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Neuroprotection by estrogen against MPP⁺-induced dopamine neuron death is mediated by ER α in primary cultures of mouse mesencephalon

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Abstract

Estrogen involvement in neuroprotection is now widely accepted, although the specific molecular and cellular mechanisms of estrogen action in neuroprotection remain unclear. This study examines estrogenic effects in a mixed population of cells in attempts to identify the contributing cells that result in estrogen-mediated neuroprotection. Utilizing primary mesencephalic neurons, we found expression of both estrogen receptor (ER α) and estrogen receptor (ER β) with a predominance of ER α on both dopamine neurons and astrocytes. We also found that 17 β -estradiol protects dopamine neurons from injury induced by the complex I inhibitor, 1-methyl-4-phenyl pyridium (MPP⁺) in a time and ER dependent manner. At least 4 hr of estrogen pre-treatment was required to elicit protection, an effect that was blocked by the ER antagonist, ICI 162,780. Moreover, ER α mediated the protection afforded by estrogen since only the ER α agonist, HPTe but not the ER β agonist, DPN protected against dopamine cell loss. Since glial cells were shown to express significant levels of ER α , we investigated a possible indirect mechanism of estrogen-mediated neuroprotection through glial cell interaction. Removal of glial cells from the cultures by application of the mitotic inhibitor, 5-Fluoro-2'-deoxyuridine significantly reduced the neuroprotective effects of estrogen. These data indicate that neuroprotection provided by estrogen against MPP⁺ toxicity is mediated by ER α and involves an interplay among at least two cell types.

Keywords

estrogen; estrogen receptor; neuroprotection; MPP⁺; mesencephalon; dopamine neuron; astrocytes

INTRODUCTION

Estrogen is known predominantly as a reproductive hormone, but its biological functions are widespread encompassing such actions as differentiation, neuronal survival and maturation (Belcher and Zsarnovszky, 2001; Beyer et al., 2003). Although the neuroprotective actions of estrogen have been extensively studied, a single mechanistic pathway has not been identified, suggesting multiple possible mechanisms. This may be due to the fact that

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estrogen actions are cell-type specific and depend on the estrogen receptor (ER) subtype targeted as well as contributing adjacent cell types. As a result, estrogen mediated neuroprotection responses may be different depending on the brain region affected. The present study examines estrogen's effects in a mixed population of mesencephalic cells, containing both neurons and glia in attempts to identify the ER and cells that elicit estrogen-mediated neuroprotection of midbrain dopamine (DA) neurons.

The nigrostriatal dopaminergic system is a target of estrogen and evidence suggests that estrogen serves as a neuroprotectant in many disorders originating in the brain, including the nigrostriatal neurodegeneration observed in Parkinson's disease (PD) (Kuppers et al., 2000). Such estrogen-mediated neuroprotection has been observed using neurotoxic agents such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) *in vivo* or its active metabolite, MPP⁺ *in vitro*, which selectively damages dopaminergic neurons in the substantia nigra similar to the cell loss observed in PD (D'Astous et al., 2006; Swada et al., 2002). Furthermore, as the brain ages and estrogen levels decline, the risk for acquiring neurodegenerative disease increases, which further substantiates the importance of understanding estrogen's role in neuronal survival (Dluzen, 2000).

The role of estrogen in regulating the differentiation and maturation of developing neurons has been characterized in dopaminergic neurons of the developing midbrain (Hutchison and Beyer, 1994; Hutchison et al., 1997; Kuppers et al., 2001). Differentiation of embryonic midbrain dopaminergic neurons involves rapid estrogen signaling via calcium and activation of the cAMP/PKA/pCREB signaling cascade concomitant with an up regulation of BDNF (Beyer and Karolczak, 2000; Ivanova et al. 2001).

Like estrogen, neurotrophic factors such as GDNF, BDNF, NGF and NT3 play a critical role in the survival and differentiation of embryonic DA neurons (Hyman et al., 1994; Lin et al., 1993; Skaper et al., 1993). Initially thought to be prevalent only in the developing brain, these same neurotrophic factors are found in substantial levels in the adult brain (Buck et al., 1987; Ho et al., 1995; Maisonpierre et al., 1990), where they play a significant role in both the protection and regeneration of neurons in response to injury (Tomac et al., 1995). Therefore, factors that regulate the production and/or release of trophic factors may have implications in the treatment of neurodegenerative disorders.

Glial cells are an important source of trophic factors and pro-inflammatory proteins, which are involved in the protection and repair of damaged neurons. In response to estrogen, microglial inflammatory mediators such as TNF- α are down regulated in astrocytes while trophic factors such as IGF-I and TGF- β 1 are up-regulated (Liao et al., 2002; Duenas et al., 1994). Similarly such responses *in vitro* have been observed in *in vivo* models of neurodegeneration. For example, Quesada and Micevych (2004) reported a decrease in DA cell loss by 17 β -estradiol and IGF-1 following 6-hydroxydopamine (6-OHDA) lesioning of the nigrostriatal dopaminergic pathway, and the IGF-1 receptor antagonist JB-1 blocked these effects. Together these studies demonstrate that estrogen positively regulates trophic factor signaling. Since glia and neurons are responsive to estrogen, it is possible that the neuroprotective mechanism of estrogen requires signal cross-talk between the two cell types.

Although there is substantial evidence demonstrating estrogen-mediated neuroprotection of dopaminergic neurons, the majority of these studies have been conducted in homogeneous cell lines that are only representative of a neuronal population. This becomes a problem when trying to identify estrogens mechanism of action because these neurons do not fully express the phenotypic properties of true neurons and are not cultured amongst other physiological cell-types, such as glia. We utilize an *in vitro* model of neurodegeneration in which mouse primary mesencephalic cultures, containing both neurons and glia, are

pretreated with 17 β -estradiol and estrogenic compounds followed by exposure to the dopaminergic neurotoxin, MPP⁺.

MATERIALS & METHODS

Primary mesencephalic cultures

Animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health.

Progenitor cells were harvested from E13.5 (vaginal plug, day 0.5) embryos from time-mated C57BL/6 mice (Harlan Sprague-Dawley; Indianapolis, IN). Pregnant mice were killed by CO₂ asphyxiation and E13.5 embryos were collected in 1x HBSS containing gentamycin. The ventral mesencephalon was dissected away from underlying tissue at the mesencephalic flexure as previously described by Studer (1997) and single cells were mechanically dissociated from pooled mesencephalic tissue with fire polished Pasteur pipettes. Cells were plated directly onto glass coverslips (12 mm diameter, Fisher) in 24-well plates (Fisher Scientific; Pittsburgh, PA). Coverslips were pretreated by washing in acid followed by heat sterilization and subsequent coating with 500 μ g/ml poly-L-ornithine for 1 hr at 37°C in 8% CO₂ followed by 10 μ g/ml fibronectin overnight at 4°C. Cells were grown in chemically defined media (DM) at a density of 160,000 cells/cm² with bFGF (20 ng/ml, Upstate Biotechnology; Lake Placid NY) and incubated at 37°C in 5% CO₂. Basal medium comprised of MEM/Ham's F-12 (Gibco/Invitrogen; Carlsbad, CA) 1/1 (v/v) supplemented with 25 mM NaHCO₃, 25 mM, glucose, 15 mM HEPES and 2 mM glutamine. Defined medium was prepared by supplementing basal medium with 100 μ g/mL human apo-transferin, 60 μ M putrescine, 5 μ g/mL insulin, 30 nM sodium selenite and 20 nM progesterone. Following this protocol, TH⁺ neurons represent 4–5% of the total number of neurons in culture, as determined by TH (1:1000, Novus Biologicals; Littleton, CO) vs. NeuN (1:1000 Chemicon; Temecula, CA) immunocytochemistry. For neuron-enriched cultures, mesencephalic cells prepared as above were treated with the mitotic inhibitor, 5-Fluoro-2'-deoxyuridine (dFUR) at a concentration of 15 μ g/mL beginning on day two for 48 hr (day of plating was considered as day 0.) Cultures were rinsed and replaced with fresh serum-free defined media prior to 17 β -estradiol treatments. Neuronal enrichment was verified by immunocytochemistry using glial fibrillary acidic protein (GFAP) (1:200, Boehringer Mannheim Corp.; Indianapolis, Ind.), MAC-1 (1:500, Serotec, Raleigh; North Carolina) and TH (1:1000 Novus Biologicals; Littleton, CO). dFUR treatment reduced glial expression by >95% whereas the number of TH expressing neurons remained the same between dFUR and non-dFUR treated coverslips. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Preparation of astroglial monolayers

Glial cultures were prepared from the midbrain of postnatal day 0–3 C57BL/6 mice (Harlan Sprague-Dawley; Indianapolis, IN) pups. A modified protocol from Ho and Blum (1997) was used for the preparation of astroglial cultures. Briefly, the meninges were removed from dissected midbrain and tissues were trypsinized (2.5 mg/ml) and treated with DNase I (10 μ g/ml) for 15 min at 37°C. Dulbecco's minimal essential medium (DMEM) (Gibco/Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) was added and the tissue suspension was centrifuged. Following resuspension in DMEM plus 10% FBS, the cell suspension was filtered through a 70 μ m nylon mesh screen (Falcon; Franklin Lakes, NJ). Cells were plated at a density of 1 x 10⁶ cells per 60mm dish and grown in 5% CO₂ at 37°C. Confluent monolayers were shaken at 37°C for 2 hr at 250 rpm to remove microglia.

The purity of the cells was assessed using antibodies directed against MAC-1 and GFAP for identification of microglia and astrocytes, respectively.

Chemicals and Treatments

17 β -Estradiol, 17 α -estradiol, 2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN) (Tocris; Ellisville, MO), and 2,2-*bis*(*p*-hydroxyphenyl)-1,1-trichloroethane (HPTE) (Cedra Corp; Austin, TX) were added to the mesencephalic cultures 24 hr, or at indicated times prior to MPP⁺ treatment and removed by replacing cultures with fresh media containing MPP⁺ for 24 hr. 1 μ M ICI182,780 (ICI) (Tocris; Ellisville, MO) was applied to cultures at least 1 hr before addition of 17 β -estradiol. MPP⁺ (0.1 M), 17 β -Estradiol (1 mM), and dFUR (25 mg/mL) were dissolved in water, 17 α -estradiol, DPN, HPTE and ICI182,780 were dissolved in ethanol at 1 mM before use and diluted in defined media to the final concentrations. Control cultures received the appropriate amount of vehicle, which did not alter dopamine neuronal survival as measured by TH immunocytochemistry. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Cell Fractionation

Subcellular fractionation analysis was performed as previously described (Jeske et al., 2004). Following homogenization by 20 strokes in a Potter-Elvehjem homogenizer in a hypotonic homogenization buffer (25 mM HEPES, 25 mM sucrose, 1.5 mM MgCl₂, 50 mM, NaCl, pH 7.2), the samples were centrifuged at 1000 x g for 5 min to remove nuclei (N) from the homogenate. The resulting post-nuclear supernatant was centrifuged at 15,000 x g for 30 min to separate cytosolic proteins (C) from cell membrane proteins (M). Equal proportion aliquots from each crude fraction were combined with 2X SDS-PAGE sample Buffer, resolved by SDS-PAGE and visualized by Western blot analysis.

SDS-Page and Western Blotting

Protein samples were boiled in SDS-PAGE loading buffer, separated on 12.5% polyacrylamide sodium dodecyl phosphate (SDS) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore; Billerica, MA). Membranes were incubated in 5% non-fat milk at room temperature for 1 hr to block non-specific antibody binding followed by overnight incubation at 4°C with the appropriate primary antibody (ER α , Santa Cruz Biotechnology, Santa Cruz, CA; ER β , Zymed Laboratories, San Francisco, CA; Flotillin-1, BD Transduction Laboratories, Lexington, KY). Membranes were washed and incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Amersham Biosciences; Buckinghamshire, England) for 1 hr at 25°C and developed using a chemiluminescence kit (Amersham Biosciences; Buckinghamshire, England).

Immunocytochemistry

Fluorescent Immunocytochemistry. Mesencephalic cultures were grown on poly L-ornithine and fibronectin coated coverslips for 5 days in defined media. Coverslips were rinsed with phosphate buffer saline (PBS), and fixed with 4% paraformaldehyde for 10 min at 25°C. Following fixation, coverslips were rinsed twice with PBS, and incubated with 5% normal goat serum, 0.5% Triton X-100 in PBS for 30 min at 25°C. Coverslips were then incubated with a combination of anti-sera directed specifically towards ER α (1: 500, MC20 Santa Cruz Biotechnology; Santa Cruz, CA), ER β (1: 500, Zymed laboratories; San Francisco, CA), TH (1: 500, Novus Biologicals; Littleton, CO) and GFAP (1: 500, Chemicon; Temecula, CA), overnight at 4°C. Coverslips were then rinsed three times and incubated with appropriate fluorescently conjugated secondary antibodies (Jackson ImmunoResearch; West Grove, PA) for 1 hr at 25°C. Following three rinses with PBS, nuclei were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; 100ng/mL) for

5 min. Coverslips were mounted onto glass microscope slides with PermaFluor aqueous mounting medium (Thermo/Shandon Immunon; Pittsburgh, PA) and dried overnight. To confirm that double labeling was not due to bleed through of fluorescent dyes, coverslips were also incubated with only one of the primary antibodies. For double-label immunofluorescence, stained cells were viewed on a Zeiss Axioplan 2 microscope and images captured using Axiovision imaging software. Confocal analysis of coverslips was carried out in conjunction with UTHSCSA Imaging Core Services, San Antonio, TX, USA.

Tyrosine Hydroxylase Immunocytochemistry: At specified times, cells were fixed in 4% paraformaldehyde for 10 min and washed in PBS. Endogenous peroxidase activity was quenched by a 10 min incubation in 0.3% H₂O₂. Non-specific antibody binding was blocked by incubation for 30 min in PBS with 5% goat serum (Vector Laboratories; Burlingame, CA) containing 0.01% Triton X-100. Cells were incubated with primary antibody (1:1000, rabbit polyclonal anti-tyrosine hydroxylase (TH) Pel-freeze; Rogers, AR) in blocking solution overnight at 4°C. Cells were washed three times with PBS and then incubated with biotinylated anti-rabbit IgG (1:500, Vector Laboratories; Burlingame, CA) for 2 hr at room temperature. Cells were washed and incubated with ExtrAvidin (1:1000) for 1 hr at room temperature. After further washes, staining was visualized with 3,3'-diaminobenzidine tetrachloride (0.05% in 0.003% H₂O₂). Coverslips were then washed and mounted on glass microscope slides in aqueous mounting medium (Thermo/Shandon Immunon; Pittsburgh, PA). Stained cells were viewed on a Zeiss Axioplan 2 microscope and images captured using Axiovision imaging software (Carl Zeiss Inc.; Göttingen, Germany). TH-ir cells were manually counted with the assistance of Stereo Investigator's meander scan function (MicroBrightField Inc.; Williston, VT) on the Zeiss Axioplan 2 microscope. The numbers of TH positive cells was counted from n=4 coverslips for each condition and was performed in triplicate.

Statistical analysis

MPP⁺ dose response and neuroprotective effects mediated by estrogenic compounds were analyzed using one-way ANOVA followed by Tukey-Kramer post-hoc comparisons. All statistical procedures were performed using GraphPad Prism v. 4 (GraphPad Software Inc., San Diego CA). In all tests, p<0.05 was defined as statistically significant.

RESULTS

Receptor expression in mouse mesencephalic cultures

To verify and characterize the expression of estrogen receptors (ER) in mouse mesencephalic cultures, subcellular fractionation of cell homogenates was performed to determine the relative distribution of the ER subtypes among the nuclear, cytosol and membrane compartments (Fig. 1A). There was expression of both ER α and ER β in the cytosolic and membrane fractions, with a predominance of ER α in the latter and low expression of either receptor in the nuclear fraction (Fig. 1A). In order to investigate whether astroglia alone could respond to 17 β -estradiol, purified astrocytes cultured from mouse mesencephalon were also analyzed for ER expression. As shown in Figure 1B, in purified astrocytes, ER α expression was limited to the cytosolic fraction with low expression in either the nuclear or membrane compartments. Equivalent proportions of each fraction were resolved by SDS-PAGE in order to make a direct comparison as to the relative abundance. This results in a lower level of protein analyzed in the membrane fraction, which explains the low level of ER expression in the membrane fraction. Expression of ER β was very low in all three fractions.

ER α and ER β expression were also visualized by double fluorescence immunocytochemistry. As shown in Figure 2 A,B, primary mesencephalic cultures

express both receptors, which appear to be localized within the same neurons. The observed ER α and ER β expression on the same neurons was not caused by fluorophore bleed-through because we still detected similar ER distribution in mesencephalic cultures stained with single ER specific antibodies (data not shown). Consistent with the Western blot data, there was abundant expression of ER α and ER β in the cytoplasm (Fig. 2. A,B). Immunostaining of purified astrocyte cultures revealed abundant expression of ER α in comparison to ER β in agreement with Western analysis and more background than cellular staining of ER α (Fig. 2. C,D). Interestingly, the DAPI counterstain suggested that most of ER α was localized to the perinuclear compartment and cytoplasm as was observed with the Western blot data (Fig. 2. C,E). The DAPI counterstain of ER β showed that the overall low level of ER β expression was mostly cytoplasmic than nuclear (Fig. 2. D,F).

To ascertain the expression of estrogen receptors on DA neurons, double label immunofluorescence was performed using TH as a dopaminergic marker. Figure 3. A shows primary mesencephalic neurons immunocytochemically labeled with TH (red) and ER α (green). Merged figures reveal double staining of ER α in the cell body of DA neurons, which was evident in all TH $^{+}$ cells. To confirm previous results of ER α expression in pure astrocyte cultures, mixed mesencephalic cultures were double labeled with GFAP (green) and ER α (red). Merged figures reveal abundant staining of ER α in astrocytes (Fig. 3. B).

Estrogen prevents MPP $^{+}$ induced loss of TH immunoreactive (TH-ir) cells in primary mesencephalic cultures

Cell counts of TH-ir DA neurons were used as a measurement of MPP $^{+}$ toxicity of DA neurons. Treatment with 1, 5 and 10 μ M MPP $^{+}$ caused a dose dependent reduction in TH-ir cells (Fig. 4, *** p <0.001 compared to vehicle control). 10 μ M MPP $^{+}$, which caused a 50% reduction in the number of TH-ir cells, was used for the remainder of the experiments.

Initial dose response experiments in the range of 10 pM to 100 nM 17 β -estradiol were conducted and no protection from MPP $^{+}$ toxicity was observed until 10 nM, which displayed full protection (data not shown). Treatments were organized into seven groups: control, 24 hr pre-treatment with 17 β -estradiol (10 nM), 24hr treatment with MPP $^{+}$ (10 μ M), and 0.25, 0.5, 1, 4 and 24 hr pre-treatment with 17 β -estradiol followed by a 24 hr treatment with MPP $^{+}$. As shown in Figure 5., treatment with 17 β -estradiol alone had no effect on the number of TH-ir neurons and was comparable to control, while treatment with 10 μ M MPP $^{+}$ significantly reduced the number of TH-ir cells by 55% (Fig. 5, *** p <0.001 compared to vehicle-treated control). An increase in TH-ir cell protection from MPP $^{+}$ toxicity was observed with increased time of 17 β -estradiol pretreatment. 17 β -estradiol pretreatment for 1, 4 and 24 hr significantly protected from MPP $^{+}$ -induced loss of TH-ir cells (Fig. 5, ## p <0.01 and ### p <0.001 compared to MPP $^{+}$ alone group). Leaving 17 β -estradiol in the culture during MPP $^{+}$ exposure did not further protect against TH-ir cell loss (data not shown). Furthermore, adding 17 β -estradiol during or post MPP $^{+}$ treatment, without pretreatment, did not protect DA neurons from MPP $^{+}$ induced cell death (data not shown).

ER α activation is required for estrogen-mediated protection of DA neurons

To determine if the estrogen-mediated protection of DA neurons was ER dependent, cultures were treated with the 17 β -estradiol stereoisomer, 17 α -estradiol and the ER α / β antagonist, ICI 182,780 (ICI). 17 α -estradiol failed to protect against MPP $^{+}$ induced neurotoxicity, whereas application of ICI (1 μ M) 1 hr prior to 17 β -estradiol exposure completely blocked the neuroprotection provided by estrogen (Fig. 6, ++ p <0.01 compared to 17 β -estradiol plus MPP $^{+}$ treatment).

To determine the contribution of the ER α or β subtype to the neuroprotective effect of estrogen, mesencephalic cultures were pre-treated with 17 β -estradiol, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), an ER α specific agonist (Roy et al., 1999), diarylpropionitrile (DPN), an ER β specific agonist (Frasor et al., 2003) or HPTE simultaneously with DPN for 24 hrs prior to exposure with MPP $^{+}$. As observed previously, 24 hr exposure of 10 μ M MPP $^{+}$ induced a significant decrease in the number of TH-ir neurons compared to vehicle control (Fig. 7, *** p <.001 compared to vehicle control). As expected, 24 hr (10 nM) completely protected against MPP $^{+}$ -induced loss of TH-ir cells (Fig. 7, ### p <0.001 compared to MPP $^{+}$ treated control). HPTE (10 nM), which had no effect on TH cell number when given alone, also protected against MPP $^{+}$ -induced loss of TH-ir cells (Fig. 7, ### p <0.001 compared to MPP $^{+}$ treated control). On the other hand, DPN afforded no significant protection from MPP $^{+}$ -induced TH cell loss and was comparable to MPP $^{+}$ treatment alone. In addition, HPTE and DPN combined did not provide any further protection than HPTE alone (data not shown), thus eliminating the role of ER β in conjunction with ER α in the neuroprotective effects of a 24 hr pre-treatment.

Glial cell input is required for estrogen-mediated protection of DA neurons

As glial cultures showed an abundant expression of ER α and little ER β and the protective effect was mediated by ER α , we examined whether the presence of glia in mesencephalic cultures was necessary to induce protection by 17 β -estradiol. 5-Fluoro-2'-deoxyuridine (dFUR), a mitotic inhibitor, was used to remove glial cells from primary mesencephalic cultures. Cells were pre-treated with 17 β -estradiol as previously described, however cultures were treated with a lower concentration of MPP $^{+}$ (5 μ M) since in the absence of glia, the sensitivity to MPP $^{+}$ -induced toxicity increases (data not shown). Consistent with the above results, MPP $^{+}$ (5 μ M) induced a 55% decrease in TH-ir neurons and treatment with 17 β -estradiol alone caused no significant change in the number of TH-ir cells (Fig. 8, ### p <0.001 compared to vehicle control). However, 24 hr pre-treatment with 17 β -estradiol followed by MPP $^{+}$ exposure no longer protected against the loss of TH-ir cells (Fig. 8, *** p <0.001 compared to vehicle-treated control). Non-dFUR treated cells pre-treated with 17 β -estradiol for 24 hr served as a positive control and showed similar protection from MPP $^{+}$ as previously observed (Fig. 8 +++ p <0.001 compared to MPP $^{+}$ treatment). The total number of TH-ir cells did not differ between dFUR and non-dFUR treatments (data not shown). These results suggest a crucial role for glia in the ER α -mediated protection of DA neurons by estrogen.

DISCUSSION

17 β -estradiol displays survival promoting and neuroprotective effects, however our studies demonstrate that its actions are highly dependent on surrounding glial cells. By counting the number of TH-ir neurons after estrogenic and MPP $^{+}$ exposure we found 17 β -estradiol and HPTE (ER α specific agonist) to be significantly neuroprotective against MPP $^{+}$ -induced DA cell loss. Pre-treatment for at least four hours was required to induce significant protection of DA neurons from MPP $^{+}$ toxicity and was comparable to a 24 hr pre-treatment. These neuroprotective effects of 17 β -estradiol were observed only when mesencephalic neurons were cultured in the presence of glia. Treating the cultures with dFUR to eliminate astrocytes completely abolished the observed estrogen-induced neuroprotection. The dependence of glia to elicit the ER α mediated neuroprotection was supported by the immunoblotting and immunofluorescence data showing abundant expression of ER α in glial cultures. These data are consistent with recent findings suggesting an interaction of estrogen with glial cells to induce neuroprotection (Dhandapani et al., 2005; Pawlak et al., 2005).

Neuroprotective studies with estrogen have demonstrated the requirement of an estrogen pre-treatment prior to neurotoxin exposure in order to display neuroprotective effects.

Results from this study support the notion that there is a time specific response of estrogen on neuronal survival, which is dependent on the duration of estrogen exposure prior to injury. In the present study, estrogen-mediated neuroprotection against MPP⁺-induced DA cell loss could be observed by 1 hr of estrogen pre-treatment with a rise in protection occurring by 4 hr and full protection by 24 hr pre-treatment. These observations do not necessarily rule out rapid membrane signaling (sec–min), since estrogen-mediated effects at the membrane may also lead to downstream signaling and regulation of gene expression, which is observable hours later.

A previous study in rat mesencephalic cultures demonstrated protective properties of 17 β -estradiol and its inactive stereoisomer, 17 α -estradiol against MPP⁺ induced toxicity (Callier et al., 2002). Neuroprotection was only observed using high concentrations of MPP⁺ (50 μ M), which is no longer selective for the DA neuron. The protection was likely due to antioxidant properties of the steroids as only concentrations in the high micromolar range (10–100 μ M) were able to induce protective effects. In addition the protection against MPP⁺ toxicity was observed in serum-supplemented and not serum-free medium, which could also contribute to the neuroprotection. In contrast, our study utilized mouse mesencephalic cultures grown in serum-free conditions and all treatments were conducted in serum-free defined media to eliminate possible protective effects by amino acids, hormones and growth factors present in serum. However, consistent with Callier et al. (2002), full protection was not observed with a 1 hr 17 β -estradiol pre-treatment as a longer pre-treatment (at least 4 hr) was necessary to induce full neuroprotection. The lower concentrations of steroid used to elicit protection, the lack of protection by 17 α -estradiol, and the blocking of neuroprotection by ICI, the non-selective ER antagonist, reduce the likelihood of an antioxidant effect of 17 β -estradiol in our studies.

A second study in rat primary mesencephalic cultures showed that low concentrations (1–10 nM) of both 17 β -estradiol and 17 α -estradiol were neuroprotective against MPP⁺-induced neuronal toxicity and both effects were completely reversed by ICI (Sawada et al., 2002). Such protection was postulated to involve a decrease in TNF- α levels (Sawada et al., 2002). This was supported by Western blot data, which showed only ER α staining and a decrease in TNF- α staining after 17 β -estradiol treatment. We found expression of ER α and ER β in the mouse mesencephalic and glial cultures by both Western blotting and immunofluorescence and the use of the ER α specific agonist, HPTE established a functional role for the observed ER α expression. Expression of both ER α and ER β mRNA has been reported in the embryonic rat mesencephalon (Lu et al., 2004; Raab et al., 1999), therefore it is unclear as to why there was no observable expression of ER β by Sawada et al. (2002).

The screening of estrogenic compounds specific to either ER α or ER β revealed HPTE, an ER α specific agonist, as a significant protectant against MPP⁺-induced DA cell loss. DPN, the ER β specific agonist afforded no protection alone or in combination with HPTE, which indicated that ER β did not play a role in the observed protection by 17 β -estradiol of TH neurons 24 hr post MPP⁺ treatment. These studies are consistent with reports of estrogen-mediated neuroprotection through ER α (Cordey and Pike, 2005; Dubal et al., 2006; Zhao et al., 2004). Studies in ischemic ER knockout mice demonstrated that unlike ER α knockouts, in ovariectomized ER β knockouts estrogen replacement no longer protected against cerebral ischemia (Dubal et al., 2001). Studies by Zhao et al. (2004) report a protective effect of PPT, another ER α selective agonist, in addition to DPN against glutamate-induced toxicity in primary hippocampal neurons.

In our mesencephalic culture system, DPN did not protect against MPP⁺-induced damage of DA neurons. However, this does not rule out a role for ER β mediated neuroprotection because although no effect of DPN was found 24 hr post MPP⁺ treatment, it remains

possible that ER α may be involved in the later stages of estrogen-mediated neuroprotection such as regeneration and survival of protected neurons. The role of ER α in neuronal survival has been studied in ER α knock out mice where the lack of ER α results in morphological deficits characterized by brain atrophy and neuronal shrinkage (Wang et al., 2003). In addition, studies have shown an immediate induction of ER α followed by a down regulation of ER α in late stage ischemic injury (24 hr), which is prevented by estrogen treatment (Dubal et al., 2006). Similarly, in this study, the initial estrogen-mediated neuroprotective actions of estrogen were ER α mediated, occurring within 24 hr of MPP $^{+}$ exposure. It will be of interest to further examine the role of ER α on neuronal survival at later times after MPP $^{+}$ exposure.

Defining the neuroprotective role of ER α and ER β becomes complex when comparing different models of neurodegeneration, which utilize different species and/or modes of inducing neuronal damage. Although the mechanisms of action reported differ in each case, the commonality is the neuroprotection afforded by estrogen. In addition, the time point at which estrogen-induced neuroprotection is measured is critical because these differences may dictate the specific role of each ER subtype. Furthermore, since estrogen appears to target multiple cell types, studying estrogen signaling in a system that allows estrogen to interact with both neuronal and non-neuronal cells would provide a more comprehensive understanding of estrogen-mediated neuroprotection.

In the present study, protection of DA neurons was abolished when mesencephalic cultures were devoid of glia, which suggested a role for glia in estrogen mediated-neuroprotection. dFUR inhibited glia cell proliferation resulting in the detachment of glia cells from mesencephalic cultures. The concentration of MPP $^{+}$ utilized in the dFUR treated mesencephalic cultures was lower than the 10 μ M MPP $^{+}$ used in experiments where glia were present in the cultures in order to achieve only 50% reduction of TH-ir neurons in the 24 hr period. In response to toxicity, glia become activated and presumably release soluble factors to protect and maintain neuronal homeostasis, which may explain why in the presence of glia, 10 μ M MPP $^{+}$ was necessary to induce a 50% reduction of TH-ir neurons and in the absence of glia, 5 μ M MPP $^{+}$ was sufficient to reduce TH-ir neurons by 50%. When the neurons depleted of glia were subjected to MPP $^{+}$ exposure, 17 β -estradiol pre-treatments no longer protected neurons from MPP $^{+}$ toxicity. The data presented here indicates an interaction of estrogen with glial cells to facilitate the observed protection of DA neurons.

Such estrogen-glial cell interactions have been studied in models of estrogen-mediated neuroprotection and suggest a distinct responsiveness of astrocytes to estrogen (Dhandapani et al., 2005; Garcia-Ovejero et al., 2005; Pawlak et al., 2005). Studies have shown signaling pathways targeted by estrogen as well as downstream neurotrophic growth factors regulated in response to estrogen. Dhandapani et al. showed that estrogen treatment increased the expression and release of both TGF- β 1 and TGF- β 2 in cortical astrocytes through the phosphatidylinositol 3-kinase PI3K-Akt signaling pathway (Dhandapani et al., 2005). Furthermore in the presence of the PI3K inhibitors, LY294002 and Wortmannin, estrogen no longer protected cortical neurons from camptothecin-induced neuronal toxicity (Dhandapani et al., 2005). Such rapid signaling suggests actions by a membrane estrogen responsive system, which has been described in mouse midbrain astroglial cultures (Pawlak et al., 2005). Estrogen was found to phosphorylate and activate both Src and ERK1/2, with maximum phosphorylation occurring at 7 and 15 min respectively, an effect that was suggested to be mediated by ER α (Pawlak et al., 2005). Indeed it is possible that estrogen acting on glial cell membrane ERs may activate signal transductions pathways to regulate downstream transcription and/or release of proteins, which act to protect against MPP $^{+}$ toxicity. Together, these studies further support the notion that estrogen-mediated

neuroprotection involves several mechanisms, direct and indirect, which when integrated together promote neuronal survival.

In summary, the current findings demonstrate that estrogen mediated neuroprotection of DA neurons involves an interplay of more than one cell type. In addition, the study indicates that estrogen effects are time dependent, suggestive of a signal transduction response regulating protein phosphorylation and/or downstream transcription. The requirement of glia to elicit estrogen effects supports an indirect estrogen action to protect DA neurons, possibly by inhibiting the endogenous pro-inflammatory and/or enhancing the protective properties of glial cells. Further studies are necessary to characterize estrogen signaling in non-neuronal cells and to determine estrogen-mediated signals between cell types resulting in the observed neuroprotection. Thus, restoring the depletion of estrogen in the aging brain may prevent the loss of estrogen mediated trophic support, thus reducing the risk for neurodegenerative diseases such as Parkinson's Disease.

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Fig. 1. Subcellular expression of estrogen receptors, ER α and ER β in mouse mesencephalic cultures and astroglia. Western Blot analysis of protein collected from E13.5 mouse mesencephalic cultures (A) and purified P1 mouse mesencephalic astrocytes (D) subjected to subcellular fractionation into nuclear (N), cytosol (C) and membrane (M) compartments. Fractions represent equal proportion aliquots from each crude fraction where the pellet fractions were resuspended in equal volumes to the cytosol. Thus they represent equal proportions of the entire sample.

Fig 2. Fluorescence immunocytochemistry of ER α and ER β . Double label Immunofluorescence staining of primary mesencephalic cultures (A,B) and pure mesencephalic glial cultures (C–F). Cultures were stained with ER α (green) or ER β (red) and the nuclei were counterstained with DAPI (blue).

Fig. 3. ER α is localized primarily on astrocytes. Immunocytochemical staining and double labeling of primary mesencephalic cultures. (A) Dopamine neurons were stained with antibodies to TH (red) and ER α (green). (B) Astrocytes were stained with antibodies to GFAP (green) and ER α (red).

Fig. 4. MPP⁺ dose-dependently reduces the number of TH-ir neurons from mesencephalic cultures. Cultures were treated with 0, 1, 5, or 10 μM MPP⁺ for 24 hr and cells were fixed. Results are presented as number of TH-ir cells, expressed as percent of control, and represent the mean ± SEM of three independent experiments. ***p<0.001 compared to vehicle control.

Fig. 5.

Time course of estrogen induced protection of DA neurons. Mouse mesencephalic cultures were divided into six treatment groups: vehicle-treated control, 60min 17 β -estradiol (10 nm), 24 hr MPP⁺ (10 μ M), and 15, 30 or 60 min 17 β -Estradiol pre-treatment followed by 24 hr MPP⁺ (10 μ M) exposure. 17 β -Estradiol was washed out prior to addition of MPP⁺. Results are presented as number of TH-ir cells, expressed as percent of control, and represent the mean $\pm$ SEM of three independent experiments. ***p<0.001 compared to vehicle control, ##p<0.01 and ###p<0.001 compared to MPP⁺ alone group.

Fig. 6.

ER involvement in protection of MPP⁺-induced loss of TH-ir cells. Mouse mesencephalic cultures were pre-treated with 17 β -Estradiol, 17 α -estradiol or ICI prior to the addition of MPP⁺ (10 μ M). ICI was added to the cultures 1 hr prior to 17 β -estradiol exposure. Results are presented as number of TH-ir cells, expressed as percent of control, and represent the mean $\pm$ SEM of three independent experiments. ***p<.001 compared to vehicle control, ##p<0.01 compared to MPP⁺ treatment and ++p<0.01 compared to 17 β -Estradiol plus MPP⁺ treatment.

Fig. 7.

Neuroprotective effect of estrogen on TH-ir is mediated by ER. Mouse mesencephalic cultures were pre-treated with 17-β-Estradiol, HPTE, or DPN for 24 hr prior to the addition of MPP⁺ (10 μM). Results are presented as number of TH-ir cells, expressed as percent of control, and represent the mean ± SEM of three independent experiments. ***p<.001 compared to vehicle control and ###p<0.001 compared to MPP⁺ treated control.

Fig. 8.

Glia involvement in estrogen-mediated protection of MPP⁺ induced loss of TH-ir neurons. Mesencephalic cultures were treated with 15 μg/mL dFUR for 48 hr, which was washed out prior to estrogen pre-treatment. Cultures were pre-treated with 17 β-Estradiol for 24 hr prior to the addition of MPP⁺ (5 μM) for 24 hr. Results are presented as number of TH-ir cells expressed as percent of control, and represent the mean ± SEM of three independent experiments. ###p<0.001 and ***p<0.001 compared to vehicle control and +++p<0.001 compared to MPP⁺ treatment.