

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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MOL Manuscript #18671

Running title page

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MOL Manuscript #18671

Abstract

The present experiments sought to determine the implication of estrogen receptors (ER α and ER β) 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 17 β -estradiol, an estrogen receptor alpha (ER α) agonist, PPT (4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)tris-phenol) or an estrogen receptor beta (ER β) agonist, Δ 3-diol (5-androsten-3 β , 17 β -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; 17 β -estradiol and PPT but not Δ 3-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 17 β -estradiol, tended to increase with PPT but not with Δ 3-diol treatments in MPTP mice. GSK3 β phosphorylation (at serine 9) was greatly reduced in MPTP mice; this was completely prevented by PPT whereas 17 β -estradiol and Δ 3-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, 17 β -estradiol and PPT increased phosphorylation of striatal Akt and GSK3 β , while the other markers measured remained unchanged. Δ 3-Diol increased GSK3 β phosphorylation less than the PPT treatment. These results suggest that a pre-treatment with estradiol promoted dopamine neuron survival by activating ER α and increasing Akt and GSK3 β 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 (Pugazhenti 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

MOL Manuscript #18671

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 ($ER\alpha$) has been implicated in the activation of the PI3K/Akt pathway (Kahlert et al., 2000; Mendez et al., 2003, 2005). Indeed, only $ER\alpha$ interacts with IGF-IR and PI3K in the brain, while estrogen receptor beta ($ER\beta$) 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.

Material and methods

Chemicals

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 17 β -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 Δ 3-diol (5-androsten-3 β , 17 β -diol, also known as 5-androstenediol, androstenediol or hermaphrodiol) was purchased from Steraloids Inc (Newport, R.I., USA). PPT is a specific ER α agonist (Stauffer et al., 2000), while Δ 3-diol preferentially binds to and activates ER β (Kuiper et al., 1997).

Animals and treatments

C57Bl/6 male mice (10-12 weeks, 25 \pm 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 17 β -estradiol, PPT or Δ 3-diol while control mice received injections of vehicle (0.9% saline with 0.3% gelatin). Concentrations used were 2 μ g per day for 17 β -estradiol and PPT, and 3 μ g per day for Δ 3-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°C). In a similar experiment, mice received estrogenic drug treatments for 10

MOL Manuscript #18671

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 C₁₈, 3 μ m, 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, Mannheim, Germany) supplemented with protease and phosphatase inhibitors (50 μ g/ml of PhenylMethylSulfonyl Fluoride, 10 μ g/ml aprotinin, 25 μ g/ml

MOL Manuscript #18671

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 GSK3 β at Serine 9 (pSer9GSK3 β abbreviated as pGSK3) were used at a dilution of 1:1000 and were obtained from Cell Signaling (Cell Signaling, MA, USA). GSK3 β monoclonal antibody was from BD Transduction (BD Pharmingen, CA, USA). Finally, β III-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 chemiluminescence 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

MOL Manuscript #18671

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.

MOL Manuscript #18671

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. 17 β -Estradiol prevented the MPTP-induced dopamine loss. Striatal dopamine concentrations of Δ 3-diol treated MPTP mice were less significantly depleted compared to intact controls than MPTP + vehicle. Δ 3-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 17 β -estradiol, PPT and Δ 3-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. 17 β -Estradiol and Δ 3-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 + Δ 3-diol than in the MPTP + PPT group.

The phosphorylated forms at serine residue 9 for GSK3 β (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 17 β -estradiol induced a significant increase in pAkt/Akt, with regards to control mice. This increase in pAkt/Akt did not reach statistical

MOL Manuscript #18671

significance ($p = 0.062$ versus control) with PPT treatment whereas $\Delta 3$ -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 GSK3 β 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). $\Delta 3$ -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 $\Delta 3$ -diol treated MPTP mice had Bcl-2/BAD ratio neither different from saline-vehicle treated mice or MPTP mice. $\Delta 3$ -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 $\Delta 3$ -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 GSK3 β (Fig. 5). $\Delta 3$ -Diol did not significantly affect the phosphorylated state of Akt ($p = 0.126$ versus control and $p = 0.0558$ versus PPT) and increased GSK3 β phosphorylation much less than the PPT treatment. There was a tight correlation between the phosphorylation levels of both Akt and GSK3 β proteins ($r = 0.87$), suggesting that there is a

MOL Manuscript #18671

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

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 ER α 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- κ B (Brunet et al., 2001) (Figure 6).

The doses and protocol of administration of 17 β -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 ER α agonist treatment protects against MPTP-

MOL Manuscript #18671

induced striatal dopamine and DOPAC depletion and that this is statistically different from the ER β agonist treated MPTP mice. In addition, 17 β -estradiol and the ER α 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 ER α 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 ER α 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 ER β activation (Carswell et al., 2004). ER α and ER β 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 ER α receptor by ER agonists could lead to transcriptional activity and to the regulation of the IGF-IR pathway. Alternatively, other ER α -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.,

MOL Manuscript #18671

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 17 β -estradiol or ER selective agonists in control animals or in moderately MPTP lesioned mice, whereas treatment with 17 β -estradiol or the ER α 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 17 β -estradiol or the ER α agonist. In contrast, the ER β agonist Δ 3-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 ER α . 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).

MOL Manuscript #18671

GSK3 β , 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). GSK3 β 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 GSK3 β 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 GSK3 β in serines, therefore inducing GSK3 β activation. This persistent activation of GSK3 β 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).

17 β -Estradiol and the ER α agonist PPT, and in a lesser extent the ER β agonist Δ 3-diol, increase the phosphorylation of GSK3 β 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 GSK3 β , the neuroprotective mechanism of 17 β -estradiol and PPT may involve the ER α mediated activation of Akt and the consecutive inhibition of GSK3 β by Akt. Therefore, we propose that inhibition of GSK3 β by an ER α mediated mechanism may be involved in the neuroprotective effect of estradiol in this model. Our findings do not exclude that ER β may also

MOL Manuscript #18671

be involved in neuroprotection. Indeed, the ER β agonist Δ 3-diol has a moderate neuroprotective effect. Although Δ 3-diol induced a moderate increase in Akt and GSK3 β phosphorylation, it is possible that ER β mediated neuroprotection may also be exerted through a different mechanism, unrelated to the activation of IGF-I signaling.

PPT completely, and 17 β -estradiol or Δ 3-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 ER β 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 (Pugazhenthii et al., 2000), both transcription factors themselves regulated by estrogen in the brain (Abraham et al., 2004). Also, Akt can induce Bcl-2 transcription (Pugazhenthii 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 GSK3 β were measured in 17 β -estradiol and PPT treated mice. Moreover, increases in the ratios pAkt/Akt and pGSK3 β /GSK3 β 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, GSK3 β . These molecules should therefore be considered as target molecules of 17 β -estradiol and PPT. Interestingly, modifications in

MOL Manuscript #18671

Akt/GSK3 β signaling are reported in individuals with schizophrenia (Emamian et al., 2004). Also, chronic haloperidol treatment in mice increases phosphorylation of Akt at Ser473 and GSK3 β at Ser9 (Emamian et al., 2004) such as reported here with 17 β -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/GSK3 β signaling.

In conclusion, the present results suggest that the activation of the PI3K/Akt/GSK3 β signaling pathway is involved in the neuroprotective effect of estradiol. This effect is mainly mediated by ER α , although our findings do not exclude a participation of ER β 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.

MOL Manuscript #18671

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MOL Manuscript #18671

References

Abraham IM, Todman MG, Korach KS and Herbison AE (2004) Critical in vivo roles for classical estrogen receptors in rapid estrogen actions on intracellular signaling in mouse brain. *Endocrinology* **145**:3055-3061.

Beaulieu JM, Sotnikova TD, Yao WD, Kockeritz L, Woodgett JR, Gainetdinov RR and Caron MG (2004) Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. *Proc Natl Acad Sci U S A* **101**:5099-5104.

Brunet A, Datta SR and Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol* **11**:297-305.

Callier S, Morissette M, Grandbois M and Di Paolo T (2000) Stereospecific prevention by 17beta-estradiol of MPTP-induced dopamine depletion in mice. *Synapse* **37**: 245-251.

Cardona-Gomez P, Perez M, Avila J, Garcia-Segura LM and Wandosell F (2004) Estradiol inhibits GSK3 and regulates interaction of estrogen receptors, GSK3, and beta-catenin in the hippocampus. *Mol Cell Neurosci* **25**:363-373.

Cardona-Gomez GP, Mendez P and Garcia-Segura LM (2002) Synergistic interaction of estradiol and insulin-like growth factor-I in the activation of PI3K/Akt signaling in the adult rat hypothalamus. *Brain Res Mol Brain Res* **107**:80-88.

MOL Manuscript #18671

Cardona-Gomez GP, Mendez P, DonCarlos LL, Azcoitia I and Garcia-Segura LM (2001) Interactions of estrogens and insulin-like growth factor-I in the brain: implications for neuroprotection. *Brain Res Brain Res Rev* **37**:320-334.

Carswell HV, Macrae IM, Gallagher L, Harrop E and Horsburgh KJ (2004) Neuroprotection by a selective estrogen receptor beta agonist in a mouse model of global ischemia. *Am J Physiol Heart Circ Physiol* **287**:H1501-H1504.

Chen G, Bower KA, Ma C, Fang S, Thiele CJ and Luo J (2004) Glycogen synthase kinase 3beta (GSK3beta) mediates 6-hydroxydopamine-induced neuronal death. *FASEB J* **18**:1162-1164.

Cohen P and Frame S (2001) The renaissance of GSK3. *Nat Rev Mol Cell Biol* **2**:769-776.

D'Astous M, Morissette M and Di Paolo T (2004) Effect of estrogen receptor agonists treatment in MPTP mice: Evidence of neuroprotection by an ER α agonist. *Neuropharmacology* **47**:1180-1188.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**:231-241.

Datta SR, Brunet A and Greenberg ME (1999) Cellular survival: a play in three Akts. *Genes Dev* **13**:2905-2927.

MOL Manuscript #18671

Dhandapani KM, Wade FM, Mahesh VB and Brann DW (2005) Astrocyte-derived transforming growth factor- β mediates the neuroprotective effects of 17 β estradiol: involvement of nonclassical genomic signaling pathways. *Endocrinology* **146**:2749-2759.

Dluzen DE and McDermott JL (2000) Neuroprotective role of estrogen upon methamphetamine and related neurotoxins within the nigrostriatal dopaminergic system. *Ann N Y Acad Sci* **914**:112-126.

Dubal DB, Zhu H, Yu J, Rau SW, Shughrue PJ, Merchenthaler I, Kindy MS and Wise PM (2001) Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. *Proc Natl Acad Sci U S A* **98**:1952-1957.

Enguita M, Degregorio-Rocasolano N, Abad A and Trullas R (2005) Glycogen synthase kinase 3 activity mediates neuronal pentraxin 1 expression and cell death induced by potassium deprivation in cerebellar granule cells. *Mol Pharmacol* **67**:1237-1246.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M and Gogos JA (2004) Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat Genet* **36**:131-137.

Gajjar TM, Anderson LI and Dluzen DE (2003) Acute effects of estrogen upon methamphetamine induced neurotoxicity of the nigrostriatal dopaminergic system. *J Neural Transm* **110**:1215-1224.

MOL Manuscript #18671

Garcia-Segura LM, Azcoitia I and DonCarlos LL (2001) Neuroprotection by estradiol. *Prog Neurobiol* **63**:29-60.

Hasbi A, O'dowd BF and George SR (2005) A G protein-coupled receptor for estrogen: the end of the search? *Mol Interv* **5**:158-161.

Hebert G, Arsaut J, Dantzer R, Demotes-Mainard J (2003) Time-course of the expression of inflammatory cytokines and matrix metalloproteinases in the striatum and mesencephalon of mice injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a dopaminergic neurotoxin. *Neurosci Lett* **349**:191-195.

Hetman M, Cavanaugh JE, Kimelman D and Xia Z (2000) Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. *J Neurosci* **20**:2567-2574.

Hsiung SC, Adlersberg M, Arango V, Mann JJ, Tamir H and Liu KP (2003) Attenuated 5-HT1A receptor signaling in brains of suicide victims: involvement of adenylyl cyclase, phosphatidylinositol 3-kinase, Akt and mitogen-activated protein kinase. *J Neurochem* **87**:182-194.

Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R and Grohe C (2000) Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. *J Biol Chem* **275**:18447-18453.

MOL Manuscript #18671

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S and Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**:863-870.

Kuppers E and Beyer C (1999) Expression of estrogen receptor-alpha and beta mRNA in the developing and adult mouse striatum. *Neurosci Lett* **276**:95-98.

LeRoith D, Werner H, Faria TN, Kato H, Adamo M and Roberts CT Jr (1993) Insulin-like growth factor receptors. Implications for nervous system function. *Ann N Y Acad Sci* **692**:22-32.

Mendez P, Azcoitia I and Garcia-Segura LM (2003) Estrogen receptor alpha forms estrogen-dependent multimolecular complexes with insulin-like growth factor receptor and phosphatidylinositol 3-kinase in the adult rat brain. *Brain Res Mol Brain Res* **112**:170-176.

Mendez P, Azcoitia I and Garcia-Segura LM (2005) Interdependence of oestrogen and insulin-like growth factor-I in the brain: potential for analysing neuroprotective mechanisms. *J Endocrinol* **185**:11-17.

Merry DE and Korsmeyer SJ (1997) Bcl-2 gene family in the nervous system. *Annu Rev Neurosci* **20**:245-267.

Picazo O, Azcoitia I, Garcia-Segura LM (2003) Neuroprotective and neurotoxic effects of estrogens. *Brain Res* **990**:20-27.

MOL Manuscript #18671

Pugazhenth S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE and Reusch JE (2000) Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem* **275**:10761-10766.

Quesada A and Micevych PE (2004) Estrogen interacts with the IGF-1 system to protect nigrostriatal dopamine and maintain motoric behavior after 6-hydroxydopamine lesions. *J Neurosci Res* **75**:107-116.

Shughrue P (2004) Estrogen attenuates the MPTP-induced loss of dopamine neurons from the mouse SNc despite a lack of estrogen receptors (ERalpha and ER beta). *Exp Neurol* **190**:468-477.

Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen BS and Katzenellenbogen JA (2000) Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *J Med Chem* **43**:4934-4947.

Vegeto E, Belcredito S, Eteri S, Ghisletti S, Brusadelli A, Meda C, Krust A, Dupont S, Ciana P, Chambon P and Maggi A (2003) Estrogen receptor-alpha mediates the brain antiinflammatory activity of estradiol. *Proc Natl Acad Sci U S A* **100**:9614-9619.

Wilson ME, Liu Y and Wise PM (2002) Estradiol enhances Akt activation in cortical explant cultures following neuronal injury. *Brain Res Mol Brain Res* **102**:48-54.

MOL Manuscript #18671

Zhao L, Wu TW and Brinton RD (2004) Estrogen receptor subtypes alpha and beta contribute to neuroprotection and increased Bcl-2 expression in primary hippocampal neurons. *Brain Res* **1010**:22-34.

Znamensky V, Akama KT, McEwen BS and Milner TA (2003) Estrogen levels regulate the subcellular distribution of phosphorylated Akt in hippocampal CA1 dendrites. *J Neurosci* **23**:2340-2347.

MOL Manuscript #18671

Footnotes

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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 -E₂), the ER α agonist PPT, the ER β agonist Δ 3-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 -E₂; \diamond P < 0.05 versus MPTP +PPT. Values are normalized to control values and represent the mean relative units (R.U.) \pm SEM of 3 mice per group. A representative example of the Western blots is shown. β III-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 -E₂), the ER α agonist PPT, the ER β agonist Δ 3-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 -E₂; \diamond P < 0.05 versus MPTP +PPT. Values are normalized to control values and represent the mean of ratio of relative units \pm 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 -E₂), the ER α agonist PPT, the

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ER β agonist Δ 3-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 + 17 β -E₂; ◇◇◇◇ $P < 0.0005$ versus MPTP +PPT. Values are normalized to control values and represent the mean of ratio of relative units \pm relative units (R.U.) \pm 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 17 β -estradiol (17 β -E₂), the ER α agonist PPT, the ER β agonist Δ 3-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 + 17 β -E₂; ◇◇◇ $P < 0.005$ versus MPTP +PPT. Values are normalized to control values and represent the mean of ratio of relative units \pm SEM of 3 mice per group. Representative examples of the Western blots are shown. β III-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 17 β -estradiol (17 β -E₂), the ER α agonist PPT or the ER β agonist Δ 3-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 -E₂; ◇◇ $P < 0.01$ versus MPTP +PPT Values are normalized to control values and represent the mean of ratio of relative units \pm 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 ER α agonists (PPT and 17β -E₂) include an activation of Akt itself, as revealed by the experiment with unlesioned animals but also an inhibition of GSK3, leading to cell survival.

Table 1: Effects of 17 β -estradiol, PPT and Δ 3-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) \pm 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 \pm 4.5	6.86 \pm 0.28	9.45 \pm 0.46
MPTP + Vehicle	76.7 \pm 10.3 ****	5.24 \pm 0.29 **	8.46 \pm 0.34
MPTP + 17 β -estradiol	106.6 \pm 7.7 †	5.76 \pm 0.34	9.42 \pm 0.48
MPTP + PPT	116.0 \pm 3.3 ††	6.45 \pm 0.29 †	10.34 \pm 0.47
MPTP + Δ 3-diol	91.5 \pm 9.6 *** §	5.39 \pm 0.47 ** §	9.93 \pm 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.

Table 2: Effects of 17 β -estradiol, PPT and Δ 3-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) \pm SEM of 6 mice per group. ANOVA global P values were for dopamine 0.101, DOPAC 0.106 and HVA 0.068.

Groups	Dopamine	DOPAC	HVA
	(ng/mg of proteins)	(ng/mg of proteins)	(ng/mg of proteins)
Vehicle	103.1 \pm 6.5	6.61 \pm 0.61	7.20 \pm 0.39
17 β -estradiol	110.6 \pm 4.7	5.70 \pm 0.27	6.81 \pm 0.50
PPT	108.5 \pm 4.8	6.76 \pm 0.36	7.34 \pm 0.47
Δ 3-diol	115.1 \pm 4.9	7.02 \pm 0.48	9.34 \pm 0.68

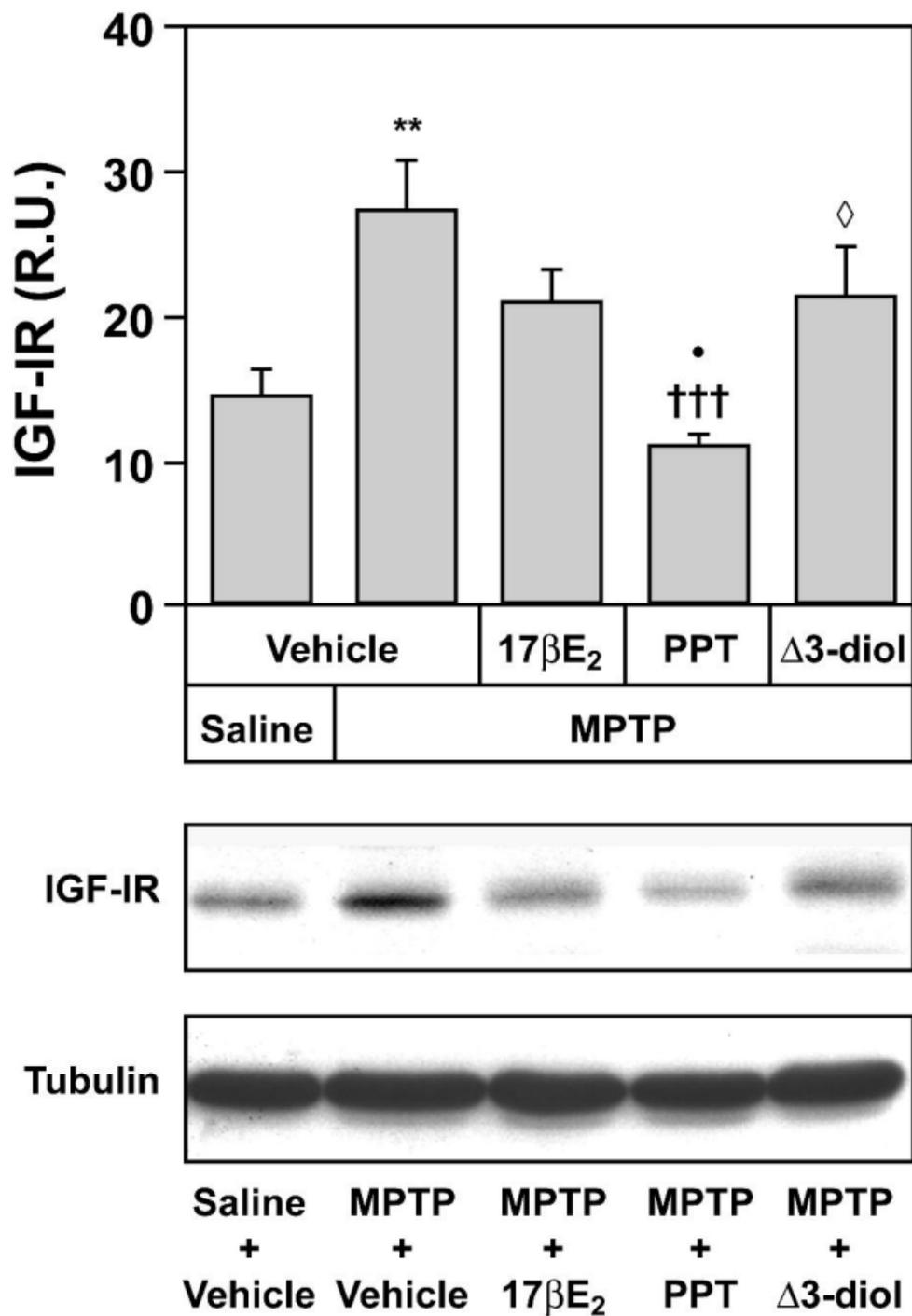


Figure 1

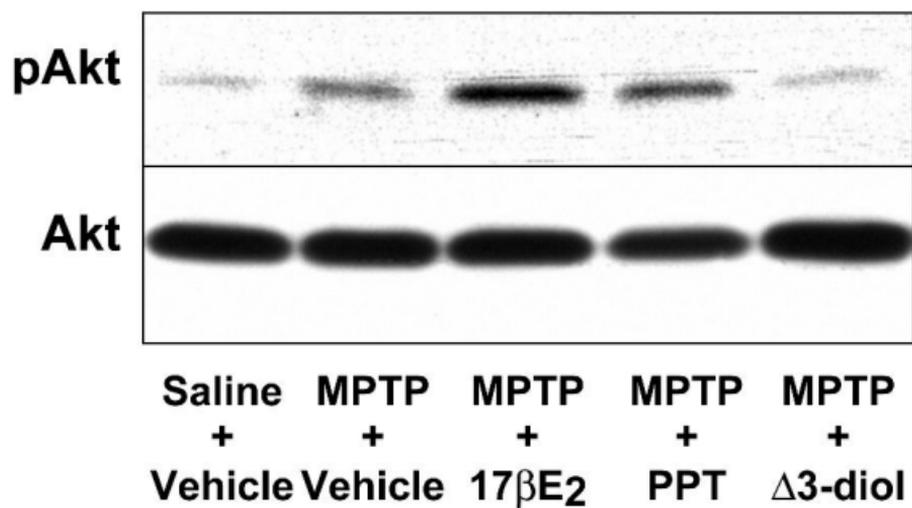
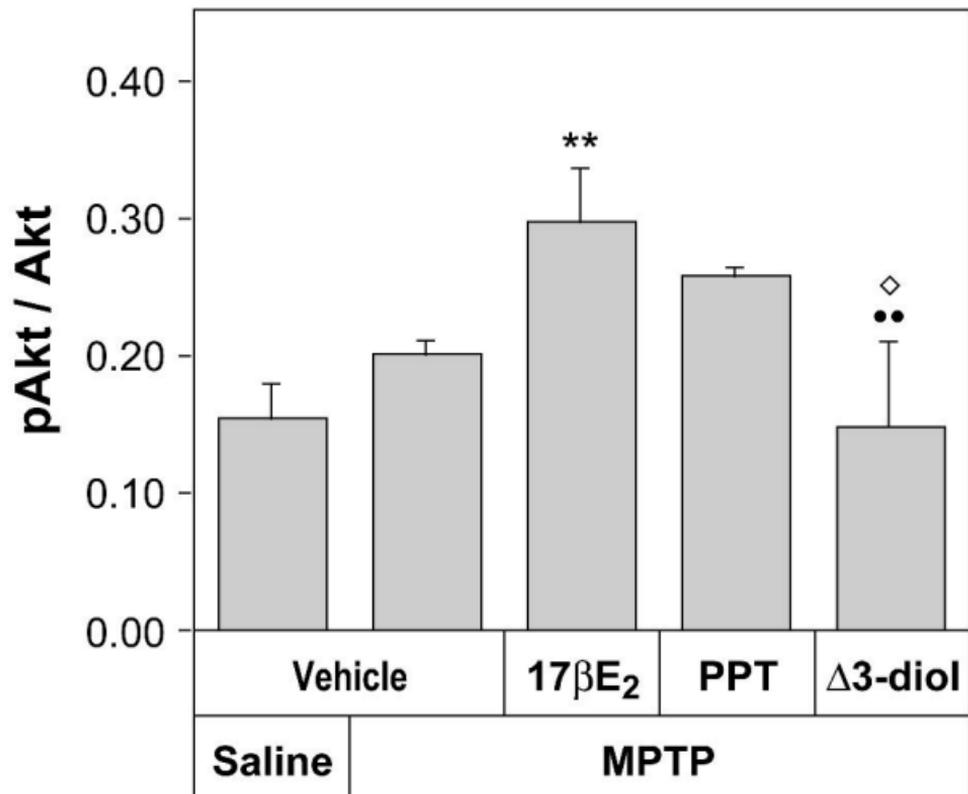


Figure 2

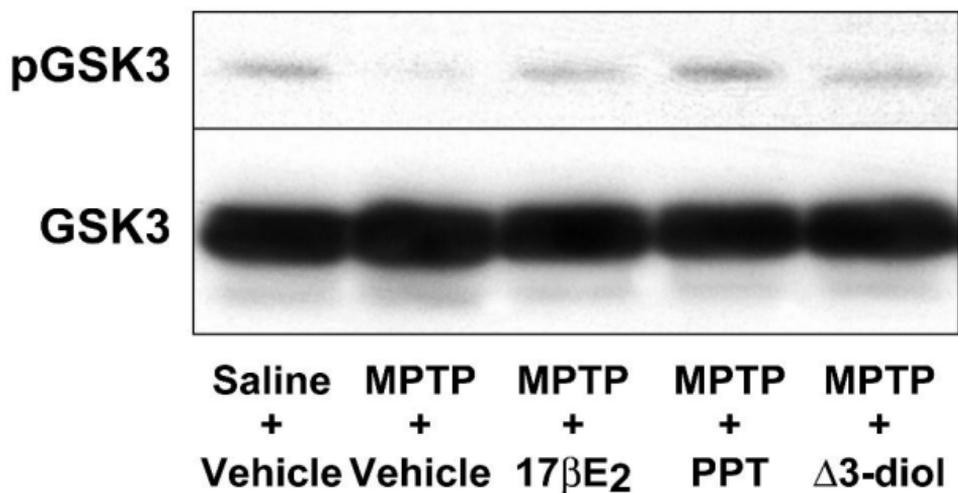
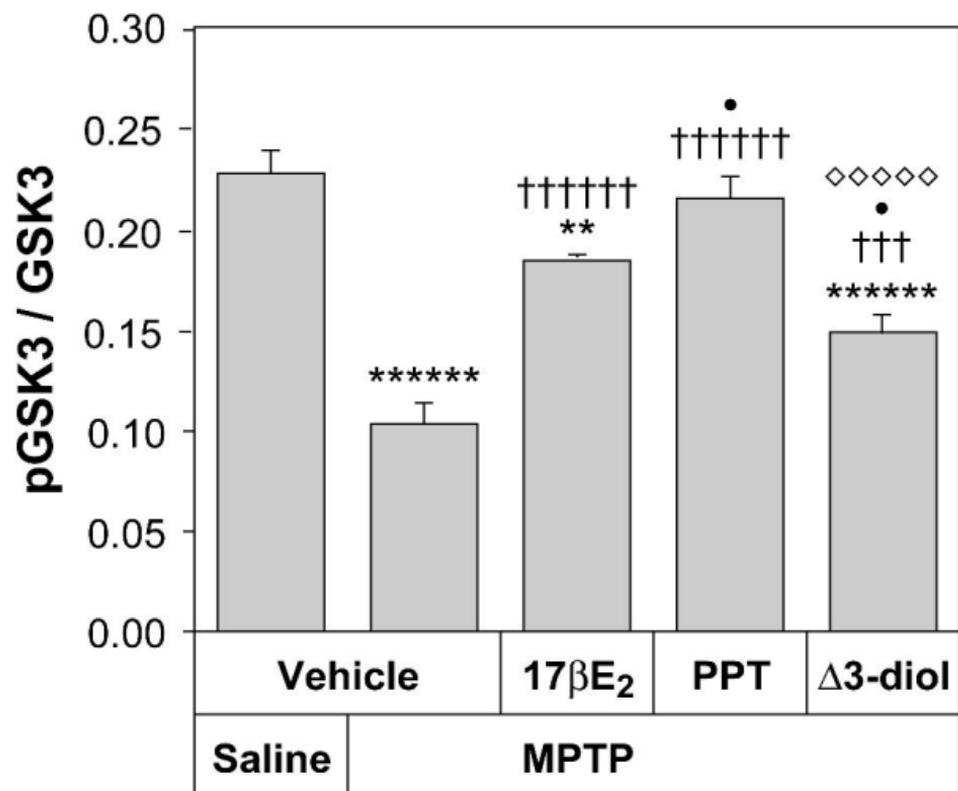


Figure 3

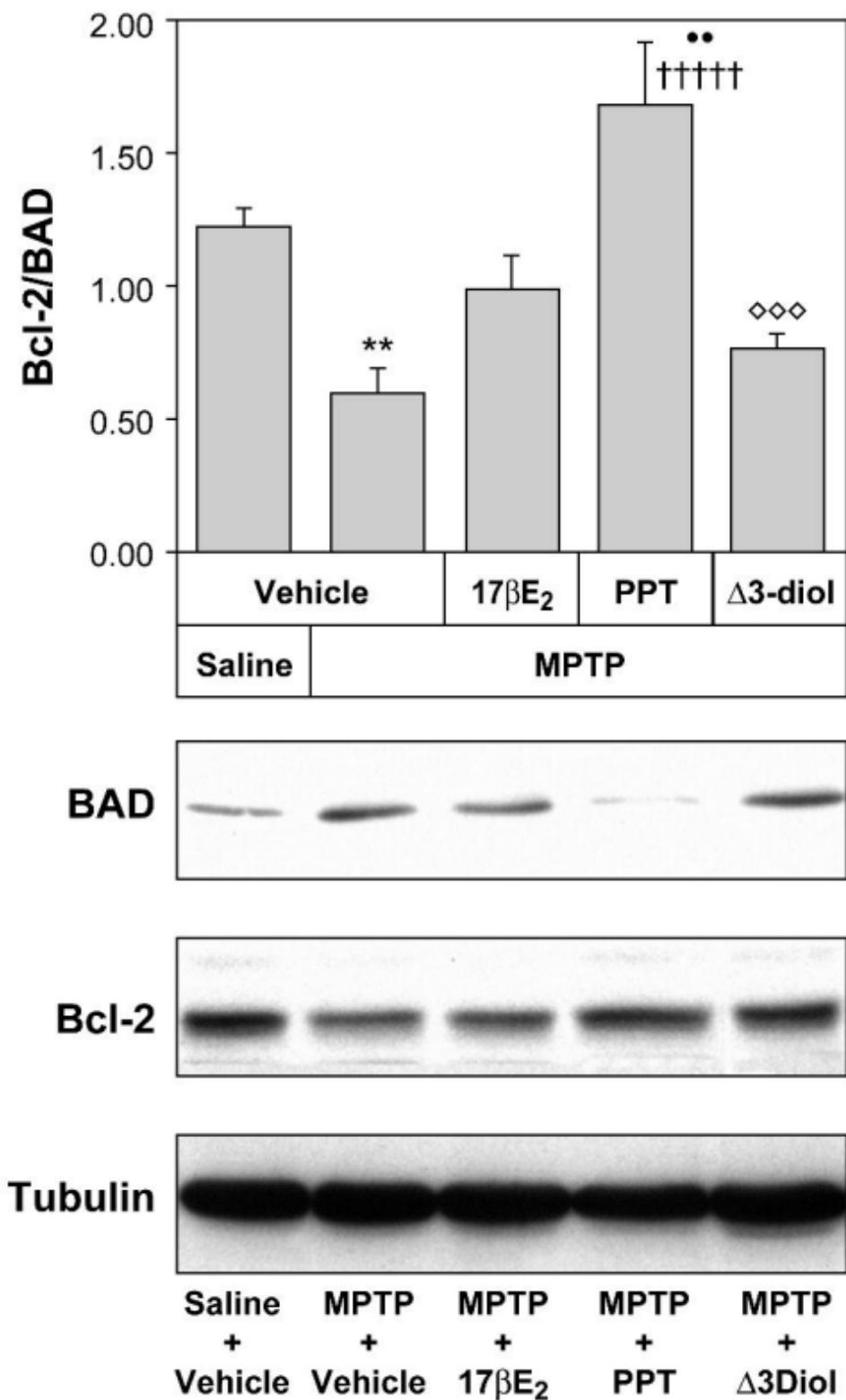


Figure 4

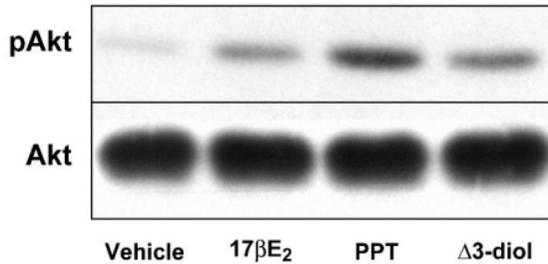
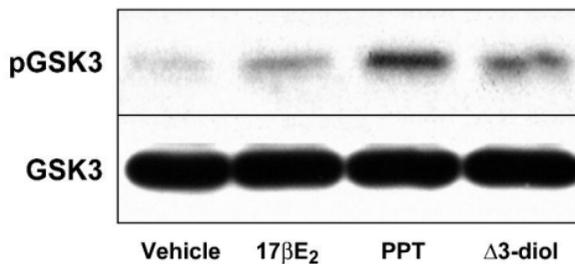
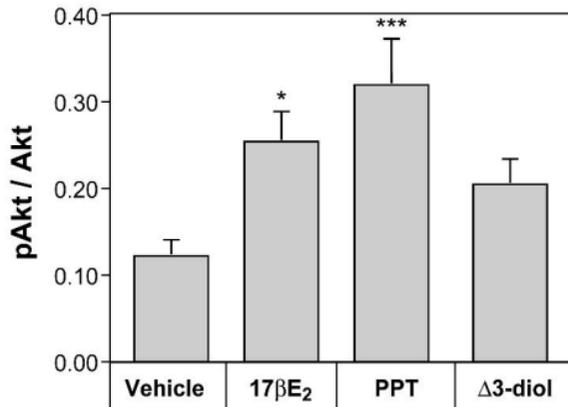
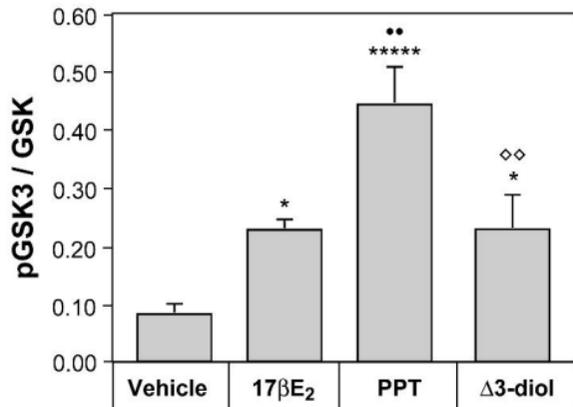


Figure 5

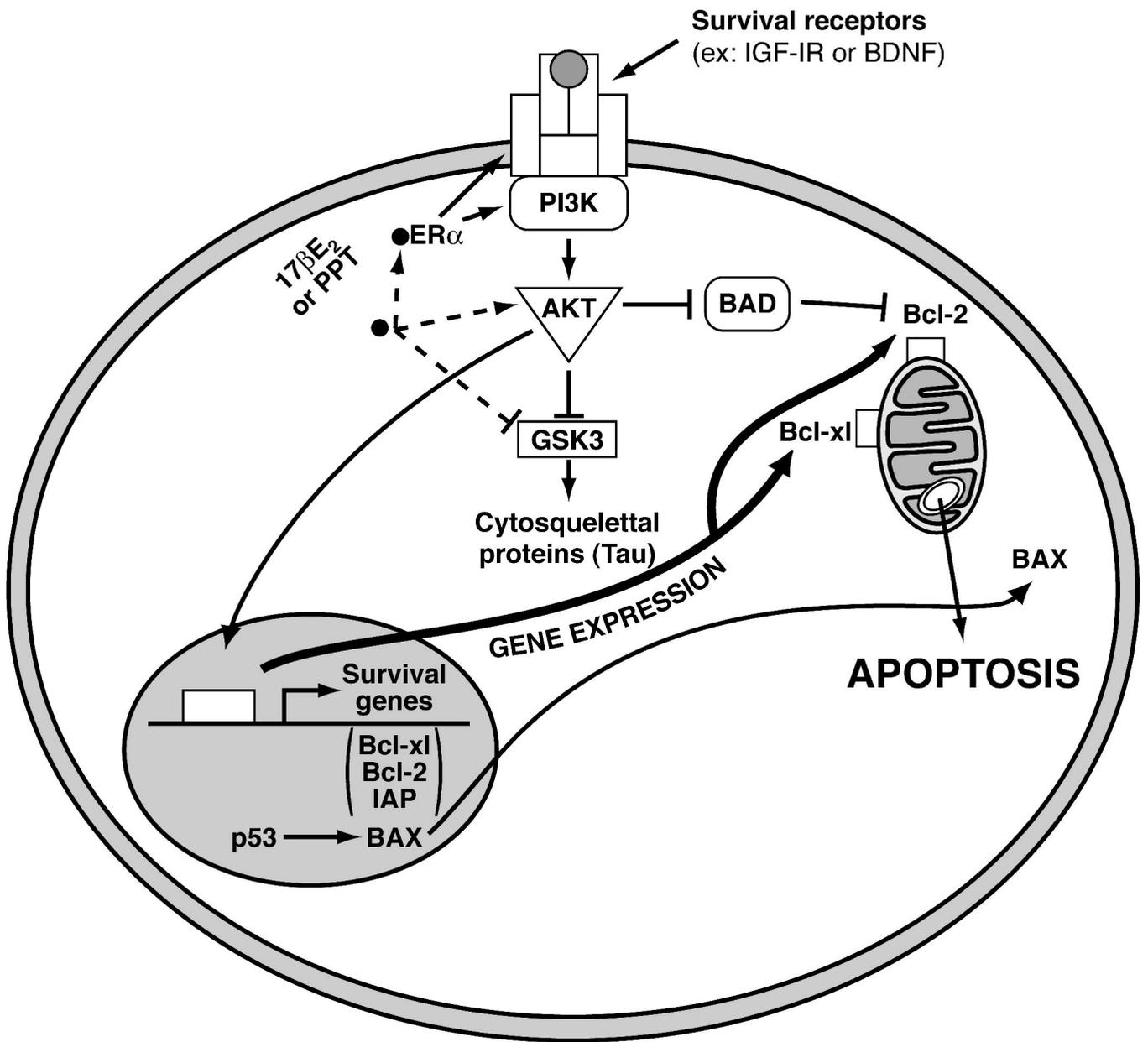


Figure 6