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Research report

Estrogen receptor subtypes alpha and beta contribute to neuroprotection and increased Bcl-2 expression in primary hippocampal neurons

Liqin Zhao, Tzu-wei Wu, Roberta Diaz Brinton*

Department of Molecular Pharmacology and Toxicology, School of Pharmacy, University of Southern California, Pharmaceutical Sciences Center, 1985 Zonal Avenue, Los Angeles, CA 90089-9121, USA
Program in Neuroscience, School of Pharmacy, University of Southern California, Pharmaceutical Sciences Center, 1985 Zonal Avenue, Los Angeles, CA 90089, USA

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Abstract

Estrogen receptor (ER) mediated neuroprotection has been demonstrated in both in vitro and in vivo model systems. However, the relative contribution by either ER subtype, ERα or ERβ, to estrogen-induced neuroprotection remains unresolved. To address this question, we investigated the impact of selective ER agonists for either ERα, PPT, or ERβ, DPN, to prevent neurodegeneration in cultured hippocampal neurons exposed to excitotoxic glutamate. Using three indicators of neuronal viability and survival, we demonstrated that both the ERα selective agonist PPT and the ERβ selective agonist DPN protected hippocampal neurons against glutamate-induced cell death in a dose-dependent manner, with the maximal response occurring at 100 pM. Further analyses showed that both PPT and DPN enhanced Bcl-2 expression in hippocampal neurons, with an efficacy comparable to their neuroprotective capacity. Collectively, the present data indicate that activation of either ERα or ERβ can promote neuroprotection in hippocampal neurons, suggesting that both receptor subtypes could be involved in estrogen neuroprotection. As ERβ is highly expressed in the brain and has little or no expression in the breast or uterus, discovery and design of ERβ selective molecules could provide a strategy for activating the beneficial effects of estrogen in the brain without activating untoward effects of estrogen in reproductive organs.

Theme: Endocrine and autonomic regulation
Topic: Neuroendocrine regulation: other

Keywords: Estrogen; Estrogen receptor; PPT; DPN; Neuroprotection; Bcl-2

1. Introduction

Two subtypes of the estrogen receptor, ERα and ERβ, exist and exhibit distinct cellular and tissue distribution patterns. Weihua et al. [50] demonstrated that ERα is highly expressed in the classical estrogen target tissues such as uterus, mammary gland, bone and cardiovascular systems, whereas ERβ is mainly expressed in non-classical tissues such as prostate, ovary and urinary tract. In the central nervous system (CNS), although both receptors similarly coexpress in some brain regions, including the preoptic area, the bed nucleus of the stria terminalis and the medial and cortical amygdaloid nuclei, some regions show selective expression such as the detection of only ERα in the ventromedial hypothalamic nucleus and subfornical organ [39]. In contrast, ERβ is predominant in the cerebral cortex and in the hippocampus in rat brain [32,39], though others show that ERα is predominant in the hippocampus in mouse [26] and human brain [46], indicating that expression of ERs may be species specific.

Estrogen and its cognate receptors can regulate gene transcription via both direct genomic and indirect genomic mechanisms. The direct genomic mechanism requires estrogen binding to the estrogen receptor, followed by the induction of conformational change in the receptor leading to dimerization and translocation of ER into the nucleus where ER interacts with estrogen response elements (EREs) located

* Corresponding author. Tel.: +1-323-442-1430; fax: +1-323-442-1489.
E-mail address: rbrinton@hsc.usc.edu (R.D. Brinton).
URL: http://www.pharmweb.usc.edu/brinton-lab.

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within the regulatory region of target genes and coadaptor proteins leading to either activation or suppression of transcription of a target gene in a promoter- and cell-specific manner [23,47]. The indirect genomic mechanism, proposed to account for the neuroprotective effects of estrogen in the brain, requires rapid activation of mitogen-activated protein kinases (MAPK) [27,30,42] and Akt signaling pathways [43], activation of a transcriptional factor cAMP-responsive element binding protein (CREB) [7,24,55], and modulation of antiapoptotic Bcl-2 family proteins [12,15,28,29].

Recent evidence reveals the requirement of ERs in mediating estrogen neuroprotection. The differential roles of estrogen receptor subtypes ERα and ERβ for neuroprotection remains unresolved. For instance, Gollapudi and Oblinger [16] showed that in PC12 cells transfected with only ERα, 17β-estradiol (E_2) enhanced the viability of cells against serum deprivation. Moreover, in studies using ERα and β knockout mice, Dubal et al. [13] found that ERα was required for the protective effects of E_2 against brain injury. However, the findings of Wang et al. [48] indicated the importance of ERβ in neuronal survival as manifested by developmental abnormalities in the brains of ERβ knockout mice. Rissman et al. [35] also proposed that ERβ plays a pivotal role for mediating E_2-induced learning and memory.

One strategy to address the relative contribution of ERα and ERβ to estrogen-inducible neuroprotection in neurons expressing both receptors is through the use of receptor selective agonists. 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT) and 2,3-bis(4-hydroxyphenyl) propionitrile (DPN) are two nonsteroidal ER subtype-selective agonists synthesized by Katzenellenbogen et al. [25,44]. PPT is a highly selective and specific ERα agonist displaying a 410-fold higher binding selectivity for ERα over ERβ and activates gene transcription only through ERα (Fig. 1) [44]. Furthermore, PPT increased progesterone receptor mRNA and suppressed experimentally induced hot flushes, both of which are known to be estrogen regulated, suggesting its estrogenic effects in the brain evoked by the activation of ERα [18]. In contrast, the estrogen receptor agonist DPN displays a 70-fold higher binding selectivity for ERβ over ERα and is a full ERβ agonist (Fig. 1) [25]. In transcriptional activation studies, DPN exhibited a 170-fold higher relative potency for ERβ over ERα [25]. Based on their ER subtype-selective characteristics, PPT and DPN offer a pharmacological strategy to elucidate the differential contributions of the two ER subtypes in estrogen-induced neuroprotection.

In the present study, using PPT and DPN, we sought to determine the potential role of ER subtypes ERα and ERβ in mediating estrogen neuroprotection and their underlying cellular mechanisms. Since glutamate excitotoxicity is thought to be one of the contributing factors to the neurodegeneration associated with Alzheimer's disease and other neurodegenerative disorders, the neuroprotective efficacy of PPT and DPN against supraphysiological glutamate-induced excitotoxicity was investigated in primary hippocampal neurons. Three independent measures of neuronal response to neurotoxic glutamate were evaluated. Furthermore, in the pursuit of the molecular mechanisms mediating PPT and DPN neuroprotection, their impact on the expression of antiapoptotic protein Bcl-2 in primary hippocampal neurons was also investigated. Results of these analyses indicate that agonists for either ERα or ERβ protect neurons against excitotoxic-induced damage and death.

2. Materials and methods

2.1. Animals

The use of animals was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Southern California (Protocol No. 10256). Pregnant Sprague–Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). They were individually housed under controlled conditions of temperature (18–24 °C), humidity (30–70%) and light (12:12-h light/dark), and received food and water ad libitum.

2.2. Chemicals

17β-Estradiol was purchased from Steraloids (Newport, Rhode Island). PPT and DPN were purchased from Tocris Cookson (Ellisville, MO). Chemicals were dissolved in analytically pure ethanol at 1 mM and diluted in culture medium to the final concentrations. The vehicle-treated cultures received the same amount of ethanol as present in the highest dose of chemicals.

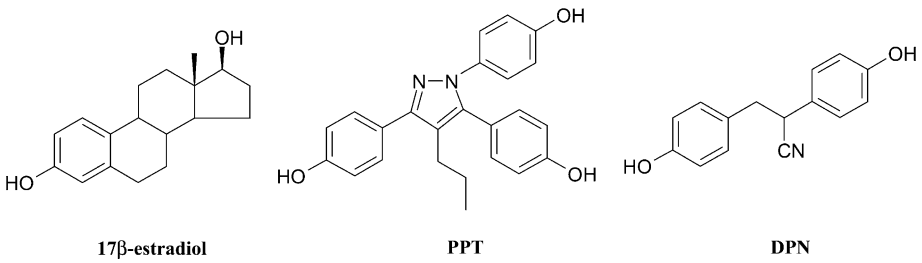

Fig. 1. Chemical structures of 17β-estradiol (E_2), PPT and DPN.

2.3. Neuronal cultures

Primary cultures of hippocampal neurons were obtained from Embryonic Day 18 (E18d) rat fetuses as previously described [6]. Briefly, after dissected from the brains of the rat fetuses, the hippocampi were treated with 0.02% trypsin in Hank's balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.34 mM $Na_2HPO_4 \cdot 7H_2O$, 10 mM glucose, and 10 mM HEPES) for 5 min at 37 °C and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. Between 2×10^4 and 4×10^4 cells were plated onto poly-D-lysine (10 μg/ml)-coated 22 mm coverslips in covered 35 mm petri dishes for morphological analysis, and 1×10^5 cells/ml were plated onto poly-D-lysine-coated 24-well, 96-well culture plates or $3-5 \times 10^5$ cells/ml onto 0.1% polyethylenimine-coated 60 mm petri dishes for biochemical analyses. Nerve cells were grown in phenol-red free Neurobasal medium (NBM, Invitrogen, Carlsbad, CA) supplemented with B27, 5 U/ml penicillin, 5 μg/ml streptomycin, 0.5 mM glutamine and 25 μM glutamate at 37 °C in a humidified 10% CO_2 atmosphere for the first 3 days and NBM without glutamate afterwards. Cultures grown in serum-free Neurobasal medium yields approximately 99.5% neurons and 0.5% glial cells.

2.4. Estrogen receptors mRNA expression with RT-PCR

Total RNA samples were isolated from rat primary hippocampal neurons plated onto 60 mm petri dishes with Trizol reagent (Invitrogen) following the manufacturer's instructions. The RNA concentration was determined by spectrophotometry. Reverse transcription reactions were performed for each RNA sample using 4 μg of total RNA with First-Strand cDNA Synthesis Kit (Amersham Bioscience, Buckinghamshire, England). RNA samples were heated to 65 °C for 5 min and cooled on ice. Samples were mixed with RT buffer containing 10 mM DDT, 20 μM each of dATP, dCTP, dGTP, and dTTP, 1 μM of oligo$(dN)_6$,and 25 units of AMV reverse transcriptase. After incubation at 37 °C for 1 h, the mixture was heated to 95 °C for 5 min prior to storage at −20 °C.

Master mixes for PCR reaction were used for each sample. The PCR reaction mixture contained forward and reverse primers (20 pmol each), dNTPs (200 μM each as final concentration), 10× PCR buffer, Taq DNA polymerase (0.5 units) (Boehringer-Mannheim, Indiana, USA), and 5 μl of RT mixture as the source of cDNA. PCR reactions were carried out with a common forward primer for both ERα and ERβ (5′-AAGAGCTGCCAGGCCTGCC-3′) and specific reverse primers for ERα (5′-TTGGCAGCTCTCATGTCTCC-3′) and ERβ (5′-GCATGCTGGGACGGCTCACCA-3′) to generate specific PCR products for both receptor subtypes in a signal PCR reaction [33]. The size of fragments amplified from ERα and ERβ was 166 and 264 base pairs, respectively. β-Actin was used as a semi-quantitative control and the primer sets were purchased from Invitrogen. The PCR cycles were 1 min 10 s at 94 °C, 1 min 10 s at 60 °C, and 1 min 55 s at 72 °C for 35 cycles.

2.5. Glutamate exposure

Seven-day-old primary hippocampal neurons were pretreated with test compounds for 48 h followed by exposure to 100 μM glutamate for 5 min at room temperature in HEPES buffer containing 100 mM NaCl, 2.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 10.0 mM glucose and 12.5 mM HEPES. Immediately following glutamate exposure, cultures were washed once with HEPES buffer and replaced with fresh Neurobasal medium containing the test compounds. Cultures were returned to the culture incubator and allowed to incubate for 24 h prior to cell viability measurements on the following day.

2.6. Measurement of LDH release

Lactate dehydrogenase (LDH) release from the cytosol of damaged cells into the culture medium after glutamate exposure was measured using a Cytotoxicity Detection Assay (Roche Diagnostics, Indianapolis, IN), which determines the LDH activity in the culture medium to enzymatically convert the lactate and NAD^+ to pyruvate and NADH. The tetrazolium salt produced in the enzymatic reaction was then reduced to red formazan in the presence of H^+, thereby allowing a colorimetric detection for neuronal membrane integrity.

Seven-day-old primary hippocampal neurons grown in 24-well plates were pretreated with test compounds for 48 h prior to exposure to 100 μM glutamate and an additional incubation with test compounds for 24 h, followed by LDH measurements on the following day. The measurement of LDH release was conducted according to the manufacturer's instructions. Briefly, 80 μl of culture medium from each well was transferred to a 96-well plate and 80 μl of Cytotoxicity Detection Reagent was added to incubate for 30 min followed by the addition of 40 μl of 1 N HCl to stop the reaction. Colorimetric absorbance was measured with a EL311SX spectrophotometer at 490 nm (Bio-Tek Instruments, Winooski, VT). The rest medium in 24-well plates was aspirated off and the protein concentration was determined using the BCA Protein Assay (Pierce Biotechnology, Rockford, IL). LDH release was normalized to protein level per well before analysis of the data. Data are normalized against the amount of LDH release from vehicle-treated control cultures receiving no glutamate. Data are presented as the mean ± S.E.M. and derived from a single experiment and are representative of at lease three separate experiments.

2.7. Measurement of ATP level

Intracellular ATP levels were determined by a luciferin/luciferase-based method with the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI), which uses ATP, a required co-factor of the luciferase reaction, producing oxyluciferin and releasing energy in the form of luminescence that is proportional to the amount of ATP present, which further signals the presence of metabolically active cells.

Seven-day-old primary hippocampal neurons seeded into solid white and clear bottom 96-well plates were pretreated with test compounds for 48 h followed by exposure to 100 μM glutamate for 5 min. Cultures were returned to fresh medium with test compounds and incubated for 24 h prior to ATP measurement. Briefly, a half volume of culture medium (100 μl/well) was aspirated off and the same volume of freshly prepared CellTiter-Glo Reagent was added to make the final volume 200 μl in each well. The resulting contents were mixed by agitation on an orbital shaker for 10 min to induce cell lysis that permitted the release of cellular ATP into the medium. The plates were then allowed to incubate at room temperature for an additional 10 min to stabilize the luminescence signal prior to luminescence detection with a Lmax microplate luminometer (Molecular Devices, Sunnyvale, CA). Data are normalized against the ATP levels in vehicle-treated control cultures receiving no glutamate. Data are presented as the mean ± S.E.M. and derived from a single experiment and are representative of at lease three separate experiments.

2.8. Assessment of live/dead cells by dual staining with calcein AM and ethidium homodimer

Cell viability was further investigated using dual staining with calcein AM and ethidium homodimer-1 (Molecular Probes, Eugene, OR). The combined use of calcein AM and ethidium homodimer provides a two-color fluorescence analysis that allows simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability, intracellular esterase activity and plasma membrane integrity, respectively. Calcein AM is a fluorogenic esterase substrate that enters the live cells through permeability and is enzymatically hydrolyzed to form the polyanionic dye calcein, which is well retained within live cells due to the intact plasma membranes and produces an intense uniform green fluorescence at 530 nm. Ethidium homodimer is excluded from live cells and only able to enter cells through compromised plasma membranes and binds to nucleic acid by intercalating between the base pairs, producing a bright red fluorescence at 645 nm. Therefore, calcein AM and ethidium homodimer serve as two indicators for the identification of live and dead cells, respectively.

To obtain the optimal dye concentration and incubation time, a progressive analysis using various concentrations of both dyes (0.1–10 μM) at various incubation time intervals (10–100 min) with live cells and dead cells (dead cells were obtained by incubation with 70% methanol for 30 min), respectively, was initially conducted. A use of 1 μM of calcein AM and 2 μM of ethidium homodimer at room temperature for 30 min was found to be the optimal condition and was used in the following experiments.

Seven-day-old primary hippocampal neurons seeded into solid black and clear bottom 96-well plates were pretreated with test compounds for 48 h before exposure to glutamate and then incubated with test compounds for an additional 24 h before assessment of the cell viability and cytotoxicity on the following day. Cultures were rinsed with phosphate-buffered saline (PBS) and incubated with the combined 1 μM calcein AM and 2 μM ethidium homodimer PBS solution at room temperature for 30 min. The fluorescence intensities were measured on a SpectraMax GEMINI EM dual-wavelength-scanning microplate spectrofluorometer (Molecular Devices) using appropriate excitation and emission filter combinations (485–530 nm for calcein AM and 530–645 nm for ethidium homodimer). Data are normalized against the values from vehicle-treated control cultures receiving no glutamate. Data are presented as the mean ± S.E.M. and derived from a single experiment and are representative of at lease three separate experiments.

For microscopic analyses, 7-day-old primary hippocampal neurons grown on glass coverslips were treated with test compounds and glutamate as described above. Cultures were then rinsed once with PBS and incubated with the combined 1 μM calcein AM and 2 μM ethidium homodimer PBS solution at room temperature for 30 min. Following incubation, the coverslip was removed from the culture dish using a fine-tipped forceps and mounted into a recording chamber covered with the dye solution. The labeled neuronal cells were viewed under a Axiovert 200 M Marianas Digital Microscopy Workstation (Intelligent Imaging Innovations, Denver, CO) using a × 40 objective.

2.9. Bcl-2 expression with Western Blot

Seven-day-old primary hippocampal neurons were pretreated with test compounds for 48 h before the cells were lysed by incubation in ice-cold lysis buffer containing: 0.005% SDS, 0.1% Igepal, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonylfluoride and protease inhibitor mixture in PBS at 4 °C for 45 min. Cell lysates were centrifuged at 10,000 rpm at 4 °C for 10 min, and the concentration of protein in the supernatant was determined using the BCA Protein Assay (Pierce Biotechnology, Rockford, IL). Twenty-five micrograms of total protein was diluted in 15 μl 2 × SDS containing sample buffer and the final volume was made 30 μl with water. After denaturalization on a hot plate at 95–100 °C for 5 min, 25 μl of the mixture was loaded per lane on 10% SDS-polyacrylamide mini-gels followed by electrophoresis at 90 V. The proteins were then electro-transferred to polyvinylidene difluoride

membranes (Millipore, Bedford, MA) from the gels. Nonspecific binding sites were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween-20 (PBS-Tween). Membranes were incubated with the primary monoclonal antibody against Bcl-2 (Zymed Laboratories, San Francisco, CA) diluted 1:250 in PBS-Tween with 1% horse serum (Vector Laboratories, Burlingame, CA) overnight at 4 °C, then incubated with the secondary horseradish peroxidase (HRP)-conjugated horse anti-mouse IgG (Vector Laboratories) diluted 1:5000 in PBS-Tween with 1% horse serum for 2 h at room temperature, and Bcl-2 proteins were visualized by developing the membranes with TMB substrate for peroxidase (Vector Laboratories). β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) level was determined to ensure equal protein loading, and high-range Precision Protein Standards (Bio-Rad Laboratories, Hercules, CA) was used to determine protein sizes. Relative intensities of bands were quantified by optical density analysis using an image digitizing software Un-Scan-It version 5.1 (Silk Scientific, Orem, UT). Data are normalized against the Bcl-2 level detected in the vehicle-treated control cultures receiving no glutamate. Data are presented as the mean ± S.E.M. for at least three independent experiments.

2.10. Statistics

Statistically significant differences between groups were determined by a one-way analysis of variance (ANOVA), followed by a Newman–Keuls post hoc analysis.

3. Results

3.1. Expression of estrogen receptors in primary cultures of rat hippocampal neurons

To ascertain the expression of estrogen receptors in primary cultures of rat hippocampal neurons used in the present study, total RNAs were isolated from hippocampal neuronal cultures following 1 to 14 days and reverse transcription PCR was performed (Fig. 2). RNA isolated from rat ovary served as positive control. cDNA synthesized from the same amount of total RNA in each sample was used as the PCR template. Optical density photometry was conducted for both RT-PCR estrogen receptor products and β-actin. Relative quantification of ERα and ERβ mRNA expression levels was normalized to β-actin. As shown in Fig. 2, the PCR amplified fragments of both ERα and ERβ cDNA were detectable 24 h after neurons were cultured. Expression levels of ERα mRNA at 4, 7, 10, and 14 days in vitro (DIV) were significantly higher than at 1 DIV (Fig. 2; 153.9 ± 13.2%, 232.3 ± 14.1%, 250.7 ± 19.3% and 219.0 ± 11.8%, respectively, relative to 1 DIV, $^{*}P<0.05$). There was a significant rise of the ERα messenger level at 4 DIV and a significant increase between 4 DIV and all time points after 7 DIV ($^{+}P<0.05$). The magnitude of increase

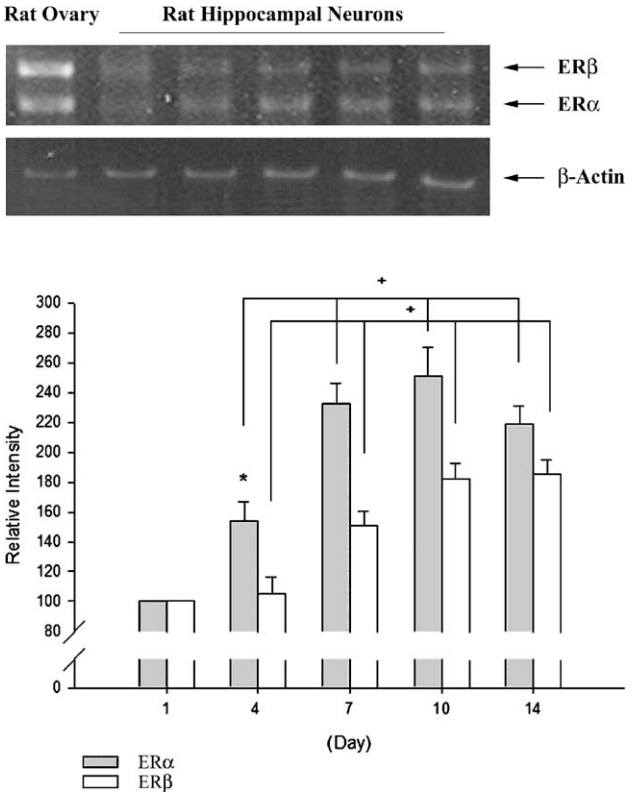

Fig. 2. Expressions of estrogen receptors in primary cultures of rat hippocampal neurons. RNA samples were collected from rat primary hippocampal neurons on 1, 4, 7, 10, and 14 day after culture and subjected to RT-PCR. Specific PCR products amplified from ERα and ERβ were indicated. RNA isolated from rat ovary tissue was used as positive control. Representative results from four separate samples are shown. $^{*}P<0.05$ compared to 1-day receptor expression, $^{+}P<0.05$ compared to 4-day receptor expression.

remained constant from 7 to 14 DIV. ERβ expressions also increased significantly at 7, 10, and 14 DIV compared to 1 and 4 DIV (Fig. 2; 150.8 ± 9.4%, 181.8 ± 11.0% and 185.2 ± 9.3, respectively, relative to 1 DIV, $^{+}P<0.05$). Again, the magnitude of the increase remained constant between 7 and 14 DIV. Based on these analyses, the subsequent biochemical analyses were performed in hippocampal neurons at 7 DIV.

3.2. Effect of PPT and DPN on glutamate-induced excitotoxicity in primary hippocampal neurons by LDH measurements

The neuroprotective efficacy and dose response profile of the ERα specific agonist PPT against excitotoxic glutamate exposure in primary hippocampal neurons was evaluated by measuring LDH release into the culture medium. Cultures were pretreated with various concentrations of PPT for 48 h prior to exposure to excitotoxic glutamate, which was followed by LDH measurement 24 h later. Exposure of primary hippocampal neurons to 100 μM glutamate for 5 min induced a significant increase in LDH release (Fig. 3;

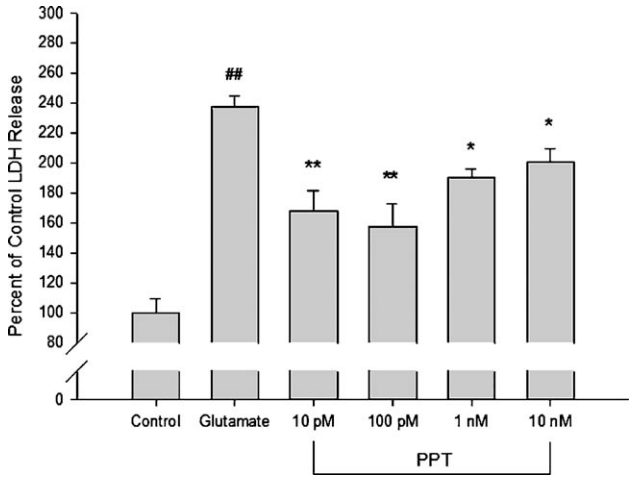

Fig. 3. Effect of PPT against glutamate-induced neurotoxicity in primary hippocampal neurons. Seven-day-old primary hippocampal neurons were treated with PPT at various concentrations for 48 h followed by a 5-min exposure to 100 μM glutamate. The amount of LDH released into the culture medium was measured 24 h later. Results are presented as the percent of LDH release from vehicle-treated control cultures and expressed as mean ± S.E.M., $n \geq 4$. Data are derived from a single experiment and are representative of at lease three separate experiments. $^{\#\#}P < 0.01$ compared to vehicle-treated control cultures, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to glutamate alone-treated cultures.

237.8 ± 6.6% relative to vehicle-treated control cultures, $^{\#\#}P < 0.01$). Pretreatment of PPT at various concentrations (10 pM to 10 nM) significantly reduced glutamate-induced LDH release (Fig. 3; 168.1 ± 13.4%, 157.5 ± 15.4%, 189.9 ± 6.1% and 200.7 ± 9.3%, respectively, relative to vehicle-treated control cultures, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to glutamate alone-treated cultures), with a rank order of efficacy of 10 pM < 100 pM>1 nM>10 nM (Fig. 3; $P < 0.05$ between 100 pM and 10 nM treatments). Based on the rank order of efficacy, the maximal effective dose 100 pM of PPT was used in the subsequent comparative analyses.

As conducted for PPT, the effect of the ERβ selective agonist DPN against glutamate-induced excitotoxicity was also evaluated by LDH release in primary hippocampal neurons. Similar to PPT, pretreatment of DPN (10 pM to 10 nM) significantly reduced 100 μM glutamate-induced LDH release with the same trend of 10 pM < 100 pM>1 nM>10 nM (Fig. 4; 171.6 ± 8.6%, 156.7 ± 8.0%, 172.4 ± 4.0% and 181.7 ± 6.4% relative to vehicle-treated control cultures, $^{**}P < 0.01$ compared to glutamate alone-treated cultures, $P < 0.05$ between 100 pM and 10 nM treatments). Based on the rank order of efficacy, the maximal effective dose 100 pM of DPN was used in the subsequent comparative analyses.

3.3. Comparison of neuroprotective efficacy of E_2, PPT and DPN against excitotoxic glutamate-induced plasma membrane damage

Primary hippocampal neurons were pretreated with estrogenic compounds for 48 h prior to glutamate exposure. LDH measurements were performed 24 h after glutamate treatment. As observed above, 5-min exposure of 100 μM glutamate in hippocampal neurons induced a significant increase of LDH release into the culture medium (Fig. 5; 168.7 ± 5.9% relative to vehicle-treated control cultures, $^{\#\#}P < 0.01$). As expected, E_2 treatment (10 nM) induced the greatest reduction in LDH release (Fig. 5; 120.0 ± 4.2% relative to vehicle-treated control cultures, $^{**}P < 0.01$ compared to glutamate alone-treated cultures). PPT (100 pM), DPN (100 pM) and PPT plus DPN treatment induced significant reduction in LDH release (Fig. 5; 141.1 ± 3.5%, 133.8 ± 3.5% and 150.5 ± 6.7%, respectively, relative to vehicle-treated control cultures, $^{**}P < 0.01$ compared to glutamate alone-treated cultures), though at a lower efficacy than E_2 (Fig. 5; $^{+}P < 0.05$ and $^{++}P < 0.01$ compared to E_2-pretreated cultures). Surprisingly, the combination of PPT and DPN was less efficacious than either PPT or DPN alone (Fig. 5; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ compared to PPT plus DPN-pretreated cultures).

3.4. Comparison of neuroprotective efficacy of E_2, PPT and DPN against excitotoxic glutamate-induced reduction in cellular metabolic activity

Primary hippocampal neurons were pretreated with estrogenic compounds for 48 h prior to glutamate exposure. Assessment of ATP was performed 24 h after glutamate treatment. As shown in Fig. 6, 5-min exposure to 100 μM glutamate in hippocampal neurons induced a significant decline in intracellular ATP level (86.2 ± 1.0% relative to vehicle-treated control cultures, $^{\#\#}P < 0.01$). As observed in

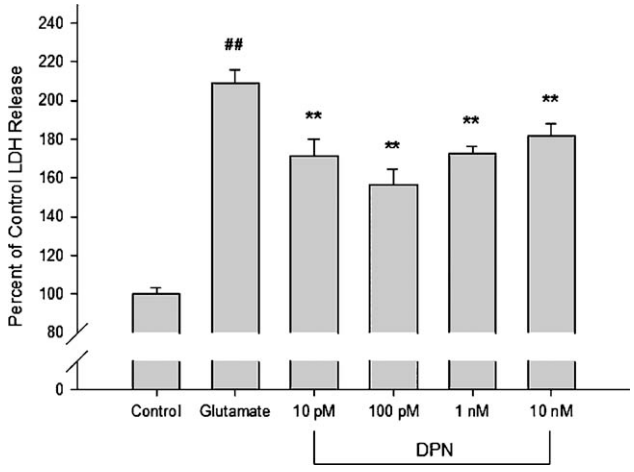

Fig. 4. Effect of DPN against glutamate-induced neurotoxicity in primary hippocampal neurons. Seven-day-old primary hippocampal neurons were treated with DPN at various concentrations for 48 h followed by a 5-min exposure to 100 μM glutamate. The amount of LDH released into the culture medium was measured 24 h later. Results are presented as the percent of LDH release from vehicle-treated control cultures and expressed as mean ± S.E.M., $n \geq 4$. Data are derived from a single experiment and are representative of at lease three separate experiments. $^{\#\#}P < 0.01$ compared to vehicle-treated control cultures, $^{**}P < 0.01$ compared to glutamate alone-treated cultures.

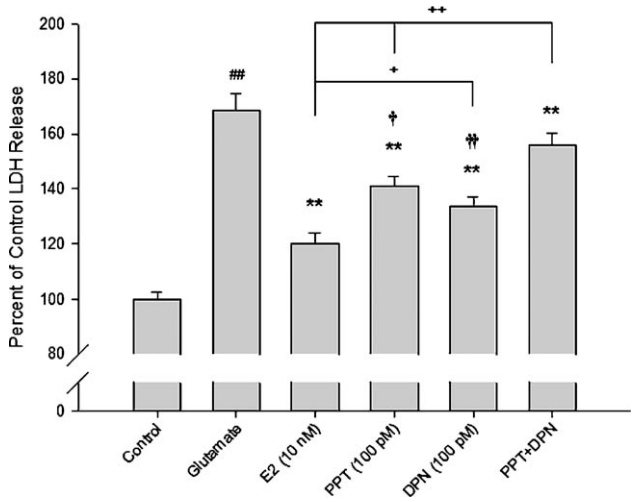

Fig. 5. Effects of E_2, PPT, DPN and PPT plus DPN against glutamate-induced neurotoxicity in primary hippocampal neurons by LDH measurements. Seven-day-old primary hippocampal neurons were treated with various compounds for 48 h followed by a 5-min exposure to 100 μM glutamate. The amount of LDH released into the culture medium was measured 24 h later. Results are presented as the percent of LDH release from vehicle-treated control cultures and expressed as mean ± S.E.M., $n \geq 8$. Data are derived from a single experiment and are representative of at lease three separate experiments. $^{\#\#}P < 0.01$ compared to vehicle-treated control cultures, $^{**}P < 0.01$ compared to glutamate alone-treated cultures, $^{+}P < 0.05$ and $^{++}P < 0.01$ compared to E_2-pretreated cultures. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ compared to PPT plus DPN-pretreated cultures.

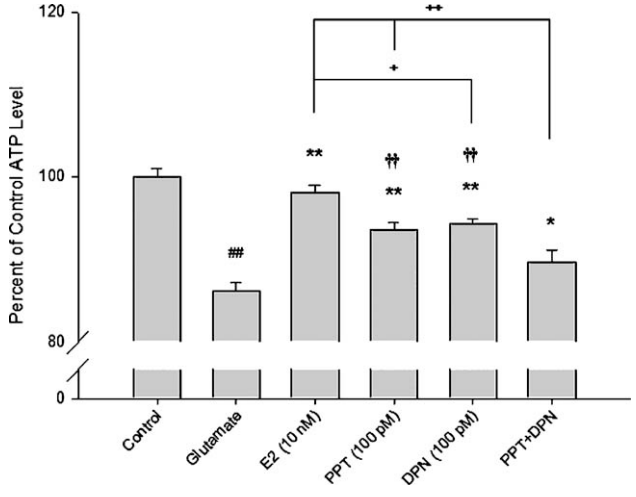

Fig. 6. Effects of E_2, PPT, DPN and PPT plus DPN against glutamate-induced neurotoxicity in primary hippocampal neurons by ATP measurements. Seven-day-old primary hippocampal neurons were treated with various compounds for 48 h followed by a 5-min exposure to 100 μM glutamate. The amount of ATP was measured by a CellTiter Glo assay 24 h later. Results are presented as the percent of ATP level in vehicle-treated control cultures and expressed as mean ± S.E.M., $n \geq 8$. Data are derived from a single experiment and are representative of at lease three separate experiments. $^{\#\#}P < 0.01$ compared to vehicle-treated control cultures, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to glutamate alone-treated cultures, $^{+}P < 0.05$ and $^{++}P < 0.01$ compared to E_2-pretreated cultures. $^{\dagger\dagger}P < 0.01$ compared to PPT plus DPN-pretreated cultures.

LDH experiments, pretreatment of cultured neurons with E_2 (10 nM) exhibited the greatest protective effect against glutamate-induced ATP decline (Fig. 6; 98.1 ± 1.0% relative to vehicle-treated control cultures, $^{**}P < 0.01$ compared to glutamate alone-treated cultures). Pretreatment of neurons

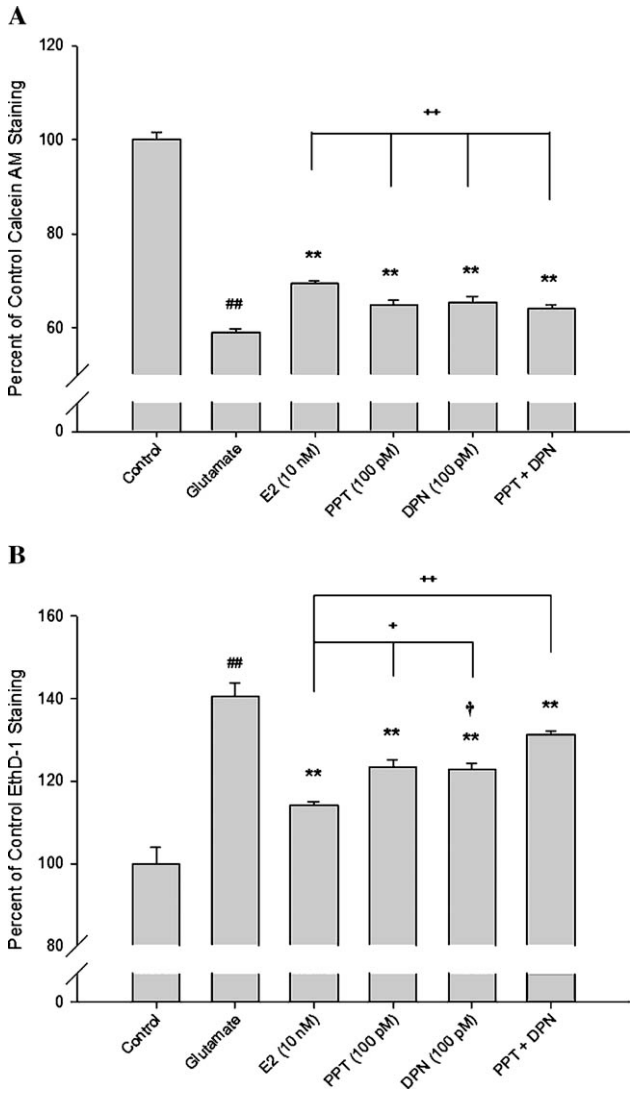

Fig. 7. Effects of E_2, PPT, DPN and PPT plus DPN against glutamate-induced neurotoxicity in primary hippocampal neurons by calcein AM/ethidium homodimer analysis. Seven-day-old primary hippocampal neurons were treated with various compounds for 48 h followed by a 5-min exposure to 100 μM glutamate. (A) The amount of fluorescence generated by calcein, green fluorescent product derived from enzymatic esteration of calcein AM by esterases in live cells, was measured at 485–530 nm. (B) The amount of fluorescence generated by ethidium homodimer, which enters cells through damaged plasma membrane and produces a red fluorescence, was measured at 530–645 nm. Results are presented as the percent of fluorescence produced in vehicle-treated control cultures and expressed as mean ± S.E.M., $n \geq 8$. Data are derived from a single experiment and are representative of at lease three separate experiments. $^{\#\#}P < 0.01$ compared to vehicle-treated control cultures, $^{**}P < 0.01$ compared to glutamate alone-treated cultures, $^{+}P < 0.05$ and $^{++}P < 0.01$ compared to E_2-pretreated cultures. $^{\dagger}P < 0.05$ compared to PPT plus DPN-pretreated cultures. EthD-1: ethidium homodimer.

with PPT (100 pM), DPN (100 pM) or PPT plus DPN significantly protected against glutamate-induced ATP decline (Fig. 6; 93.6 ± 0.9%, 94.4 ± 0.5% and 89.7 ± 1.4%, respectively, relative to vehicle-treated control cultures, $*P<0.05$ and $**P<0.01$ compared to glutamate alone-treated cultures), but at a lower efficacy than E_2 (Fig. 6; $^{+}P<0.05$ and $^{++}P<0.01$ compared to E_2-pretreated cultures). Similar to the observation in LDH measurements, coadministration of PPT and DPN was less efficacious than either PPT or DPN alone (Fig. 6; $^{\dagger\dagger}P<0.01$ compared to PPT plus DPN-pretreated cultures).

3.5. Comparison of neuroprotective efficacy of E_2, PPT and DPN against excitoxotic glutamate-induced reduction in intracellular esterase activity and plasma membrane integrity

To confirm the results obtained based on LDH release and ATP measurements, simultaneous assessment of cell viability and cytotoxicity by dual staining with calcein AM (staining live cells) and ethidium homodimer (staining dead cells) was conducted in primary hippocampal neurons.

Primary hippocampal neurons were pretreated with estrogenic compounds for 48 h prior to exposure to excitotoxic glutamate. Biochemical assessment and morphological analysis of live and dead cells were conducted 24 h following glutamate treatment. As shown in Fig. 7, 5-min exposure of 100 μM glutamate in primary hippocampal neurons induced a significant decrease in cell viability (Fig. 7A; 59.0 ± 0.9% relative to vehicle-treated control cultures, $^{\#\#}P<0.01$) and an increase in cell death (Fig. 7B; 140.6 ± 3.0% relative to vehicle-treated control cultures, $^{\#\#}P<0.01$). Consistent with the results from LDH and ATP experiments, pretreatment with E_2 (10 nM) induced a significant protective effect against glutamate-induced decline in cell viability (Fig. 7A; 69.4 ± 0.6% relative to vehicle-treated control cultures, $**P<0.01$ compared to glutamate alone-treated cultures) and cell death (Fig. 7B; 114.0 ± 1.0% relative to vehicle-treated control cultures, $**P<0.01$ compared to glutamate alone-treated cultures). PPT (100 pM), DPN (100 pM) or PPT plus DPN treatment were less efficacious than E2 (Fig. 7A; 65.0 ± 0.9%, 65.3 ± 1.4% and 64.1 ± 0.8%, respectively, relative to vehicle-treated control cultures; Fig. 7B; 123.4 ± 1.7%, 122.8 ± 1.6% and 131.4 ± 0.7%, respectively, relative to vehicle-treated control cultures, $**P<0.01$ compared to glutamate alone-treated cultures, $^{+}P<0.05$ and $^{++}P<0.01$ compared to E_2-pretreated cultures). Treatment of neurons with the combination of PPT plus DPN was slightly less effective against glutamate-induced decrease in neuron viability and survival than either PPT or DPN treatment alone (Fig. 7B; $^{\dagger}P<0.05$ compared to PPT plus DPN-pretreated cultures).

The quantitative biochemical analyses were confirmed by microscopic observation of calcein AM/ethidium homodimer staining shown in Fig. 8. Live cells stained with

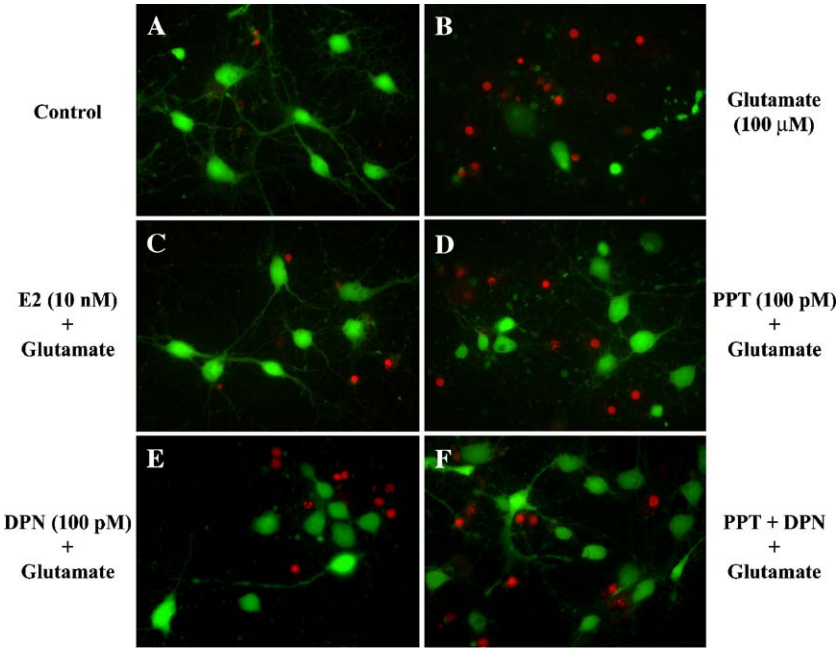

Fig. 8. Representative calcein AM/ethidium homodimer fluorescence micrographs of primary hippocampal neurons. Seven-day-old primary hippocampal neurons were treated with E_2, PPT, DPN or PPT plus DPN for 48 h prior to a 5-min exposure to 100 μM glutamate, followed by a two-color staining with calcein AM and ethidium homodimer 24 h after. Green fluorescence is produced by enzymatic product of calcein AM in live cells. Red fluorescence is produced by ethidium homodimer in dead cells. (A) vehicle-treated control cultures; (B) glutamate alone-treated cultures; (C) E_2-pretreated cultures followed by glutamate exposure; (D) PPT-pretreated cultures followed by glutamate exposure; (E) DPN-pretreated cultures followed by glutamate exposure; (F) PPT plus DPN-pretreated cultures followed by glutamate exposure. EthD-1: ethidium homodimer.

calcein AM are shown in green, whereas dead cells labeled with ethidium homodimer are shown in red. Results of these analyses demonstrate an increase in ethidium homodimer labeling in neurons treated with 100 μM glutamate which was markedly diminished in neurons pretreated with either E_2, PPT, DPN or the combination of PPT and DPN.

3.6. Effect of E_2, PPT and DPN on the expression of antiapoptotic protein Bcl-2

Previous studies from our laboratory and others indicate that upregulation of the antiapoptotic protein Bcl-2 is an obligatory mechanistic pathway leading to estrogen neuroprotection [12,15,29]. To determine whether the selective ERα and ERβ agonists promote expression of this antiapoptotic protein, Western Blot analysis was conducted to determine the protein level of Bcl-2. Primary hippocampal neurons were pretreated with estrogenic compounds for 48 h followed by Western blot measurement. Consistent with the neuroprotection analyses, E_2 pretreatment (10 nM) induced the greatest increase in Bcl-2 expression in hippocampal neurons (Fig. 9; $137.2 \pm 4.1\%$ relative to vehicle-treated control cultures, $^{**}P < 0.01$). PPT (100 pM), DPN (100 pM) and PPT plus DPN treatment also induced a significant increase in Bcl-2 level in neurons (Fig. 9; $121.3 \pm 5.6\%$, $123.0 \pm 3.3\%$ and $109.7 \pm 2.3\%$, respectively, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to vehicle-treated control cultures). Consistent with the results obtained from neuroprotection evaluation, PPT, DPN and PPT plus DPN treatment were less efficacious compared to E_2, which was paralleled in a diminished increase in Bcl-2 expression (Fig. 9; $^{++}P < 0.01$ compared to E_2-pretreated cultures; $^{\dagger}P < 0.01$ compared to PPT plus DPN-pretreated cultures).

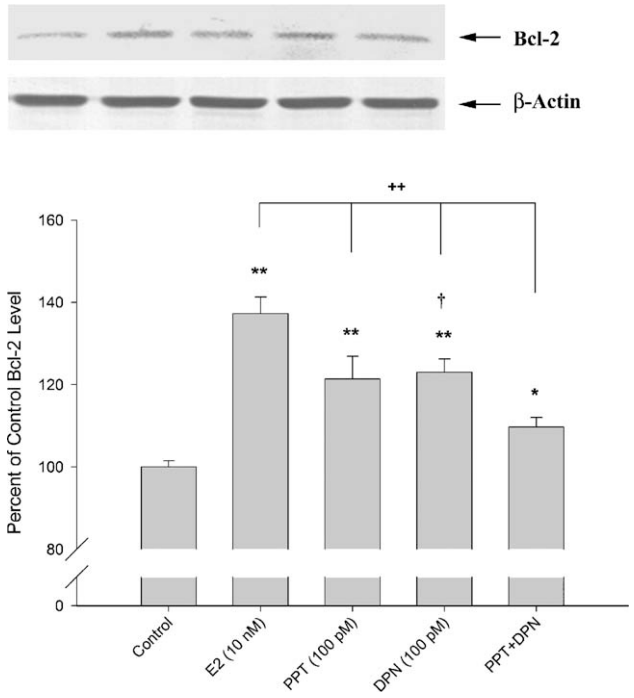

Fig. 9. Bcl-2 expression induced by E_2, PPT, DPN and PPT plus DPN in primary hippocampal neurons. Seven-day-old primary hippocampal neurons were treated with various compounds for 48 h followed by Bcl-2 protein level determination with Western Blot analysis. Results are presented as the percent of Bcl-2 level in vehicle-treated control cultures and expressed as mean ± S.E.M., $n \geq 6$. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to vehicle-treated control cultures, $^{++}P < 0.01$ compared to E_2-pretreated cultures. $^{\dagger}P < 0.05$ compared to PPT plus DPN-pretreated cultures.

4. Discussion

In the present study, the neuroprotective efficacy of two estrogen receptor agonists selective for ERα, PPT, and ERβ, DPN were investigated. Using three indicators of neuroprotection that rely on different aspects of neuronal viability (LDH release, intracellular ATP level, and calcein AM/ethidium homodimer live/dead cell staining), both the ERα, PPT, and ERβ agonist DPN induced significant protection against glutamate-induced neurotoxicity. Furthermore, PPT and DPN significantly increased expression of the antiapoptotic protein Bcl-2 in primary hippocampal neurons. These results indicate that both estrogen receptor subtypes ERα and ERβ can contribute to estrogen-induced neuroprotection. Our data are consistent with recent studies from Dorsa and colleagues who reported that estrogen-mediated neuroprotection against β-amyloid toxicity and oxidative stress is present in a clonal cell line, HT22 cells, that stably express either ERα or ERβ [14].

4.1. Comparison of neuroprotective efficacy of E_2, PPT and DPN against glutamate-induced neurotoxicity

In the current study, multiple neuronal viability and neurotoxicity analyses were conducted to evaluate the neuroprotective efficacy of E_2, PPT and DPN. Results from these analyses consistently demonstrated that E_2 exerted the greatest neuroprotective effect, which was significantly higher than PPT, DPN or PPT plus DPN-induced neuroprotection. However, it should be noted that E_2 induced the greatest neuroprotection at 100 times the optimal concentration required for PPT and DPN. The present data from PPT and DPN suggest that both ER subtypes might be involved in the mechanisms underlying estrogen neuroprotection. Surprisingly, coadministration of PPT and DPN was less efficacious than either PPT or DPN alone, which is most likely due to the result of a yin/yang relationship of two ER subtypes proposed by Weihua et al. [50] where one ER receptor may antagonize the actions of the other. The different ratio and distinct function of homodimer and heterodimer induced by coadministration of PPT and DPN may be another possibility to account for the reduced efficacy exerted by the combination of both agonists.

4.2. Involvement of ERα and ERβ in estrogen neuroprotection

While our data indicate that both ERα and ERβ can contribute to protection against excitotoxicity, the specific contributions of these two ER subtypes for estrogen-induced neuroprotection in vivo remains unresolved. Recent studies through the use of estrogen receptor (ERα, ERβ or ERα/ERβ) knockout mice have advanced our understanding of the biological roles of ERα and ERβ in the brain. For instance, in an in vivo ischemia model, Dubal et al. [13] demonstrated that ERα, not ERβ, is required for the protective effects of E_2 against ischemia-induced injury. In contrast, Gustafsson's group demonstrated that ERβ is crucial for brain development as evidenced by developmental abnormalities in the brains of ERβ knockout mice [48]. The brains of ERβ knockout mice exhibited an increase in apoptotic cells in the ventricular zones of the cerebral cortex that resulted in abnormalities in brain development [49]. Rissman et al. [35] also found that ERβ gene disruption impairs optimal spatial learning in female mice, suggesting the role of ERβ in mediating learning and memory exerted by E_2.

As to the mechanisms underlying ERs-mediated estrogen neuroprotection, recent studies in hippocampal neurons showed that both ERα and ERβ are expressed in non-nuclear regions. Adams et al. [1] found that ERα is expressed at synapse while Zhang et al. [53] demonstrated that ERβ is expressed in both cytoplasmic and neuronal processes, suggesting that ERs may also carry out non-classical functions in mediating estrogen action in hippocampal neurons.

4.3. Impact of E_2, PPT and DPN on the expression of antiapoptotic protein Bcl-2

Proapoptotic and antiapoptotic Bcl-2 family proteins play a central role in regulating programmed cell death [10] and have been associated with various neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) [3,19,37,38,45]. It is well documented that overexpression of antiapoptotic Bcl-2 prevents the release of cytochrome *c* from mitochondria that further activates the caspase cascade leading to apoptotic cell death after cytotoxic insults [10,34]. Moreover, Bcl-2 localized in the mitochondria can function to promote survival factor capable of suppressing mitochondrial membrane potential depolarization [11], ATP depletion [22] and lipid peroxidation triggered by cytotoxic stimuli such as oxidative stress [20].

Recent studies have proposed that upregulation of the antiapoptotic protein Bcl-2 is a potential mechanistic pathway leading to E_2 neuroprotection. In earlier studies, Garcia-Segura et al. [15] found that Bcl-2 expression in neurons was decreased in ovariectomized rats and that E_2 treatment induced a dose-dependent reversal to increase Bcl-2 expression following ovariectomy. In disease model paradigms, Dubal et al. [12] demonstrated that in a cerebral ischemia model, E_2 prevented the ischemic injury-induced downregulation of Bcl-2 expression. Bcl-2 was also implicated in E_2-induced regulation of mitochondrial calcium tolerability. Studies from our laboratory showed that E_2-induced attenuation of the rise in bulk-free calcium ions in response to excitotoxic glutamate, which has been shown to be the initial event involved in E_2-induced neuroprotection, is correlated to an increase in Bcl-2 expression, which could enhance the mitochondrial tolerability of increased calcium [29].

The present data indicate that pretreatment with both PPT and DPN significantly increased Bcl-2 expression in hippocampal neurons, suggesting that both ERα and ERβ can induce Bcl-2 upregulation. Furthermore, the magnitude of Bcl-2 upregulation was comparable to the efficacy for promoting neuron survival suggesting that Bcl-2 expression could serve as an indicator of neuroprotective efficacy.

4.4. Functional significance for the development of therapeutic interventions for the treatment of neurodegenerative diseases

The present data suggest that both ERα and ERβ can contribute to neuroprotection exerted by estrogen, which raises the possibility of developing novel estrogen alternatives for the brain. Estrogen therapy (ET) can reverse the memory/cognitive deficits associated with a loss in circulating estrogen as occurs in naturally or surgically induced menopause [21,31]. Moreover, ET has been associated with a reduced risk of Alzheimer's disease [51,52], although the recent Women's Health Initiative Memory Study (WHIMS) reported that coadministration of estrogen and medroxyprogesterone acetate increased the risk for probable dementia in postmenopausal women aged 65 years or older [41].

Despite the encouraging epidemiological data indicating a reduced risk of developing Alzheimer's disease in women who have received ET, only 25% of eligible postmenopausal women elect to receive prescribed ET and, of that, about 50% discontinue use within the first year of therapy, and more than 70% of those for whom it has been prescribed are not compliant [4]. The principle reason women forego ET is the fear of developing breast cancer [17]. Thus, estrogen alternatives that could exert estrogen agonist properties in brain and bone, while exerting estrogen antagonist action in the breast and uterus, have attracted considerable attention [2,36,54]. Development of selective estrogen receptor modulators for the brain (NeuroSERMs) could potentially benefit menopausal women who are at risk for cognitive aging and neurodegenerative disorders [5].

An issue that remains unresolved is the biochemical basis for the tissue specificity of the action of selective estrogen receptor modulators (SERMs). Estrogenic or anti-estrogenic profiles of biologic response of SERMs in distinct tissues depend on the estrogen receptor subtypes expression, the conformation of the SERM-receptor complex, the estrogen-receptor-signaling pathway, and the tissue co-activator and

co-repressor composition, all of which impose complexity and challenge for the development of an ideal NeuroSERM for the brain [5].

The observation that two ER subtypes have distinct distribution patterns in different tissues, specifically in the CNS and peripheral system, predicts their functional preference in specific tissues. Both receptors are highly expressed in the brain, though ERβ has a higher level of expression in regions such as the basal forebrain and cerebral cortex [9,40] while ERα has a higher level in the hippocampus in human [46], suggesting both receptors could contribute to learning and memory function in the brain, which is further indicated by the present study that both receptors can contribute to estrogen neuroprotection. Meanwhile, little or no expression of ERβ is detected in reproductive tissues such as the uterus, where ERα has been shown to mediate the sexual characteristics of estrogen effect [39], which is consistent with the earlier findings from ERα knockout mice in which estrogen was unable to stimulate significant uterine weight gain [8] and is further confirmed by the later findings using PPT [18]. PPT was shown to be as efficacious as 17-ethinyl-17-estradiol, an ER subtype nonselective ligand, in increasing uterine weight and upregulating complement three gene expression in an in vivo model, suggesting that stimulation of ERα is sufficient to elicit a full uterotrophic response and that ERβ is not required for these estrogenic effects [18]. Taken together, these findings make it plausible to discover and design ERβ selective molecules to exert estrogenic action specifically in the brain, although this might be at the cost of lower potency due to the lack of activation of ERα in the brain. With minimal peripheral side effects, these ERβ selective agonists could be used to promote memory function and protection against neurodegeneration associated with Alzheimer's disease.

In conclusion, the present data indicate that both ERα and ERβ agonists can exert significant neuroprotection in primary hippocampal neurons, and that their neuroprotective efficacy is correlated with the magnitude of upregulation of the anti-apoptotic protein Bcl-2. This study raises the possibility for the development of novel pharmacological interventions against neuronal deficits and neurodegenerative disorders associated with aging. Specifically, taking advantage of the differential distributions of the two estrogen receptor subtypes in the CNS and peripheral system, discovery and design of ERβ selective molecules could provide a strategy for activating the beneficial effects of estrogen in the brain without activating untoward effects of estrogen in the breast or uterus.

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