Molecular Pharmacology Fast Forward. Published on January 24, 2006 as doi:10.1124/mol.105.018671

MOL Manuscript #18671

Title page

Implication of the PI3K/Akt signaling pathway in the neuroprotective effect of estradiol in the striatum of MPTP mice.

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Molecular Pharmacology Fast Forward. Published on January 24, 2006 as DOI: 10.1124/mol.105.018671
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Running title page

a) Running title: PI3K/Akt in estradiol neuroprotection

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c) Number of text pages: 30

Number of tables: 2

Number of figures: 6

Number of references:40

Number of words in the Abstract: 250

Number of words in the Introduction: 390

Number of words in the Discussion: 1552

d) Abbreviations: ER α, estrogen receptor alpha; ER β, estrogen receptor beta; IGF-I, insulin growth factor; IGF-IR, IGF-I receptor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PPT, 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)tris-phenol; Δ3-diol, 5-androsten-3β, 17β-diol; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3 kinase; Akt, protein kinase B; GSK3β, glycogen synthase kinase 3β; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid, HPLC, high performance liquid chromatography, SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; pAkt or pSer473Akt, phosphorylated Akt at serine 473; pGSK3 or pSer9GSK3β, phosphorylated GSK3β at serine 9.

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Abstract

The present experiments sought to determine the implication of estrogen receptors (ERα and ERβ) and their interaction with insulin-like growth factor receptor (IGF-IR) signaling pathways in neuroprotection by estradiol against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity. C57Bl/6 male mice were pre-treated for 5 days with 17β-estradiol, an estrogen receptor alpha (ERα) agonist, PPT (4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)tris-phenol) or an estrogen receptor beta (ERβ) agonist, Δ3-diol (5-androsten-3β, 17β-diol). On day 5, mice received MPTP (9 mg/kg) or saline injections and estrogenic treatments were continued for 5 more days. MPTP decreased striatal dopamine, measured by HPLC, to 59% of control values; 17β-estradiol and PPT but not Δ3-diol protected against this depletion. MPTP increased IGF-IR measured by Western blot, which was prevented by PPT. The phosphorylation of Akt (at serine 473), an essential mediator of IGF-I neuroprotective actions, increased following 17β-estradiol, tended to increase with PPT but not with Δ3-diol treatments in MPTP mice. GSK3β phosphorylation (at serine 9) was greatly reduced in MPTP mice; this was completely prevented by PPT whereas 17β-estradiol and Δ3-diol treatments were less effective. The ratio between the levels of striatal Bcl-2 and BAD proteins, two apoptotic regulators, decreased after MPTP treatment. This effect was effectively prevented only in the animals treated with PPT. In non-lesioned mice, 17β-estradiol and PPT increased phosphorylation of striatal Akt and GSK3β, while the other markers measured remained unchanged. Δ3-Diol increased GSK3β phosphorylation less than the PPT treatment. These results suggest that a pre-treatment with estradiol promoted dopamine neuron survival by activating ERα and increasing Akt and GSK3β phosphorylation.

Introduction

Many studies have demonstrated the neuroprotective effects of estradiol *in vivo* against neurotoxins of the nigrostriatal dopaminergic system (Callier et al., 2000; Dluzen and McDermott, 2000; D'Astous et al., 2004). The molecular mechanisms implicated in the neuroprotection are still to be described. The aim of the present experiment was to investigate the possible implication of the insulin-like growth factor (IGF-I) signaling pathway in the neuroprotective effects of estradiol since there is a great interdependence between the actions of estradiol, IGF-I and their respective receptors. Indeed, these molecules interact with one another, via their receptors, and are involved in cross talking through different signaling pathways (Kahlert et al., 2000). These molecules interact to positively affect neuronal differentiation, neurogenesis, synaptic plasticity, neuroendocrine regulation and also neuroprotection (Cardona-Gomez et al., 2001; Garcia-Segura et al., 2001).

Intracellular signaling of IGF-I receptors (IGF-IR) is mediated by the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 kinase (PI3K) pathways (LeRoith et al., 1993; Cardona-Gomez et al., 2002). PI3K promotes the phosphorylation and activation of Akt (also known as protein kinase B), a general mediator of cell survival (Datta et al., 1997). Therefore, activation of IGF-IR leads to activation of PI3K and Akt. Akt can inhibit apoptosis induced by several stimuli in multiple cell types, acting on various factors influencing cell death, such as members of the Bcl-2 family. Akt regulates Bcl-2 levels (Pugazhenthi et al., 2000) and can phosphorylate and inactivate the pro-apoptotic protein BAD (Datta et al., 1997). Furthermore, Akt inhibits glycogen synthase kinase 3 (GSK3) activity by increasing its phosphorylation on

serine 9 and 21 (Cohen and Frame, 2001). In turn, inhibition of GSK3 is associated with the activation of survival pathways in neurons (Hetman et al., 2000).

The specific estrogen receptor alpha (ERα) has been implicated in the activation of the PI3K/Akt pathway (Kahlert et al., 2000; Mendez et al., 2003, 2005). Indeed, only ERα interacts with IGF-IR and PI3K in the brain, while estrogen receptor beta (ERβ) does not participate in such complexes (Mendez et al., 2005). This interaction might represent a way by which estradiol affects IGF-I signaling on the brain. In the present experiments, we sought if neuroprotection by estradiol against MPTP is mediated by the activation of the PI3K/Akt pathway. Moreover, with specific ER agonists, we determined if the protective effects of estradiol are dependent on the subtype of receptor.

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Material and methods

Chemicals

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 17β-estradiol were purchased from Sigma Chemical (St-Louis, MO, USA), 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)tris-phenol (PPT) from Tocris (Ellisville, MO, USA) and Δ3-diol (5-androsten-3β, 17β-diol, also known as 5-androstenediol, androstenediol or hermaphrodiol) was purchased from Steraloids Inc (Newport, R.I., USA). PPT is a specific ERα agonist (Stauffer et al., 2000), while Δ3-diol preferentially binds to and activates ERβ (Kuiper et al., 1997).

Animals and treatments

C57Bl/6 male mice (10-12 weeks, 25 ± 2g) were purchased from Charles River Laboratories (Canada). Mice were randomly assigned in groups of eight animals. Each group received a 5- day pre-treatment of estrogen receptor agonists or vehicle prior to MPTP injections. The pre-treatment consisted of two daily subcutaneous injections (in the dorsal part of the neck) of 17β-estradiol, PPT or Δ3-diol while control mice received injections of vehicle (0.9% saline with 0.3% gelatin). Concentrations used were 2 μg per day for 17β-estradiol and PPT, and 3 μg per day for Δ3-diol such as in our previous publication (D'Astous et al., 2004). On day 5, mice received four injections of MPTP (9 mg/kg, intraperitoneal) at 2 hours interval, while the control group received saline solution. The treatments (estrogenic compounds or vehicle) were continued until day 10 and the next day, mice were decapitated, brains were quickly removed and frozen in isopentane (- 40^{o}C). In a similar experiment, mice received estrogenic drug treatments for 10

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days at the same concentrations as described above, while one group received the vehicle. These groups served as control for the estrogenic treatments and were referred to as intact (non-lesioned) groups since no MPTP lesion was induced in these animals.

The Laval University Animal Care Committee approved all the animal studies. All efforts were made to minimize animal suffering and to reduce the number of mice used.

Striatal biogenic amines determination

The concentrations of dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by high performance liquid chromatography (HPLC) with electrochemical detection. Supernatants of striatal tissue were directly injected into the chromatograph consisting of a Waters 717 plus autosampler automatic injector, a Waters 515 pump equipped with a Beckman C-18 column (Waters Nova-Pak C_{18}, 3µm, 3.9 mm x 150 cm), a BAS LC-4C electrochemical detector and a glassy carbon electrode. The mobile phase consisted of 0.025 M citric acid, 1.7 mM 1-heptane-sulfonic acid and 10% methanol, in filtered distilled water, delivered at a flow rate of 1 ml/min. The final pH of 4.1 was obtained by addition of NaOH. The electrochemical potential was set at 0.8V with respect to an Ag/AgCl reference electrode, as described previously (D'Astous et al., 2004).

Western blot

Striata were dissected and homogenized in lysis buffer (150mM NaCl, 20mM Tris HCl, 10% glycerol, 5mM EDTA, 1% NP-40, Roche, Mannhein, Germany) supplemented with protease and phosphatase inhibitors (50µg/ml of PhenylMethylSulfonyl Fluoride, 10µg/ml aprotinin, 25µg/ml

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leupeptin and 100nM orthovanadate, all from Sigma, St. Louis, MO, USA). Homogenates were allowed to solubilize for 30 minutes on ice and centrifuged at 21000 x g for 10 min. Protein content of the supernatant was measured with a modified Bradford assay (BioRad, Munchen, Germany).

Proteins were resolved using sodium dodecylsulphate-polyacrylamide gel electrophoresis (10-12% SDS-PAGE) with a Mini-Protean system (Bio-Rad, Hercules, CA, USA) and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk diluted in 0.05% Tween-20 Tris-buffered saline and incubated overnight with the primary antibodies. The antibodies against IGF-IR (C20; diluted 1:1000), BAD (H168, diluted 1:1000) and Akt (H136, diluted 1:2000) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The monoclonal antibody against Bcl-2 (clone 124, diluted 1:500) was purchased from Dako (DAKO A/S, Denmark). Both phospho-specific antibodies against phosphorylated Akt at Serine 473 (pSer473Akt abbreviated as pAkt) and phosphorylated GSK3β at Serine 9 (pSer9GSK3β abbreviated as pGSK3) were used at a dilution of 1:1000 and were obtained from Cell Signaling (Cell Signaling, MA, USA). GSK3β monoclonal antibody was from BD Transduction (BD Pharmingen, CA, USA). Finally, βIII- tubulin antibody was from Promega (Madison, WI, USA). After incubation with the primary antibody, the membranes were washed and incubated with HRP-coupled secondary antibodies (Jackson Immunoresearch, PE, USA; diluted 1:10 000). Immunoreactive bands were detected using an enhanced chemiluminiscence system (ECL, Amersham Pharmacia Biotech, UK). When needed, membranes were stripped using a commercial solution purchased from Chemicon (Chemicon, CA, USA). Films were analyzed using the Molecular Dynamics Image Quant software version 3.22 (Computing

densitometer model 300A). For Bcl-2, Bad, IGF-IR, GSK3 and Akt, the density of each band was normalized to its respective loading control (β-III-Tubulin). For pAkt and pGSK3 the total levels of the kinase (Akt or GSK3) were used for normalization. In order to minimize inter-assay variations, samples from all animal groups, in each experiment, were processed in parallel.

Statistical analysis

Statistical comparisons of data were evaluated using a one-way analysis of variance (ANOVA) using Statview 4.51 for Macintosh Computer software, followed by a post-hoc analysis with the Fisher probability of least significant difference test. Coefficient of correlations and significance of the degree of linear relationship between the variables were determined using a simple regression model using the Statview software. A $p<0.05$ was required for the results to be considered statistically significant.

Results

A MPTP dose of 9 mg/kg gave a moderate depletion of striatal dopamine and its metabolites; vehicle-treated MPTP mice had dopamine depleted to 59% of the control animals (Table 1). PPT showed a clear protective effect against MPTP-induced striatal dopamine and DOPAC depletion. 17β-Estradiol prevented the MPTP-induced dopamine loss. Striatal dopamine concentrations of Δ3-diol treated MPTP mice were less significantly depleted compared to intact controls than MPTP + vehicle. Δ3-diol treated MPTP mice had significantly less striatal dopamine and DOPAC concentrations than the MPTP + PPT treated mice. Striatal concentrations of dopamine, DOPAC and HVA of unlesioned mice remained unchanged by the 17β-estradiol, PPT and Δ3-diol treatments (Table 2).

Administration of MPTP led to a significant increase in the concentrations of striatal IGF-IR (Fig. 1). Pre-treatment with PPT prevented the increase of IGF-IR levels, which were significantly lower than MPTP mice. 17β-Estradiol and Δ3-diol treated MPTP mice had levels not different from controls or vehicle-treated MPTP mice. Striatal IGF-IR levels were significantly higher in the MPTP + Δ3-diol than in the MPTP + PPT group.

The phosphorylated forms at serine residue 9 for GSK3β (pGSK3) and at serine residue 473 of Akt (pAkt) were also measured in these groups relative to their unphosphorylated form. Striatal Akt levels remained unchanged following MPTP lesion or estrogenic treatments (Fig. 2). However, in these MPTP mice, pre-treatment with 17β-estradiol induced a significant increase in pAkt/Akt, with regards to control mice. This increase in pAkt/Akt did not reach statistical

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significance ($p = 0.062$ versus control) with PPT treatment whereas Δ3-diol treated MPTP mice had lower pAkt/Akt levels than either 17β-estradiol or PPT treated MPTP mice.

MPTP administration induced a large reduction in the levels of phosphorylated GSK3β compared to the control group (Fig. 3). 17β-Estradiol and PPT pre-treatments prevented this decrease; pGSK3/GSK3 concentrations were significantly higher than the vehicle-treated MPTP group. Moreover, PPT completely spared the decrease of this protein, which was equal to control levels (Fig. 3). Δ3-Diol treated MPTP mice had a small increase of pGSK3/GSK3 compared to vehicle-treated MPTP mice and these levels were lower than estradiol or PPT treated MPTP mice.

Two different markers of apoptosis were measured, Bcl-2 and BAD. The Bcl-2/BAD ratios showed a significant effect of lesion and treatments. MPTP treatment decreased this ratio, compared to saline-vehicle treated mice and PPT prevented it. 17β-Estradiol and Δ3-diol treated MPTP mice had Bcl-2/BAD ratio neither different from saline-vehicle treated mice or MPTP mice. Δ3-diol treated MPTP mice had the Bcl-2/BAD ratio lower than the MPTP + PPT treated mice.

In unlesioned animals, administration of 17β-estradiol, PPT or Δ3-diol left unchanged the striatal IGF-IR, BAD or Bcl-2 levels (data not shown). 17β-Estradiol and PPT induced an increase in the phosphorylation of Akt and GSK3β (Fig. 5). Δ3-Diol did not significantly affect the phosphorylated state of Akt ($p= 0.126$ versus control and $p = 0.0558$ versus PPT) and increased GSK3β phosphorylation much less than the PPT treatment. There was a tight correlation between the phosphorylation levels of both Akt and GSK3β proteins ($r=0.87$), suggesting that there is a

functional relationship in response to the estrogenic compounds between these two kinases in mice striatum.

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Discussion

Although estrogen receptors are known to be involved in the neuroprotective mechanism of estradiol, high pharmacological concentrations of the hormone are necessary to exert neuroprotection in different experimental models of brain injury (Picazo et al, 2003). This suggests that non-classical mechanisms of action, such as the activation of membrane-associated signaling, are involved in these estrogen receptor-mediated effects. Indeed, high doses of estradiol are necessary to activate the brain PI3K/Akt signaling pathway (Cardona-Gomez et al., 2002) and ERα appears to be involved in this effect (Mendez et al., 2003; Cardona-Gomez et al., 2004). Thus, the participation of estrogen receptors in the neuroprotective mechanism may be mediated by the activation of membrane signaling and not by the direct regulation of transcription by binding to estrogen response elements in DNA. The present study investigated if the PI3K/Akt pathway of signalization is implicated in the neuroprotection following treatment with estrogen agonists. The PI3K/Akt pathway, one of the signaling pathways downstream of IGF-IR, is often linked to cell survival (Datta et al., 1999). Indeed, Akt is a major regulator of cell survival, since it presents regulatory activity on many molecules such as BAD, GSK3 (both known to be pro-apoptotic factors) and on transcription factors such as NF-κB (Brunet et al., 2001) (Figure 6).

The doses and protocol of administration of 17β-estradiol and PPT used in this study have been previously shown to prevent MPTP-induced striatal dopamine depletion (D'Astous et al., 2004). Therefore, this is an adequate experimental design to test whether the neuroprotective effect of estradiol and estrogenic ligands is correlated with a modification of the PI3K/Akt signaling pathway. The present study confirms that an ERα agonist treatment protects against MPTP-

induced striatal dopamine and DOPAC depletion and that this is statistically different from the ERβ agonist treated MPTP mice. In addition, 17β-estradiol and the ERα ligand PPT modulate the expression of IGF-IR. This finding is in agreement with previous studies showing that estradiol and IGF-I co-regulate each other, and their cognate receptors in the brain (Cardona-Gomez et al., 2001).

Since IGF-IR is coupled to two different signaling pathways leading to cell survival (PI3K/Akt and MAP kinases) (LeRoith et al., 1993; Cardona-Gomez et al., 2002), it is fair to assume that an augmentation in the expression of this receptor contributes positively to cell changes in response to toxic damages. It already has been shown that ERα is the only estrogen receptor to co-precipitate with IGF-IR (Kahlert et al., 2000; Mendez et al., 2003). Moreover, neuroprotection by estrogens has been linked to ERα activation in different models of toxicity (Dubal et al., 2001; Vegeto et al., 2003; D'Astous et al., 2004). However, in some experimental models, neuroprotection by estradiol is mediated by ERβ activation (Carswell et al., 2004). ERα and ERβ are detected in the mice striatum (Kuppers and Beyer, 1999) and are shown to remain unchanged after vehicle/MPTP or estradiol/MPTP treatments (Shughrue, 2004). Therefore although scarce, activation of ERα receptor by ER agonists could lead to transcriptional activity and to the regulation of the IGF-IR pathway. Alternatively, other ERα-like receptors may convey the ER agonist signal (Hasbi et al., 2005).

Downstream of IGF-IR are the signaling molecules PI3K and Akt, which are both regulated by estrogens (Cardona-Gomez et al., 2002, 2004). It has been demonstrated that estradiol activates Akt in the hippocampus and cortex by increasing its phosphorylation (Cardona-Gomez et al.,

2002; Wilson et al., 2002; Znamensky et al., 2003). This could be another way by which estradiol protects cells against damage.

We did not detect significant changes in Akt after treatment with 17β-estradiol or ER selective agonists in control animals or in moderately MPTP lesioned mice, whereas treatment with 17β-estradiol or the ERα agonist PPT led to important and significant increases in its phosphorylation. Moreover, in intact animals, we showed an increase in Akt phosphorylation after treatment with either 17β-estradiol or the ERα agonist. In contrast, the ERβ agonist Δ3-diol left the phosphorylation of Akt unchanged in both MPTP lesioned and unlesioned mice. This could represent a mechanism by which an estrogenic pre-treatment leads to a positive modulation of cell survival by the activation of ERα. Moreover, this increase in the phosphorylation and activation of pro-survival factors could explain why estradiol pre-treatment is necessary to obtain neuroprotection in other neurodegenerative models (Gajjar et al., 2003).

This is the first report linking estradiol striatal dopamine MPTP neuroprotection in mice with IGF-I and Akt signaling pathways. Nevertheless, supporting our findings, Dhandapani et al. (2005) recently reported that transforming growth factor-β mediates the neuroprotective effect of estradiol and involves Akt phosphorylation in cultures of primary rat cortical astrocytes. In addition, estrogen was reported to interact with the IGF-I system to protect nigrostriatal dopamine and maintain motor behavior in 6-hydroxydopamine lesioned rats (Quesada and Micevych, 2004).

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GSK3β, another molecule studied in the present experiment, may affect neuronal survival by different mechanisms such as the regulation of glucose metabolism (Brunet et al., 2001), phosphorylation of microtubule-associated proteins, or interaction with transcription factors (Cardona-Gomez et al., 2004). GSK3β activity is negatively regulated by the phosphorylation of some of its serines, while phosphorylation of tyrosine residues leads to its activation (Cohen and Frame, 2001). Activation of GSK3β results in neuronal apoptosis (Enguita et al., 2005) and is shown to mediate striatal toxin-induced neuronal death (Chen et al., 2004), while its inhibition promotes neuronal survival (Cohen and Frame, 2001). Our results indicate that MPTP induces a persistent reduction in the phosphorylation of striatal GSK3β in serines, therefore inducing GSK3β activation. This persistent activation of GSK3β suggests that striatal neuronal death may persist for several days after MPTP treatment. This is in agreement with the persistent expression of striatal inflammatory cytokines of mice several days after the administration of MPTP (Hebert et al., 2003) as well as with the persistent decrease in the Bcl-2/BAD ratio observed in the present study, an indication of the activation of proapoptotic signaling, since Bcl-2 is an anti-apoptotic factor, while BAD is pro-apoptotic (Merry and Korsmeyer, 1997).

17β-Estradiol and the ERα agonist PPT, and in a lesser extent the ERβ agonist Δ3-diol, increase the phosphorylation of GSK3β in serine 9 and, therefore, contribute to its inhibition in the striatum of intact and MPTP-lesioned animals. Since Akt is one of the kinases that inactivates GSK3β, the neuroprotective mechanism of 17β-estradiol and PPT may involve the ERα mediated activation of Akt and the consecutive inhibition of GSK3β by Akt. Therefore, we propose that inhibition of GSK3β by an ERα mediated mechanism may be involved in the neuroprotective effect of estradiol in this model. Our findings do not exclude that ERβ may also

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be involved in neuroprotection. Indeed, the ERβ agonist Δ3-diol has a moderate neuroprotective effect. Although Δ3-diol induced a moderate increase in Akt and GSK3β phosphorylation, it is possible that ERβ mediated neuroprotection may also be exerted through a different mechanism, unrelated to the activation of IGF-I signaling.

PPT completely, and 17β-estradiol or Δ3-diol partially, overcame the decrease in the Bcl-2/BAD ratio induced by MPTP, therefore positively regulating cell survival. An *in vitro* study has demonstrated that PPT and DPN (an ERβ agonist) modulate Bcl-2 levels and promote cell survival in primary hippocampal neurons (Zhao et al., 2004). Bcl-2 expression can be modulated by activation of ERE and CREB (Pugazhenthi et al., 2000), both transcription factors themselves regulated by estrogen in the brain (Abraham et al., 2004). Also, Akt can induce Bcl-2 transcription (Pugazhenthi et al., 2000). Moreover, Bcl-2 is negatively regulated by BAD.

Many intracellular molecules measured, such as IGF-IR, BAD and Bcl-2, were not affected by treatments with estrogen agonists in unlesioned animals. However, important increases in the phosphorylation of both Akt and GSK3β were measured in 17β-estradiol and PPT treated mice. Moreover, increases in the ratios pAkt/Akt and pGSK3β/GSK3β in intact and lesioned animals revealed that changes in these molecules are in favor of cell survival, since both ratios are markers of survival. These changes could indicate which parameters are activated first or are more sensitive to estrogen agonist treatments. We suggest that pre-treatment with these molecules contribute to the priming of the survival pathway, both by activating an anti-apoptotic molecule, Akt, but also by inhibiting a pro-apoptotic molecule, GSK3β. These molecules should therefore be considered as target molecules of 17β-estradiol and PPT. Interestingly, modifications in

Akt/GSK3β signaling are reported in individuals with schizophrenia (Emamian et al., 2004). Also, chronic haloperidol treatment in mice increases phosphorylation of Akt at Ser473 and GSK3βat Ser9 (Emamian et al., 2004) such as reported here with 17β-estradiol and PPT. In addition, attenuated 5-HT_{1A} receptor signaling involving reduced Akt activity is observed in the occipital cortex of depressed suicide victims (Hsiung et al., 2003). Furthermore, lithium salts used in the treatment of depression in humans are shown to antagonize dopamine-dependent behaviors mediated by an Akt/GSK3 signaling cascade in mice (Beaulieu et al., 2004). Hence, the neuroprotective and neuromodulatory activity of estrogens in animal models and humans may share a common mechanism by affecting Akt/GSK3β signaling.

In conclusion, the present results suggest that the activation of the PI3K/Akt/GSK3β signaling pathway is involved in the neuroprotective effect of estradiol. This effect is mainly mediated by ERα, although our findings do not exclude a participation of ERβ in the neuroprotective effects of the hormone. Moreover, results from the unlesioned animals support the beneficial role of estradiol pre-treatment, by increasing the activity of signaling pathways implicated in cell survival.

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Acknowledgements

We thank Laurent Gregoire for assistance in the statistical evaluation of the results.

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Footnotes

This research was supported by grants from the Canadian Institutes of Health Research (CIHR) to TDP and from Ministerio de Ciencia y Tecnología, Spain (SAF 2002-00652) to LMGS. MD held a CIHR studentship.

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Molecular Pharmacology Fast Forward. Published on January 24, 2006 as DOI: 10.1124/mol.105.018671
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Legends for figures

Figure 1: Effect of estrogen agonist treatments on IGF-IR levels measured by Western blot in C57Bl/6 male mice treated with MPTP as compared to intact control (Saline +Vehicle) animals. Mice were treated with 17β-estradiol (17β-E_2), the ERα agonist PPT, the ERβ agonist Δ3-diol or vehicle for 10 days and MPTP mice received 4 injections of MPTP (9 mg/kg) on day 5. ANOVA global P value was 0.014 and individual group comparisons were ∗∗ $P < 0.01$ versus control; †††$P < 0.005$ versus MPTP + vehicle; •$P < 0.05$ versus MPTP + 17β-E_2; ◊ $P < 0.05$ versus MPTP +PPT. Values are normalized to control values and represent the mean relative units (R.U.) ± SEM of 3 mice per group. A representative example of the Western blots is shown. βIII-tubulin was used as a loading control.

Figure 2: Effect of estrogen agonist treatments on phosphorylated Akt/Akt levels measured by Western blot in C57Bl/6 male mice treated with MPTP as compared to intact control (Saline +Vehicle) animals. Mice were treated with 17β-estradiol (17β-E_2), the ERα agonist PPT, the ERβ agonist Δ3-diol or vehicle for 10 days and MPTP mice received 4 injections of MPTP (9 mg/kg) on day 5. ANOVA global P value was 0.049 and individual group comparisons were ∗∗ $P < 0.01$ versus control; •• $P < 0.01$ versus MPTP + 17β-E_2; ◊ $P < 0.05$ versus MPTP +PPT. Values are normalized to control values and represent the mean of ratio of relative units ± SEM of 3 mice per group. A representative example of the Western blots is shown.

Figure 3: Effect of estrogen agonist treatments on phosphorylated GSK3/GSK3 measured by Western blot in C57Bl/6 male mice treated with MPTP as compared to intact control (Saline +Vehicle) animals. Mice were treated with 17β-estradiol (17β-E_2), the ERα agonist PPT, the

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ERβ agonist Δ3-diol or vehicle for 10 days and MPTP mice received 4 injections of MPTP (9 mg/kg) on day 5. ANOVA global P value was < 0.0001 and individual group comparisons were ∗ P < 0.05, ∗∗ P < 0.01, ∗∗∗ P < 0.005 and ∗∗∗∗∗∗ P < 0.0001 versus control; †††P < 0.005, ††††††P < 0.0001 versus MPTP + vehicle; • P < 0.05 versus MPTP + 17β-E_2; ◊◊◊◊◊ P < 0.0005 versus MPTP +PPT. Values are normalized to control values and represent the mean of ratio of relative units ± relative units (R.U.) ± SEM of 3 mice per group. A representative example of the Western blots is shown.

Figure 4: Effects of estrogen agonist treatments on the ratio of levels of anti-apoptotic Bcl-2 on pro-apoptotic BAD, measured by Western blot in C57Bl/6 male mice treated with MPTP as compared to intact control (Saline +Vehicle) animals. Mice were treated with 17β-estradiol (17β-E_2), the ERα agonist PPT, the ERβ agonist Δ3-diol or vehicle for 10 days and MPTP-treated mice received 4 injections of MPTP (9 mg/kg) on day 5. ANOVA global P value was 0.005 and individual group comparisons were ∗∗ P < 0.01 versus control; †††††P < 0.0005 versus MPTP + vehicle; •• P < 0.01 versus MPTP + 17β-E_2; ◊◊◊ P < 0.005 versus MPTP +PPT. Values are normalized to control values and represent the mean of ratio of relative units ± SEM of 3 mice per group. Representative examples of the Western blots are shown. βIII-tubulin was used as a loading control.

Figure 5: Effects of estrogen agonist treatments on phosphorylated GSK3/GSK3, phosphorylated Akt/Akt measured by Western blot in intact C57Bl/6 male mice. Mice were treated with 17β-estradiol (17β-E_2), the ERα agonist PPT or the ERβ agonist Δ3-diol for 10 days while control animals received the vehicle only. ANOVA global P value were for pGSK3/GSK 0.003,

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pAkt/Akt 0.024 and individual group comparisons were ∗ $P < 0.05$, ∗∗∗ $P < 0.005$ and ∗∗∗∗∗ $P < 0.0005$ versus control; •• $P < 0.01$ versus 17β-E_2; ◊◊ $P < 0.01$ versus MPTP +PPT Values are normalized to control values and represent the mean of ratio of relative units ± SEM of 3 mice per group. Representative examples of the Western blots are shown.

Figure 6: Schematic representation of the PI3K/Akt signaling pathway and possible interaction of estrogenic compounds with some of the signaling molecules, based on findings of the present experiments. Possible interactions of ERα agonists (PPT and 17β-E_2) include an activation of Akt itself, as revealed by the experiment with unlesioned animals but also an inhibition of GSK3, leading to cell survival.

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Table 1: Effects of 17β-estradiol, PPT and Δ3-diol treatments on striatal catecholamine concentrations in C57Bl/6 male mice lesioned with MPTP (9 mg/kg) as compared to intact control (Saline +Vehicle treated) and vehicle treated MPTP animals. Values are the mean (ng/mg of protein) ± SEM of 6-9 mice per group. ANOVA global P values were for dopamine 0.005, DOPAC 0.007 and HVA 0.319.

Groups	**Dopamine** (ng/mg of proteins)	**DOPAC** (ng/mg of proteins)	**HVA** (ng/mg of proteins)
Saline + Vehicle	129.6 ± 4.5	6.86 ± 0.28	9.45 ± 0.46
MPTP + Vehicle	76.7 ± 10.3 ****	5.24 ± 0.29 **	8.46 ± 0.34
MPTP +17β-estradiol	106.6 ± 7.7 †	5.76 ± 0.34	9.42 ± 0.48
MPTP + PPT	116.0 ± 3.3 ††	6.45 ± 0.29 †	10.34 ± 0.47
MPTP +Δ3-diol	91.5 ± 9.6 *** §	5.39 ± 0.47 ** §	9.93 ± 0.73

** P < 0.01, *** P < 0.005 and **** P < 0.0005 versus Intact + Vehicle; † P < 0.05 and †† P < 0.0005 versus MPTP + vehicle; § P < 0.05 versus MPTP + PPT.

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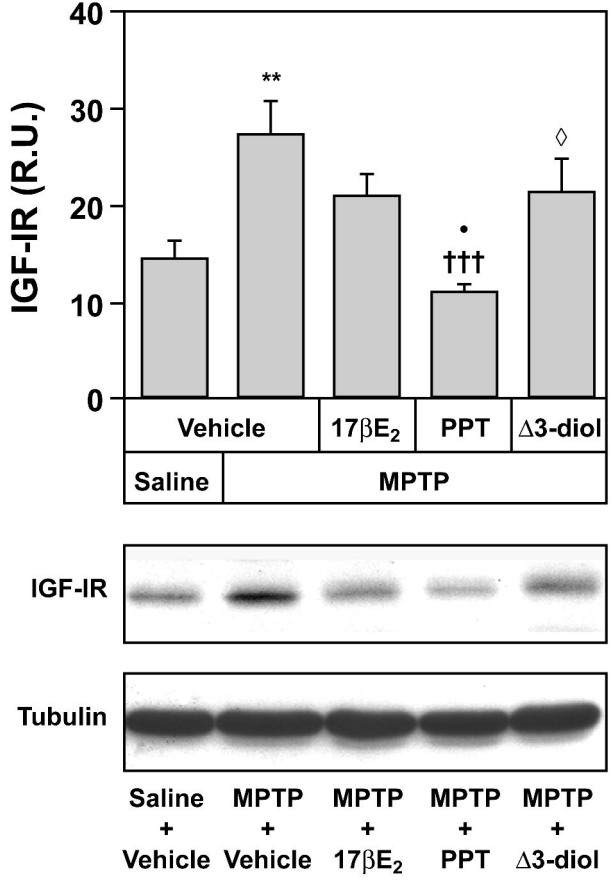
Table 2: Effects of 17β-estradiol, PPT and Δ3-diol treatments for 10 days in intact C57Bl/6 male mice on striatal catecholamine concentrations as compared to control (Vehicle-treated) animals. Values are the mean (ng/mg of protein) ± SEM of 6 mice per group. ANOVA global P values were for dopamine 0.101, DOPAC 0.106 and HVA 0.068.

Groups	**Dopamine** (ng/mg of proteins)	**DOPAC** (ng/mg of proteins)	**HVA** (ng/mg of proteins)
Vehicle	103.1 ± 6.5	6.61 ± 0.61	7.20 ± 0.39
17β-estradiol	110.6 ± 4.7	5.70 ± 0.27	6.81 ± 0.50
PPT	108.5 ± 4.8	6.76 ± 0.36	7.34 ± 0.47
Δ3-diol	115.1 ± 4.9	7.02 ± 0.48	9.34 ± 0.68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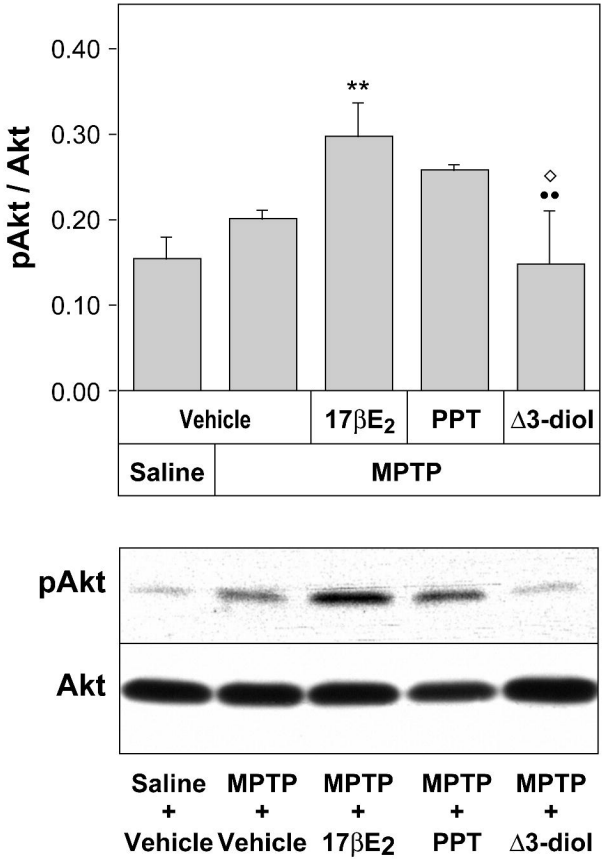
Tubulin
Saline + Vehicle
MPTP + Vehicle
MPTP + 17βE2
MPTP + PPT
MPTP + Δ3-diol

Figure 1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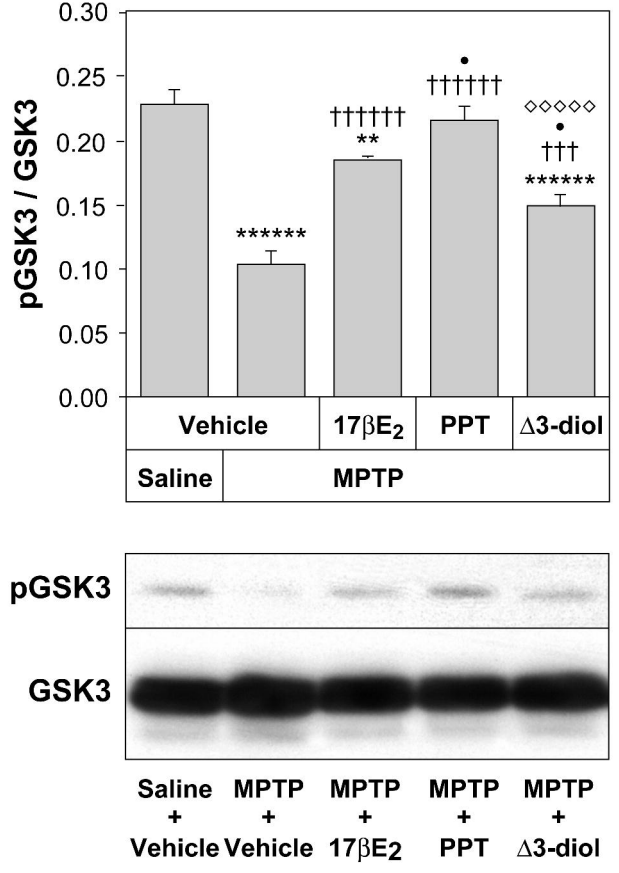
MPTP + Δ3-diol

Figure 2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MPTP + Vehicle
MPTP + 17βE2
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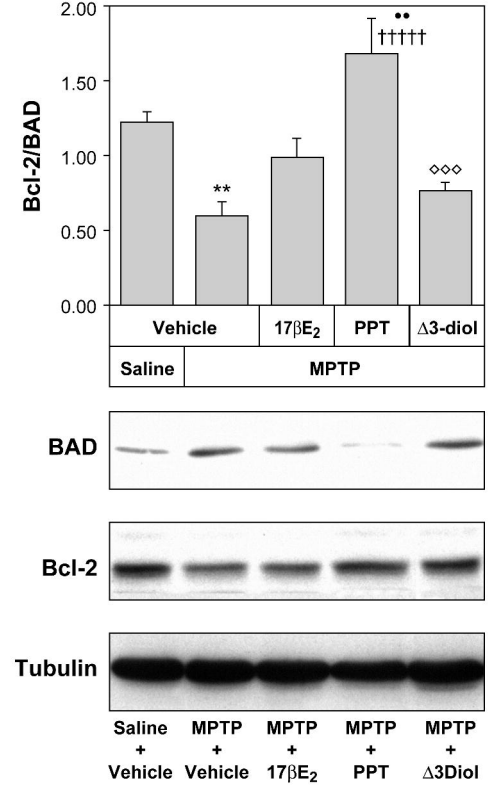
MPTP + Δ3-diol

Figure 3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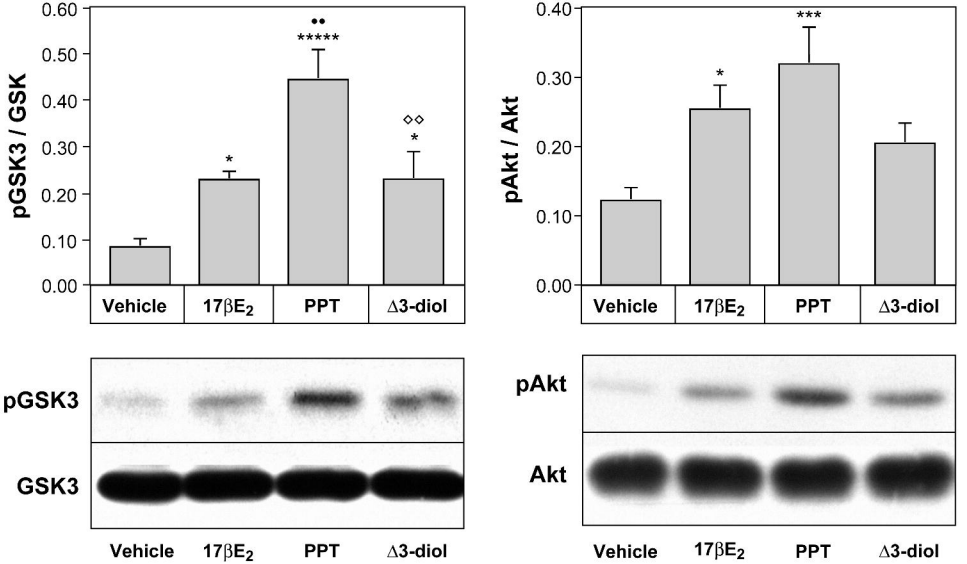
MPTP + 17βE2
MPTP + PPT
MPTP + Δ3Diol

Figure 4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PPT
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Δ3-diol

Figure 5

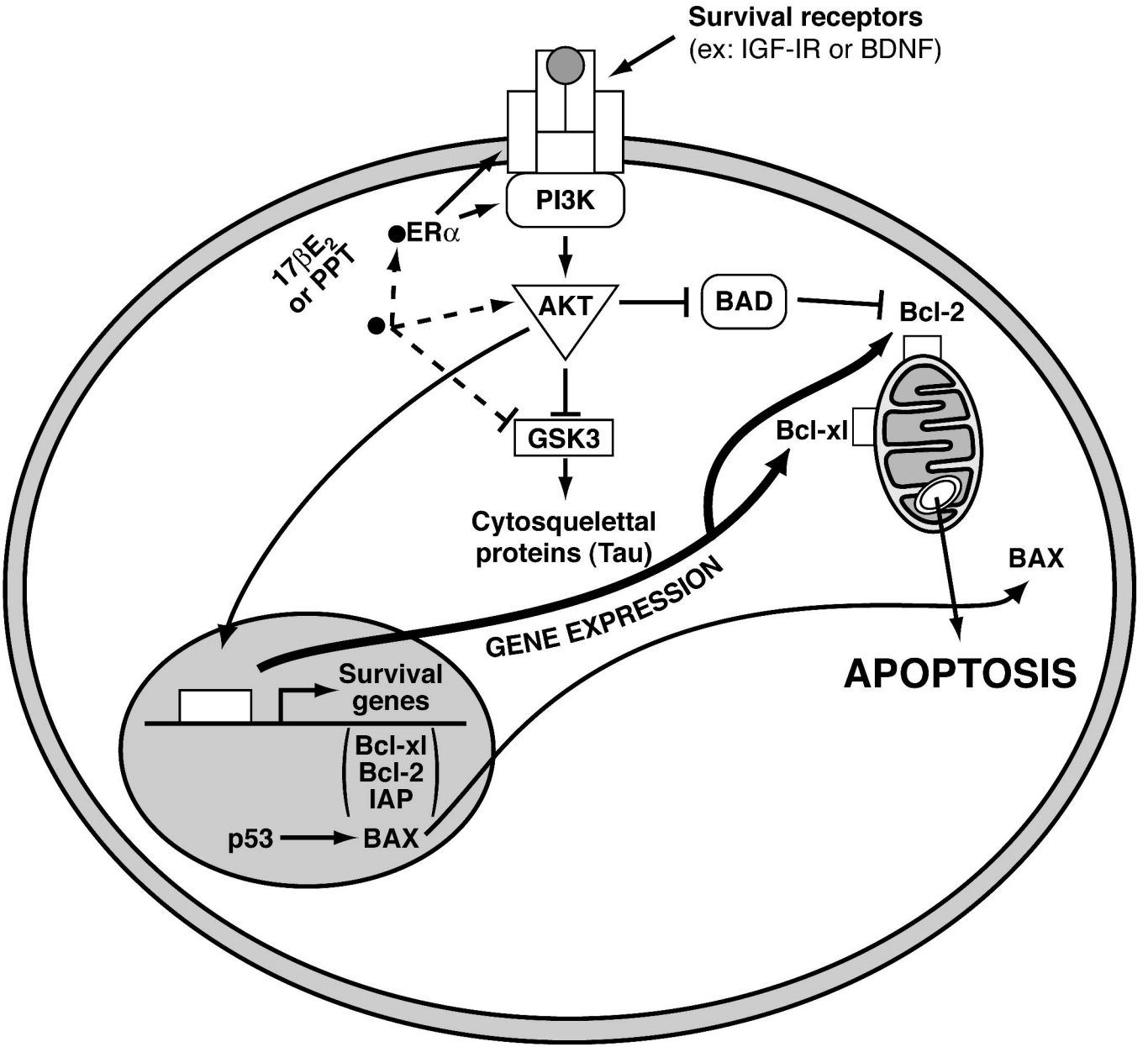
Survival receptors
(ex: IGF-IR or BDNF)
PI3K
ERα
17βE2
or PPT
AKT
BAD
Bcl-2
GSK3
Bcl-xl
Cytosquelettal
proteins (Tau)
BAX
GENE EXPRESSION
APOPTOSIS
Survival
genes
Bcl-xl
Bcl-2
IAP
p53
BAX

Figure 6