Journal of Neurochemistry, 2002, **82**, 674–682

Estrogen-mediated neuroprotection against β-amyloid toxicity requires expression of estrogen receptor α or β and activation of the MAPK pathway

Jennifer L. Fitzpatrick,*[,1] Amy L. Mize,*[,1] Christian B. Wade,* Julie A. Harris,† Robert A. Shapiro‡ and Daniel M. Dorsa*[,‡]

**Department of Pharmacology, †Graduate Program in Neurobiology and Behavior, and ‡Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, Washington, USA*

Abstract

It is well documented that estrogen can activate rapid signaling pathways in a variety of cell types. These non-classical effects of estrogen have been reported to be important for cell survival after exposure to a variety of neurotoxic insults. Since direct evidence of the ability of the estrogen receptors (ERs) α and/or β to mediate such responses is lacking, the hippocampal-derived cell line HT22 was stably transfected with either ERα (HTERα) or ERβ (HTERβ). In HTERα and HTERβ cells, but not untransfected cells, an increase in ERK2 phosphorylation was measured within 15 min of 17β-estradiol treatment. The ER antagonist ICI 182, 780 (1 μM) and the MEK inhibitor, PD98059 (50 μM) blocked this increase in ERK2 phosphorylation. Treatment of HT22, HTERα and HTERβ cells with the β-amyloid peptide (25–35) (10 μM) resulted in a significant decrease in cell viability. Pre-treatment for 15 min with 10 nM 17β-estradiol resulted in a 50% increase in the number of living cells in HTERα and HTERβ cells, but not in HT22 cells. Finally, ICI 182, 780 and PD98059 prevented 17β-estradiol-mediated protection. This study demonstrates that both ERα and ERβ can couple to rapid signaling events that mediate estrogen-elicited neuroprotection.

Keywords: β-amyloid, ERK2, estrogen receptor, estrogen, HT22, neuroprotection.

J. Neurochem. (2002) **82,** 674–682.

The estrogen receptors belong to the steroid hormone receptor superfamily and function as ligand-activated transcription factors (MacGregor and Jordan 1998). Upon binding estrogen, they undergo conformational changes that result in DNA binding and regulation of gene transcription (MacGregor and Jordan 1998). Estrogen treatment regulates the expression of many genes throughout the brain. This includes those which encode products involved in apoptosis (Singer *et al*. 1998; Linford *et al*. 2001), axonal regeneration (Tanzer and Jones 1997), or general trophic support (Sohrabji *et al*. 1995). Thus, estrogen regulates the expression of genes involved in proliferation and neuronal survival.

In addition to regulating gene transcription, estrogen initiates second messenger signaling events, including mobilization of intracellular calcium (Morley *et al*. 1992), production of cAMP (Razandi *et al*. 1999), Akt (Ahmad *et al*. 1999) and activation of the mitogen-activated protein kinases (MAPK), ERK1 and ERK2 (Watters *et al*. 1997; Singh *et al*. 1999). Several lines of evidence suggest estrogen neuroprotection may be mediated by rapid intracellular signaling events rather than ERE-mediated gene transcription. For example, activation of protein kinase A, protein kinase C, and MAPK have been linked to neuroprotection in various cellular model systems (Singer *et al*. 1999; Rydel and Greene 1988; Watters *et al*. 1997). However, it is not clear whether the nuclear receptors mediate these rapid

Received March 4, 2002; revised manuscript received April 22, 2002; accepted April 30, 2002.

Address correspondence and reprint requests to Dr Jennifer Fitzpatrick, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201, USA. E-mail: fretlanj@ohsu.edu

[1]These authors contributed equally to this manuscript.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ER, estrogen receptor; ERK, extracellular regulated kinase; FBS, fetal bovine serum; HTERα or HTERβ, hippocampal-derived cell line HT22 stably transfected ERα or ERβ; MEK, MAPK kinase; MES, 2-(*N*-morpholine) ethane sulfonic acid; NSE, neuronal specific-enolase; PBSA, phosphate-buffered saline + 5% bovine serum albumin; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate.

events and whether this activation of signaling pathways is sufficient to elicit neuroprotection.

Studies in various systems have shown that estrogen protects neurons from insults including β-amyloid peptide (Green *et al*. 1996), serum-deprivation (Gollapudi and Oblinger 1999a), excitotoxicity (Singer *et al*. 1998), and oxidative stress (Moosmann and Behl 1999). Not only is exogenous estradiol protective, but estrogen receptor (ER) expression is increased at sites of injury (Garcia-Segura *et al*. 2001), suggesting a role for estrogen receptors in mediating neuroprotection. Although estrogen exerts direct effects on neurons, the molecular events that mediate estrogen neuroprotection are only beginning to be elucidated.

An increasing body of evidence implicates the cloned estrogen receptors, ERα and ERβ, in mediating estrogen-induced neuroprotection. Gollapudi and Oblinger have found that estradiol exposure attenuates serum-deprivation toxicity in PC12 cells transfected with ERα, but not those transfected with an empty vector (Gollapudi and Oblinger 1999b). In addition, in an *in vivo* model of ischemia, ERα was found to mediate the protective effects of physiological concentrations of estradiol in brain injury (Dubal *et al*. 2001). The role of ERβ, however, in neuroprotection remains controversial.

Recent reports suggest transfected estrogen receptors (ERα and β) initiate rapid signaling in CHO cells (Razandi *et al*. 1999) and Rat-2 fibroblast cells (Wade *et al*. 2001). Comparable evidence showing that ERα or β function similarly in a neuronal cell system or that their introduction confers estrogen-mediated neuroprotection is lacking. Their individual contributions cannot easily be evaluated in primary cultures since coexpression of both receptor isoforms in primary neurons and throughout the brain is likely. In the present study, hippocampal-derived HT22 cells were stably transfected with ERα or ERβ to characterize the role of each receptor in mediating the activation of signaling pathways and in neuroprotection.

Materials and methods

Chemicals

17β-Estradiol and PD98059 were purchased from Sigma Chemical Company (St Louis, MO, USA). ICI 182, 780 was purchased from Tocris Cookson (Ballwin, MO, USA). β-Amyloid peptide (fragment 25–35) was purchased from Promega (Madison, WI, USA).

Cell culture

HT22 cells were given as a kind gift from Dr Pamela Maher (The Scripps Research Institute, La Jolla, CA, USA). These cells were grown on 100 mm tissue culture dishes and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) media supplemented to 5% fetal bovine serum (FBS) and 1% Pen-Strep (Gem Cell, Woodland, CA, USA) at 37°C in a 5% CO_2 atmosphere. Cell density was maintained ≤ 70% confluency as described previously (Maher and Davis 1996).

Stable transfection of HT22 cells

HT22 cells were grown to approximately 60% confluence before being transfected with the lipid transfection reagent, TransFast (Promega). pCDNA3.1-hygromycin (7.3 μg) containing either the full-length human ERα cDNA (Kumar *et al*. 1987) (a gift from Dr Pierre Chambon, Strasbourg, France) or rat ERβ cDNA (Kuiper *et al*. 1996) (a gift from George Kuiper, Karolinska Institute, Sweden) was added at a 1 : 1 ratio with Transfast per 100 mm plate. Media was changed 24 h later and hygromycin (125 μg/mL) was added to the media 72 h after transfection for selection of estrogen receptor-expressing clones. Single colonies were isolated after the 10th day of growth in selective conditioned media and tested for receptor expression by immunoblotting.

Immunocytochemistry

Rat-2 fibroblast and HT22 cells (5×10^5) were plated on glass coverslips coated with poly-L-ornithine and incubated overnight in 24-well culture plates. The cultures were washed three times in cold (1x) phosphate-buffered saline (PBS) and immersed in fixative containing PBS, 2% paraformaldehyde, 0.1% glutaraldehyde, 0.12 M sucrose, and 0.5% Triton X-100 for 1 min followed by three washes. Reactive aldehyde groups were quenched in 50 mM ammonium chloride at room temperature (25°C) for 1 h. Cells were rinsed again in PBS and non-specific binding was blocked by incubating in PBS + 5% bovine serum albumin (PBSA) for 1 h at room temperature. The primary antibody, mouse monoclonal neuronal specific-enolase (NSE) (Monosan Products, Burlingame, CA, USA) was added to cells at a concentration of 1 : 200 in 1% PBSA and incubated overnight at 4°C. Following five 12-min washes in PBS, the cultures were incubated in goat anti-mouse AlexaFluor 488 (Molecular Probes, Eugene, OR, USA) at 10 μg/mL in 1% PBSA for 1 h at room temperature. In order to intensify the signal, cells were also incubated in a tertiary antibody, donkey anti-goat AlexaFluor 488 at 10 μg/mL in 1% PBSA for 1 h at room temperature. Cultures were washed five times in PBS. The coverslips were removed and allowed to air dry, then inverted onto glass slides, mounted with Vectashield (Vector, Burlingame, CA, USA) and sealed. Images were acquired on a Bio-Rad MRC600 confocal microscope.

Immunoblotting

Cells were rinsed with ice-cold PBS buffer, scraped into immunoprecipitation buffer (1 M HEPES, 0.1 M EGTA, 0.5 M EDTA, 0.5 M Na^+ pyrophosphate, 1 M NaF, 1 mM $NaVO_4$, 9 mM NaCl) and incubated on ice for at least 5 min. The samples were then sonicated for 2 min followed by centrifugation at 20 800 *g* for 10 min. The supernatant was transferred to a new tube and protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA). The protein samples were diluted in laemmeli sodium dodecyl sulfate (SDS) sample buffer and 15 μg of protein were loaded per well and resolved by electrophoresis on 4–12% Bis-Tris precast gels (Invitrogen, Carlsbad, CA, USA) in 2-(*N*-morpholine) ethane sulfonic acid (MES) running buffer (50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA) as described by the manufacturer. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked in 5% non-fat dry milk in T-TBS (Tris-buffered saline containing 0.2% Tween-20) for 1 h at room temperature. Clones were tested for ERα and ERβ expression using

a mouse anti-ERα antibody AB-15 (1 : 500, Neomarkers, Fremont, CA, USA) or rabbit anti-ERβ antibody (1 : 1000, Upstate Biotechnology, Lake Placid, NY, USA). Secondary goat anti-mouse antibodies (1 : 2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-rabbit antibodies (1 : 2000, Santa Cruz Biotechnology) conjugated to horseradish peroxidase were used for detection by enhanced chemiluminescence (NEN, Boston, MA, USA) on film.

Activation of ERK2

Cells were grown to 70–80% confluence on 100 mm plates. Eighteen hours before treatment the media was replaced with phenol red-free DMEM not supplemented with FBS. Cells were treated with ethanol vehicle (0.1% final concentration), 17β-estradiol (10 nM), PD98059 (50 μM), or ICI 182, 780 (1 μM) for the indicated times. The media was removed and the cells were washed in ice-cold PBS. Immunoblotting was performed as described above, except that equal volumes of protein extracts were loaded and resolved on 4–12% Bis-Tris precast gels (Invitrogen). ERK2 phosphorylation was detected using mouse anti-phospho-p44/42 MAPK (1 : 2000, Santa Cruz Biotechnology) that recognizes phospho-THR202 and THR204 forms of ERK1/2. Phosphorylation at these sites has been correlated with increased activity (Payne *et al*. 1991; Robbins and Cobb 1992; Robbins *et al*. 1993). Total ERK2 was detected using rabbit anti-ERK2 (1 : 10 000, Santa Cruz Biotechnology). Secondary goat anti-mouse antibodies (1 : 5000, Santa Cruz Biotechnology) or goat anti-rabbit antibodies (1 : 5000) conjugated to horseradish peroxidase were used for detection by enhanced chemiluminescence (NEN) on film. The resulting film samples were scanned and analyzed with an image analysis program (NIH IMAGE, Scion Corporation, Frederick, MD, USA). Data are presented as a ratio of phospho-ERK2/total ERK2 in the sample, normalized to vehicle-treated samples.

Cytotoxicity assay

Cell treatments

Cells were grown to 70–80% confluence in 12-well plates. Twenty-four hours before treatment the media was replaced with phenol red free DMEM supplemented with charcoal-stripped FBS (1%). β-Amyloid peptide 25–35 was suspended in sterile PBS and incubated for 1 h at 37°C immediately before use. β-Amyloid peptide was then diluted to a final concentration of 10 μM in culture media and cells were exposed for 24 h. 17β-Estradiol was initially dissolved in 95% ethanol at a concentration of 1 mM and diluted to the appropriate concentration (10 nM) in culture media. Exposure to 17β-estradiol was initiated 15 min prior to β-amyloid peptide addition. Ethanol was used at a final concentration of 0.1% as a vehicle control. This concentration of ethanol had no effect on cell viability or β-amyloid peptide toxicity. ICI 182, 780 and PD98059 were made as 1000 × stocks in 100% DMSO and were added to cells 15 min prior to 17β-estradiol exposure.

Calcein AM

Following incubation of cells in β-amyloid peptide, cells were rinsed once with PBS and incubated with 1 μM Calcein AM dye (Molecular Probes) at 37°C for 15 min, washed twice with PBS and coverslips applied. Green fluorescent cells are the product of mitochondrial cleavage of Calcein AM and were observed using a Nikon Optiphot 2 microscope with the EF-D fluorescence attachment and G-1B and DM510 filters and counted as living cells.

Statistical analysis

The significance of differences among groups was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $p < 0.05$ was considered significant and each group consisted of 6–12 wells or plates. All values are expressed as mean ± SEM.

Results

Generation of HT22 stable transfectants

The mouse hippocampal-derived cell line, HT22, which lacks functional estrogen receptors (Behl *et al*. 1995; Green *et al*. 1998; Manthey *et al*. 2001) was used for transfection studies. Immunocytochemistry was performed to detect expression of NSE, a neuron-specific marker, in HT22 cells and Rat-2 fibroblasts. Figure 1(a) shows that HT22 cells maintain a neuronal morphology and express NSE. Rat-2 fibroblast cells showed only non-specific background staining that appeared when the cells were incubated with secondary antibody only (data not shown). HT22 cells were stably transfected with plasmids containing either the full-length human ERα or the full-length rat ERβ cDNA. After transfection of ERα or ERβ, immunoreactive protein of the appropriate molecular weight was detected in HTERα and HTERβ cells by immunoblotting (Fig. 1b).

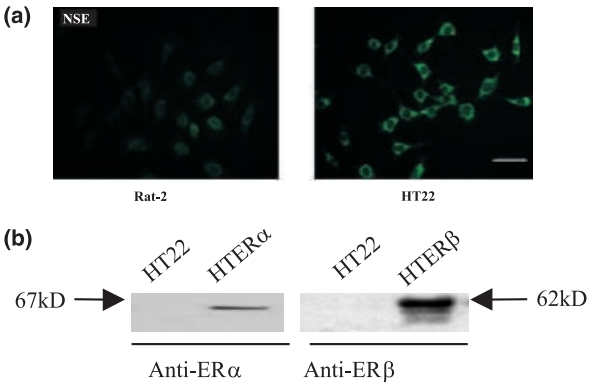

Fig. 1 Generation of ERα or ERβ stably expressing HT22 neuronal cells. (a) Confocal microscopy of HT22 cells and Rat-2 fibroblast cells after immunocytochemistry was performed using a monoclonal antibody directed against the gamma gamma subunit of neuronal specific enolase (NSE). (b) Fifteen micrograms of cell lysates from untreated HT22, HTERα, and HTERβ cells were analyzed by immunoblotting using monoclonal mouse anti-ERα antibody (Neomarkers, Lab Vision, Fremont, CA, Ab-15, 1 : 500) or polyclonal rabbit anti-ERβ antibody (Upstate, Lake Placid, NY, 1 : 1000).

ERα or ERβ is required for estrogen to induce ERK2 phosphorylation

Activation of the MAPK pathway has been implicated as one potential mediator of estrogen-induced neuroprotection (Singer *et al*. 1999; Kuroki *et al*. 2001). To determine if ERα or ERβ can mediate rapid activation of the MAPK pathway in HTERα and HTERβ cells, we tested the ability of 10 nM 17β-estradiol to increase ERK2 phosphorylation. Figure 2(a) shows that ERK2 phosphorylation is increased within 15 min and returns to basal levels by 30 min in cells expressing either ERα or ERβ. Under these culture conditions, no increase in ERK2 phosphorylation was observed in untransfected HT22 cells, suggesting that expression of either ERα or ERβ is required for estrogen to increase ERK2 phosphorylation.

To further demonstrate that the transfected estrogen receptors are mediating the ability of estrogen to increase ERK2 phosphorylation, we tested the ability of the estrogen receptor antagonist ICI 182, 780 to block ERK2 phosphorylation. ICI 182, 780 (1 μM), significantly inhibited the increase in ERK2 phosphorylation by 17β-estradiol in both HTERα and HTERβ cells (Fig. 3). These data, together with the lack of ERK2 phosphorylation in response to 17β-estradiol in untransfected HT22 cells, demonstrate that either ERα or ERβ can mediate the increase in ERK2 phosphorylation by 17β-estradiol in a neuronal cell model expressing either ERα or ERβ.

To determine whether the increase in ERK2 phosphorylation was initiated by MEK, cells were pretreated with the inhibitor PD98059 (50 μM) for 15 min prior to 17β-estradiol

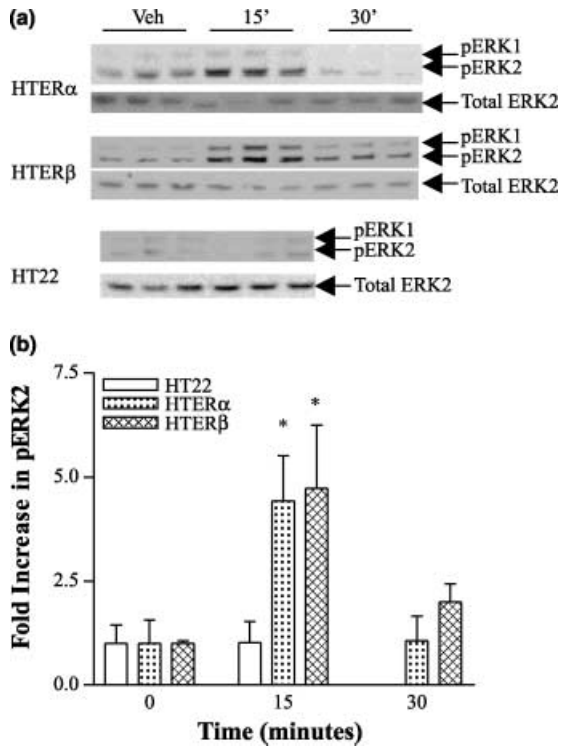

Fig. 2 17β-Estradiol treatment increases ERK2 phosphorylation in HTERα and HTERβ cells. Lysates from HTERα and HTERβ cells treated with 10 nM 17β-estradiol for 15, or 30 min were analyzed by immunoblotting for changes in phosphorylation of ERK2. (a) A representative immunoblot measuring phosphorylated and total ERK2. Equal volumes of cell lysates were probed with an anti-phospho-specific p42/44 MAPK (ERK1/ERK2) antibody. Total ERK2 was detected using an anti-ERK2 antibody that recognizes both phosphorylated and unphosphorylated ERK2. (b) Relative amounts of phosphorylated ERK2 were determined from densitometric scanning of ECL-exposed film. Data is represented as fold increase in relative phosphorylated ERK2 as compared to vehicle treatment ± SEM. All experiments were performed at least three times in triplicate; $^{*}p \leq 0.05$, as determined by one-way ANOVA.

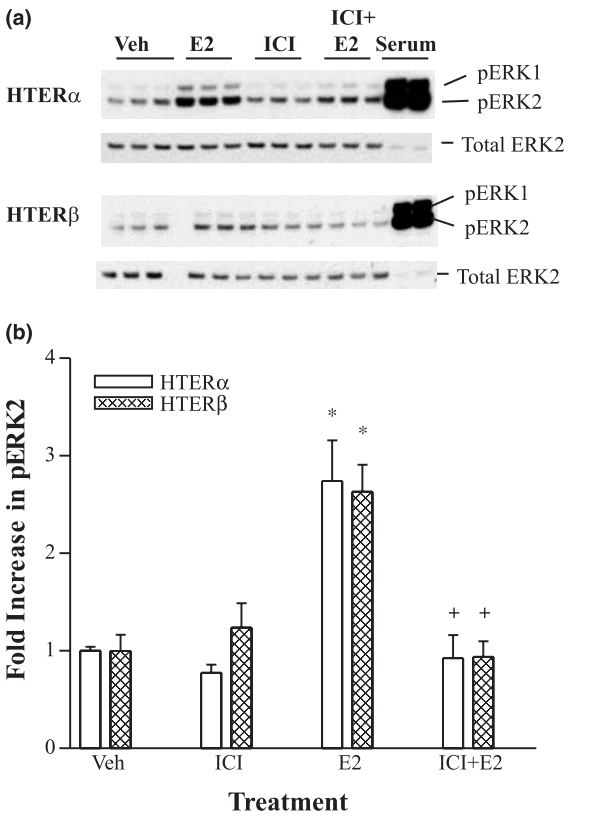

Fig. 3 The ER antagonist, ICI 182, 780 blocks the increase in ERK2 phosphorylation induced by 17β-estradiol in HTERα and HTERβ cells. HTERα and HTERβ cells were treated with vehicle, 10 nM 17β-estradiol, 1 μM ICI 182,780, or 17β-estradiol and ICI 182, 780 for 15 min and analyzed as described in Fig. 2. (a) A representative immunoblot probing for phosphorylated and total ERK2 using anti-phospho-specific p42/44 MAPK (ERK1/ERK2) antibody and anti-ERK2 antibody. (b) Relative amounts of phosphorylated ERK2 were determined and represented as described in Fig. 2. Experiments were performed at least three times in triplicate; $^{*}p \leq 0.05$ versus vehicle-treated cells and $^{+}p \leq 0.05$ versus 17β-estradiol-treated cells as determined by one-way ANOVA.

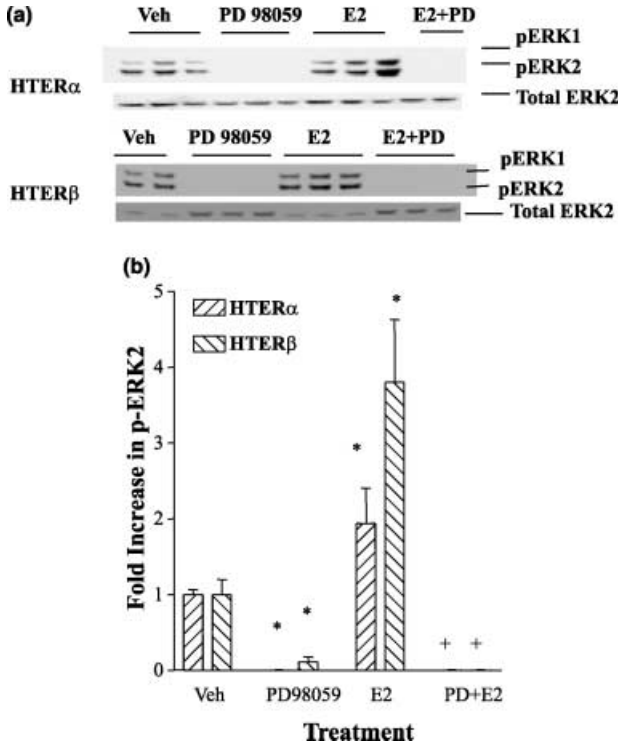

Fig. 4 The MEK inhibitor, PD98059, blocks the increase in ERK2 phosphorylation induced by 17β-estradiol in HTERα and HTERβ cells. HTERα and HTERβ cells were pretreated for 15 min with 50 μM PD98059 as indicated, followed by treatment with vehicle or 10 nM 17β-estradiol for 15 min. Cellular protein was isolated and analyzed by immunoblotting as described in Fig. 2. (a) A representative immunoblot measuring phosphorylated and total ERK2 using antiphospho-specific p42/44 MAPK (ERK1/ERK2) antibody and anti-ERK2 as described previously. (b) Relative amounts of phosphorylated ERK2 were determined and represented as described previously. Experiments were performed at least three times in triplicate; $^{*}p \leq 0.05$ versus vehicle-treated cells and $^{+}p \leq 0.05$ versus 17β-estradiol-treated cells as determined by one-way ANOVA.

treatment. PD98059 completely blocked the increase in ERK2 phosphorylation by 17β-estradiol (Fig. 4), suggesting that estrogen requires MEK and possibly other upstream signaling molecules of the MAPK pathway to increase ERK2 phosphorylation. PD98059 also increased total ERK2 levels in HTERβ cells as compared to vehicle treatment and 17β-estradiol treatment (Fig. 4a). This observation was not seen in HT22 or HTERα cells, but was reproducible in HTERβ cells.

Estrogen-mediated neuroprotection against β-amyloid toxicity requires expression of ERα or ERβ

Figure 5(a) shows that HT22, HTERα and HTERβ cells are sensitive to β-amyloid peptide exposure, in which greater than 50% of cells were killed after a 24-h treatment. HT22, HTERα and HTERβ cells were treated with 10 nM 17β-estradiol for 15 min prior to β-amyloid peptide exposure to determine if a short pretreatment with the hormone would elicit neuroprotection. In HTERα and HTERβ cells, a 15-min pretreatment with 17β-estradiol significantly increased cellular viability as evidenced by Calcein AM staining (Figs 5a and b). By contrast, at this dose of estrogen HT22 cells were not protected from β-amyloid toxicity. Figure 5(b) is a representative experiment depicting the abundance of living cells (green) treated with β-amyloid peptide after a 15-min pretreatment with 17β-estradiol or vehicle.

The conditions used to measure neuroprotection were initially different than those used to measure estrogen activation of ERK2. When measuring ERK2 activation cells are grown in serum-free media overnight, however, these cells do not live in serum-free media for more than 36 h. Therefore, in neuroprotection experiments the cells were maintained in 1% charcoal-stripped media. To ensure that under these conditions 17β-estradiol could indeed activate ERK2, western blots were performed in cells that had been maintained in 1% charcoal-stripped media. Figure 5(c) shows that and increase in ERK2 phosphorylation is observed in HTERα and HTERβ cells.

In order to confirm that the neuroprotective effect of 17β-estradiol was dependent on estrogen receptors, cells were pretreated with ICI 182, 780. While ICI 182, 780 (1 μM) had no cytotoxic or neuroprotective effects on its own (data not shown), it blocked the ability of 17β-estradiol to increase living cell numbers (Figs 6a and b), demonstrating that the protective effects of estrogen occurred in an estrogen receptor-dependent manner. Figure 6(b) is a representative experiment depicting the abundance of living cells (green) treated with β-amyloid peptide in the presence and absence of 17β-estradiol, and the reduction of living cells observed after β-amyloid peptide treatment in the presence of estrogen and ICI 182, 780.

In order to determine if 17β-estradiol-mediated neuroprotection was dependent on activation of MAPK, cellular cytotoxicity was determined in the presence and absence of PD98059. While PD98059 (50 μM) had no effect on cytotoxicity alone, it abolished the ability of 17β-estradiol to increase living cell number (Figs 7a and b). This data suggests that the protective effects of estrogen are occurring through a MAPK-dependent pathway. Figure 7(b) is a representative experiment depicting the abundance of living cells (green) treated with β-amyloid peptide in the presence and absence of 17β-estradiol, and the reduction of living cells observed after β-amyloid peptide treatment in the presence of estrogen and PD98059.

Discussion

Although it has been suggested that estrogen is beneficial in the prevention of Alzheimer's disease (Paganini-hill and

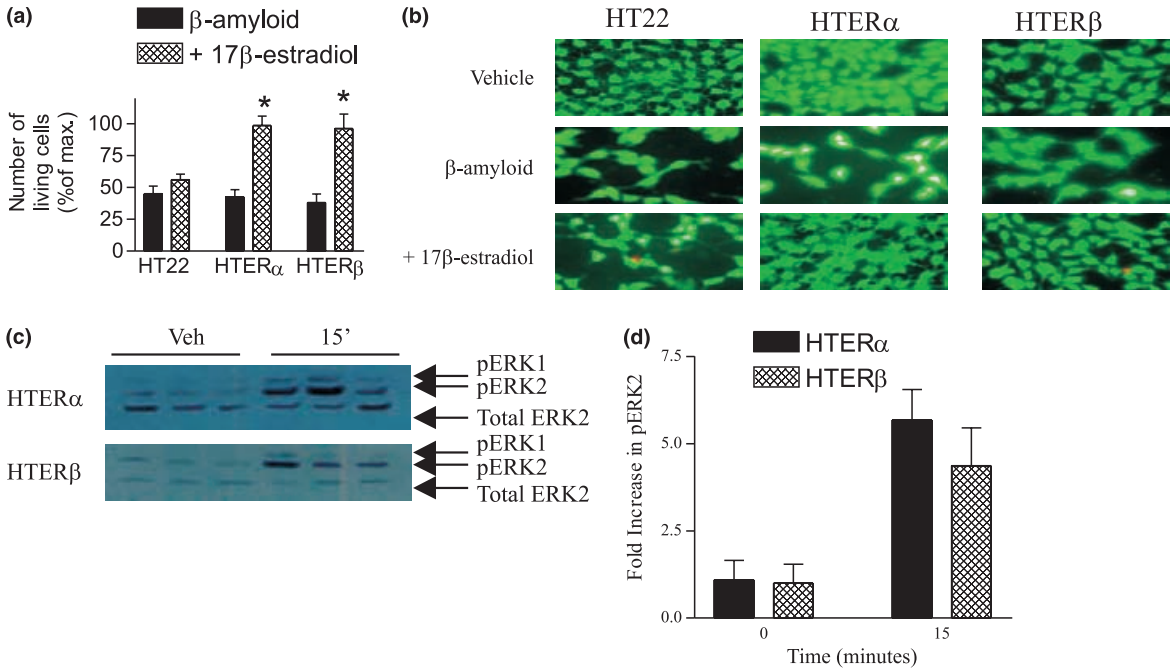

Fig. 5 Fifteen-minute pretreatment with 17β-estradiol protects HTERα and HTERβ cells from β-amyloid peptide toxicity. HT22, HTERα and HTERβ cells were treated with 17β-estradiol (10 nM) for 15 min, followed by β-amyloid peptide (10 μM) for 24 h. Cellular viability was assessed by Calcein AM staining. The number of living cells was assessed by fluorescence with Calcein AM. (a) Numbers of living cells are expressed as percentage of vehicle-treated controls. All results represent the mean ± SEM from three to four separate plates. *Statistically different from β-amyloid peptide-treated cells, $p < 0.05$. (b) Representative micrograph showing the presence of living cells as compared to vehicle-treated controls. (c) A representative western blot measuring phosphorylated ERK1/2 and total ERK2. (d) Relative amounts of ERK2 were determined as described in Figure 2.

Henderson 1994), it is not known whether this involves increasing neuronal survival in Alzheimer's patients. In addition, the underlying molecular mechanism by which estrogen might act to enhance neuronal survival is not clearly understood. Previous studies have shown that high concentrations of estrogen can protect against β-amyloid peptide induced cell death in the ER-negative HT22 cells (Behl *et al*. 1995; Green *et al*. 1998), suggesting that estrogen can protect neurons through ER-independent pathways, potentially involving its antioxidant properties at supraphysiological concentrations.

However, other reports suggest that physiological concentrations of estrogen act through an ER-dependent mechanism to protect against neuronal cell death. For example, estrogen activation of MAPK through the estrogen receptors provides neuroprotection in primary neuronal cultures against a variety of toxic insults including quinolinic acid toxicity (Kuroki *et al*. 2001) and glutamate excitotoxicity (Singer *et al*. 1999). It is likely that ERα and ERβ are coexpressed in various primary neuronal preparations, or are endogenously coexpressed in many cell lines, making it difficult to study the role of the individual receptor subtype in mediating neuroprotection. Therefore, a neuronal cell model that expresses only ERα or ERβ was generated in order to determine the possible contribution of each receptor in activation of signaling pathways and in neuroprotection.

In this study, the contribution of ERα and ERβ in mediating neuroprotection against β-amyloid peptide toxicity was examined. Cells were pretreated with 17β-estradiol followed by β-amyloid peptide for 24 h. Physiological concentrations of 17β-estradiol (10 nM) were protective against β-amyloid peptide in HTERα and HTERβ cells, but not in HT22 cells. This suggests that under the culture conditions used, estrogen receptor expression is required for low concentrations of estrogen to be neuroprotective against β-amyloid peptide toxicity. A supraphysiological concentration of 17β-estradiol (1 μM) was neuroprotective in HT22, HTERα and HTERβ cells (data not presented) an effect previously attributed to antioxidant properties of the molecule (Maher and Davis 1996). Neuroprotection was blocked by ICI 182,780, further demonstrating that ERα or ERβ is required for low doses of 17β-estradiol to elicit neuroprotection. Studies have observed neuroprotection in the presence of transcriptional inhibitors (Goodman *et al*. 1996; Regan and Guo 1997; Sawada *et al*. 1998) emphasizing that other more transient events are crucial for estrogen to be neuroprotective. In this and other studies, pretreatment for periods of 24 h or longer are also

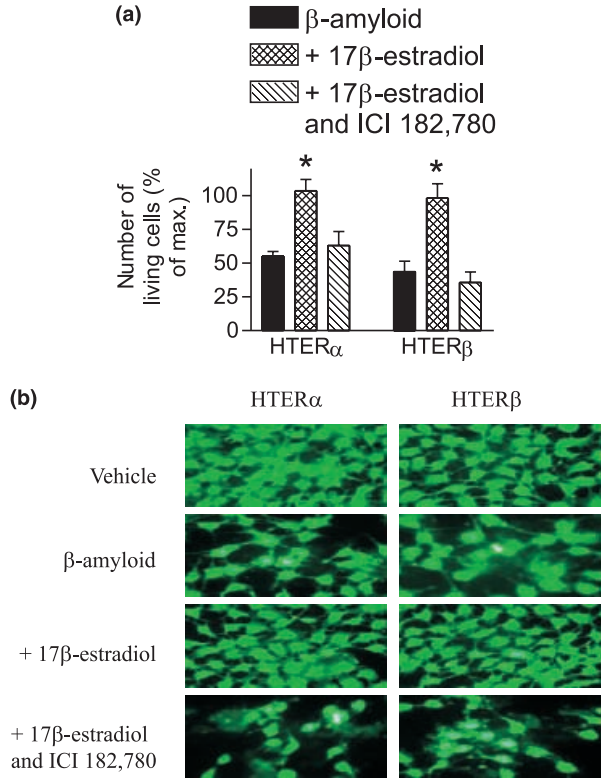

Fig. 6 ICI 182, 780 blocks 17β-estradiol-mediated neuroprotection against β-amyloid peptide induced toxicity in HTERα, and HTERβ cells. HTERα and HTERβ cells were treated with 17β-estradiol (10 nM) for 15 min in the presence and absence of ICI 182, 780 (1 μM), followed by β-amyloid peptide (10 μM) for 24 h. Cellular viability was assessed as described and presented in Fig. 2.

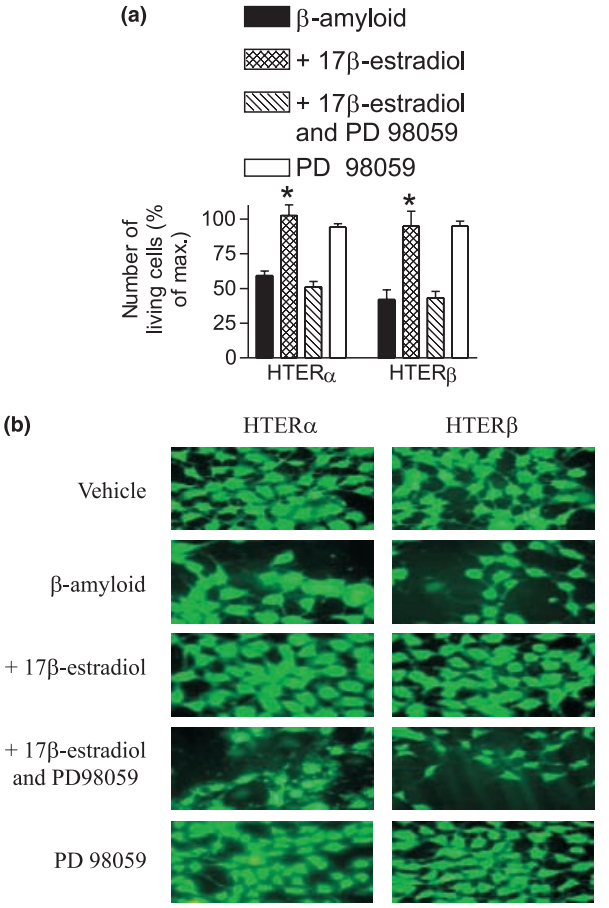

Fig. 7 PD98059 blocks 17β-estradiol-mediated neuroprotection against β-amyloid peptide induced toxicity in HTERα, and HTERβ cells. HTERα and HTERβ cells were treated with 17β-estradiol (10 nM) for 15 min in the presence and absence of PD98059 (50 μM), followed by β-amyloid peptide (10 μM) for 24 h. Cellular viability was assessed as described in Fig. 2.

protective (data not shown). Thus acute and long-term neuroprotection by estrogen is likely to exhibit components that may be mediated by different mechanisms.

It is well documented that 17β-estradiol can activate the MAPK pathway and studies by Razandi *et al*. (1999) and Wade *et al*. (2001) demonstrated that transient or stable transfection of either ERα or ERβ can mediate this rapid activation. Estradiol activation of MAPK has been described in neocortical explants (Singh *et al*. 1999) and primary cortical neurons (Singer *et al*. 1999), while 17β-estradiol-BSA, a membrane impermeable estrogen, has been shown to activate this pathway in SK-N-SH cells (Watters *et al*. 1997). Activation of this pathway is rapid, with phosphorylation of ERK2 occurring within 5–15 min after estradiol exposure.

Here we document that MAPK activation requires the expression of ERα or ERβ in HT22 cells, and the ER antagonist, ICI 182,780, blocks this activation. The MEK inhibitor, PD98059, also blocked MAPK activation by estrogen suggesting that active MEK and perhaps other upstream kinases of the MAPK pathway are involved. The time course of MAPK activation was similar for ERα and ERβ expressing cells in which maximal activation was seen at 15 min, and this response returned to basal levels by 30 min.

In contrast to these studies, it was recently reported that estrogen treatment leads to MAPK phosphorylation in HT22 cells (Manthey *et al*. 2001). ICI 182, 780 failed to block the effects of estrogen indicating that activation of MAPK was independent of estrogen receptor expression. Under our culture conditions, wild-type HT22 cells treated with 10 nM estrogen fail to activate MAPK ($n = 10$). Estrogen receptor expression is necessary in order to detect MAPK phosphorylation. There are many notable differences between this study and our own. Importantly, Manthey *et al*. (2001) use very low density cultures, reportedly only 30–40% of that used here. Perhaps at low density, HT22 cells express elements, which are capable of mediating estrogen-induced MAPK activation. The failure of ICI 182, 780 to block these effects suggests that ERα or ERβ are not involved under these conditions.

The time frame of MAPK activation correlates to the pretreatment time required to see neuroprotection. Indeed, PD98059 blocked neuroprotection and ERK2 activation suggesting that transient phosphorylation of MAPK results in estrogen's ability to be neuroprotective. It is possible that estrogen is eventually activating transcription of genes important for neuroprotection. This has been shown to occur in SK-N-BE2C cells transfected with ERα. In these cells, the membrane impermeable E2-BSA acted through non-genomic mechanisms at the cell membrane to activate an ERE-luciferase reporter. The events were proven to act through the classical estrogen receptor at the cell membrane, as ICI 182, 780 blocked the transcriptional effects (Vasudevan *et al*. 2001). Alternatively, genes responding to activation of the MAPK pathway, independent of ERE activity may also be induced. Therefore, it is possible that a 15-min pretreatment with estrogen is neuroprotective due to the subsequent transcription of multiple genes. (i.e. a non-classical pathway for estrogen activation of transcription).

Neuroprotection through an ERα dependent pathway was not surprising, as several studies have supported a role for ERα in neuroprotection. Gollapudi and Oblinger found that estradiol exposure attenuates serum-deprivation toxicity in PC12 cells transfected with ERα, but not those transfected with a control plasmid (Gollapudi and Oblinger 1999b). In addition, in an *in vivo* model of ischemia, ERα was found to be the critical link in mediating the protective effects of physiological concentrations of estradiol in brain injury (Dubal *et al*. 2001). A recent report has shown that 1.8 nM estrogen is neuroprotective against β-amyloid peptide toxicity in HT22 cells stably transfected with ERα (Kim *et al*. 2001). These effects of 17β-estradiol were also blocked by ICI 182,780, further indicating the need for ERα in order to manifest protection. These studies were conducted in a cotreatment paradigm for 24 h, and the requirement for rapid signaling events was not studied.

The role of ERβ in mediating neuroprotection is less well documented. For example, in studies by Dubal *et al*. (2001) ERβ was not sufficient for protection against ischemia in an *in vivo* model. In addition, Kim *et al*. (2001) did not observe neuroprotection against β-amyloid peptide toxicity in HT22 cells stably transfected with ERβ. In the current study, however, we observe pronounced neuroprotection in HTERβ cells. An explanation for the discrepancy could be due to differences in the treatment paradigms. Kim *et al*. report that estrogen is not neuroprotective in HTERβ cells following an 18-h treatment, whereas here we report that estrogen is neuroprotective following a 15-minute pretreatment. In support of our findings, studies by Sawada *et al*. (2000) demonstrated estrogen to be neuroprotective in mesencephalon dopaminergic neurons, which exclusively express ERβ. *In vivo*, Wang *et al*. (2001) demonstrated the importance of ERβ in the survival of neurons throughout the brain by measuring a neuronal deficit in ERβ knockout mice. In this study, the neuronal loss was increased with age suggesting a role for ERβ in the prevention of neurodegenerative diseases.

Our studies clearly show that ERα and ERβ can mediate rapid activation of MAPK in stably expressing HT22 cells, and that this activation is necessary for neuroprotection against β-amyloid peptide toxicity. HTERα and HTERβ cells rapidly activate MAPK within 15 min of exposure to 10 nM 17β-estradiol, and these effects are blocked by pretreatment with the ER antagonist ICI 182, 780 and the MEK inhibitor PD98059. 17β-Estradiol provided neuroprotection against β-amyloid peptide toxicity in a similar time frame. Neuroprotection was blocked by ICI 182, 780 and PD98059, further demonstrating that estrogen activation of MAPK via the estrogen receptor is necessary for 17β-estradiol to be neuroprotective. This is the first demonstration that either ERα or ERβ can mediate estrogen neuroprotection in a neuronal cell model, suggesting that activation of either receptor may be a target for increasing neuroprotection in neurodegenerative diseases.

Acknowledgements

The authors wish to thank Pierre Chambon (Strasbourg, France) for the use of the human ERα cDNA, George Kuiper (Karolinska Institute, Sweden) for the rat ERβ cDNA, and Nephi Stella for critical review of the manuscript. Work was supported by NIH grant T32 ST32NS07332-10 and F32 NS42495-01 (JLF), NIH grant T32 AG00057 (ALM), NRSA T32 G07270 from NIGMS (CBW), a project in the University of Washington Alzheimer's Center AG 05136–18, and NIH NS20311 (DMD).

References

Ahmad S., Singh N. and Glazer R. (1999) Role of AKT1 in 17β-estradiol- and insulin-like growth factor I (IGF-1)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. *Biochem. Pharmacol.* **58**, 425–430.

Behl C., Widmann M., Trapp T. and Holsboer F. (1995) 17-Beta estradiol protects neurons from oxidative stress-induced cell death *in vitro*. *Biochem. Biophys. Res. Comm.* **216**, 473–482.

Dubal D. B., Zhu H. YuJ., Rau S. W., Shughrue P. J., Merchenthaler I., Kindy M. S. and Wise P. M. (2001) Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. *Proc. Natl Acad. Sci. USA* **98**, 1952–1957.

Garcia-Segura L. M., Azcoitia I. and DonCarlos L. L. (2001) Neuroprotection by estradiol. *Prog. Neurobiol.* **63**, 29–60.

Gollapudi L. and Oblinger M. M. (1999a) Estrogen and NGF synergistically protect terminally differentiated, ERalpha-transfected PC12 cells from apoptosis. *J. Neurosci. Res.* **56**, 471–481.

Gollapudi L. and Oblinger M. M. (1999b) Stable transfection of PC12 cells with estrogen receptor (ERalpha): protective effects of estrogen on cell survival after serum deprivation. *J. Neurosci. Res.* **56**, 99–108.

Goodman Y., Bruce A. J., Chang B. and Mattson M. P. (1996) Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury, and amyloid beta peptide toxicity in hippocampal neurons. *J. Neurochem.* **66**, 1836–1844.

Green P. S., Gridley K. E. and Simpkins J. W. (1996) Estradiol protects against beta-amyloid peptide (25–35)-induced toxicity in SK-N-SH human neuroblastoma cells. *Neurosci. Lett.* **218**, 165–168.

Green P. S., Gridley K. E. and Simpkins J. W. (1998) Nuclear estrogen receptor-independent neuroprotection by estratrienes: a novel interaction with glutathione. *Neuroscience* **84**, 7–10.

Kim H., Bang O. Y., Jung M. W., Ha S. D., Hong H. S., Huh K., Kim S. U. and Mook-Jung I. (2001) Neuroprotective effects of estrogen against beta-amyloid peptide toxicity are mediated by estrogen receptors in cultured neuronal cells. *Neurosci. Lett.* **302**, 58–62.

Kuiper G. G., Enmark E., Pelto-Huikko M., Nilsson S. and Gustaffson J. A. (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc. Natl Acad. Sci. USA* **93**, 5925–5930.

Kumar V., Green S., Stack G., Berry M., Jin J. R. and Chambon P. (1987) Functional domains of the human estrogen receptor. *Cell* **51**, 941–951.

Kuroki Y., Fukushima K., Kanda Y., Mizuno K. and Watanabe Y. (2001) Neuroprotection by estrogen via extracellular signal-regulated kinase against quinolinic acid-induced cell death in the rat hippocampus. *Eur. J. Neurosci.* **13**, 472–476.

Linford N. J., Yang Y., Cook D. G. and Dorsa D. M. (2001) Neuronal apoptosis resulting from high doses of isoflavone genistein: role for calcium and P42/44 mitogen-activated protein kinase. *J. Pharmacol. Exp. Ther.* **299**, 1–9.

MacGregor J. I. and Jordan V. C. (1998) Basic guide to the mechanisms of antiestrogen action. *Pharmacol. Rev.* **50**, 151–196.

Maher P. and Davis J. B. (1996) The role of monoamine metabolism in oxidative glutamate toxicity. *J. Neurosci.* **16**, 6394–6401.

Manthey D., Heck S., Engert S. and Behl C. (2001) Estrogen induces a rapid secretion of amyloid β precursoe protein via the mitogen-activated protein kinase pathway. *Eur. J. Biochem.* **268**, 4285–4291.

Moosmann B. and Behl C. (1999) The antioxidant neuroprotective effects of estrogens and phenolic compounds are independent from their estrogenic properties. *Proc. Natl Acad. Sci. USA* **96**, 8867–8872.

Morley P., Whitfield J. F., Vanderhyden B. C., Tsang B. K. and Schwartz J. L. (1992) A new, nongenomic estrogen action: the rapid release of intracellular calcium. *Endocrinology* **131**, 1305–1312.

Paganini-hill A. and Henderson V. W. (1994) Estrogen deficiency and risk of Alzheimer's disease in women. *Am. J. Epidemiol.* **140**, 256–261.

Payne D. M., Rossomando A. J., Martino P., Erickson A. K., Her J. H., Shabanowitz J., Hunt D. F., Weber M. J. and Sturgill T. W. (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J.* **10**, 885–892.

Razandi M., Pedram A., Greene G. L. and Levin E. R. (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERα and ERβ expressed in Chinese hamster ovary cells. *Mol. Endocrinol.* **13**, 307–319.

Regan R. F. and Guo Y. (1997) Estrogens attenuate neuronal injury due to hemoglobin, chemical hypoxia, and excitatory amino acids in murine cortical cultures. *Brain Res.* **764**, 133–140.

Robbins D. J. and Cobb M. H. (1992) Extracellular signal-regulated kinases 2 autophosphorylaes on a subset of peptides phosphorylated in intact cells in response to insulin and nerve growth factor: anaylsis by peptide mapping. *Mol. Biol. Cell* **3**, 299–308.

Robbins D. J., Zhen E., Owaki H., Vanderbilt C. A., Ebert D., Geppert T. D. and Cobb M. H. (1993) Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 *in vitro*. *J. Biol. Chem.* **268**, 5097–5106.

Rydel R. E. and Greene L. A. (1988) cAMP analogs promote survival and neurite outgrowth in cultures of rat sympathetic and sensory neurons independently of nerve growth factor. *Proc. Natl Acad. Sci. USA* **85**, 1257–1261.

Sawada H., Ibi M., Kihara T., Urushitani M., Akaiki A. and Shimohama S. (1998) Estradiol protects mesencephalic dopaminergic neurons from oxidative stress-induced neuronal death. *J. Neurosci. Res.* **54**, 707–719.

Sawada H., Ibi M., Kihara T., Urushitani M., Honda K., Nakanishi M., Akaiki A. and Shimohama S. (2000) Mechanisms of antiapoptotic effects of estogens in nigral dopaminergic neurons. *FASEB J.* **14**, 1202–1214.

Singer C. A., Rogers K. L. and Dorsa D. M. (1998) Modulation of Bcl-2 expression: a potential component of estrogen protection in NT2 neurons. *Neuroreport* **9**, 2565–2568.

Singer C. A., Figueroa-Masot X. A., Batchelor R. H. and Dorsa D. M. (1999) The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *J. Neurosci.* **19**, 2455–2463.

Singh M., Setalo G., Guan X., Warren M. and Toran A. C. (1999) Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. *J. Neurosci.* **19**, 1179–1188.

Sohrabji F., Miranda R. C. and Toran-Allerand C. D. (1995) Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. *Proc. Natl Acad. Sci.* **92**, 11110–11114.

Tanzer L. and Jones K. J. (1997) Gonadal steroid regulation of hamster facial nerve regeneration: effects of dihydrotestosterone and estradiol. *Exp. Neurol.* **146**, 258–264.

Vasudevan N., Kow L. M. and Pfaff D. W. (2001) Early membrane estrogenic effects required for full expression of slower genomic actions in a nerve cell line. *Proc. Natl Acad. Sci. USA* **98**, 12267–12271.

Wade C. B., Robinson S., Shapiro R. A. and Dorsa D. M. (2001) Estrogen receptor ERα and ERβ exhibit unique pharmacologic properties when coupled to activation of the mitogen-activated protein kinase pathway. *Endocrinology* **142**, 2336–2342.

Wang L., Andersson S., Warner M. and Gustafsson J. A. (2001) Morphological abnormalities in the brains of estrogen receptor beta knockout mice. *Proc. Natl Acad. Sci. USA* **98**, 2792–2796.

Watters J. J., Campbell J. S., Cunningham M. J., Krebs E. G. and Dorsa D. M. (1997) Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signaling cascade and c-fos immediate early gene transcription. *Endocrinology* **138**, 4030–4033.