-initiated” pathway, in which the membrane-associated ERs could activate different protein kinase cascades, such as the phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) pathway (Hayashi and Yamaguchi, 2008). The PI3K/Akt pathway is correlated with neuronal cell survival and cell death, transducing mitogenic signals that promote proliferation and inhibit differentiation of adult neural progenitors (Peltier et al., 2007). Downstream, Akt targets also include several members of the Bcl-2 family, critical checkpoints in the mitochondrial pathway of apoptosis, and are comprised of both pro- (Bax) and anti-apoptotic (Bcl-2) molecules (Parcellier et al., 2008). Furthermore, activation of Akt could also induce transcriptional activity as these kinases have the possibility to translocate to the nucleus where they can activate transcription factors such as CREB, which induces the expression of Bcl-2 (Hetman and Gozdz, 2004; Parcellier et al., 2008). As a transcription factor, CREB plays an important role in the modulation of proliferation, differentiation, survival or death of neurons, and it is normally phosphorylated at Ser133 and activated. Several studies have shown the levels of total or phosphorylated CREB in the hippocampus of old mice and rats are reduced, and these linked to the impairments of synaptic plasticity and memory during aging (Saura and Valero, 2011). Moreover, the activation of CREB triggers the transcription of many genes associated with memory, learning, neuroprotection, synaptic transmission, neuronal survival, cell differentiation and axonal growth (Freitas et al., 2013). The best known transcriptional target of CREB is brain-derived neurotrophic factor (BDNF), which is known to emerge as an important synaptic modulator of synaptogenesis and synaptic plasticity (Lu, 2003; Poo, 2001). BDNF is crucial for the consolidation of long term memory (Cunha et al., 2010) and has been reported to be involved in hippocampal-dependent learning (Tyler et al., 2002). In the current study, we found the PI3K/Akt, CREB and BDNF were activated after the ERs agonists' treatment in the hippocampus of T2DM mice. Based on these, we speculate ERα and ERβ might improve the diabetic cognitive impairment through the membrane-associated ERs mediated signals. Although current results show no significant changes in the nuclear ERα and ERβ proteins expression, we will investigate further whether they are involved in diabetic cognitive disorders.

Overall, our study demonstrates the contributions of ERα and ERβ to cognition, neurogenesis and apoptosis in T2DM mice. These appear to be the membrane ERα and/or ERβ activation lead to PI3K/Akt, CREB and BDNF enhancement, which increases neurogenesis, prevents apoptosis and ameliorates memory impairment in T2DM mice. It is worth noting that ERs activation did not completely rescue the damage of estrogen-deficient diabetic mice, which may be related to failure to inhibit their hyperglycemia. Although ERs activation could improve glucose metabolism in the brain, sustained peripheral hyperglycemia is still damaging to the central nervous system. We will explore the effects of estrogen receptor agonists in combination with hypoglycemic agents on estrogen-deficient diabetic mice in further study. In any case, it is certain that ERα and ERβ play an important role in the estrogen-deficient T2DM and ERs agonists might be beneficial for it.

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Statement of interest

All of the authors do not have financial interests to disclose.

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