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The Impact of Gender and Estrogen on Striatal Dopaminergic Neurotoxicity

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ABSTRACT: The reproductive properties of estrogen are well established, but it is now evident that this steroid hormone has substantial modulatory capabilities in nonreproductive systems. For example, estrogen may be neuroprotective as Alzheimer's disease progresses more slowly in women receiving hormone replacement therapy, and Parkinson's disease affects more men than women. Gender affects both the functional and biochemical responses of the nigral-striatal pathway to dopaminergically active compounds. To begin to evaluate the possible neuroprotective effects of estrogen in this pathway, we first determined if gender affected the dopaminergic striatal neurotoxicity induced by two different neurotoxicants, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and methamphetamine (METH). Both agents induced greater neurotoxicity in males than females as evidenced by greater striatal dopamine (DA) depletions. An examination of striatal levels of 1-methyl-4-phenylpyridium ion (MPP⁺) following MPTP treatment established that the observed gender differences were not due to metabolic/pharmacokinetic variables. The neurotoxicity of MPTP was then examined in ovariectomized (OVX) mice. Estrogen replacement reduced the DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) depletions as well as the glial fibrillary acidic protein (GFAP) elevation induced by MPTP, which indicates that estrogen has neuroprotective properties in this model of striatal dopaminergic neurotoxicity. Surprisingly, estrogen supplementation did not protect against the neurotoxic effects of MPTP in intact 2-yr-old intact female mice, suggesting that low endogenous levels of estrogen may provide neuroprotection.

INTRODUCTION

The rapidly increasing age of the population and the recent focus on hormone-like effects of many xenobiotics have led to a renewed interest in both the reproductive and nonreproductive actions of estrogen.¹⁻⁵ The protective or age-retardive actions of estrogen are well established for multiple body systems including skin, bone and teeth, colon, blood vessels and heart.⁶⁻⁸

Several lines of evidence also suggest estrogen is neuroprotective. Clinical and epidemiological studies indicate more men than women develop Parkinson's disease and that the progression and severity of Alzheimer's disease is blunted in women receiving hormone replacement therapy.⁹⁻¹¹ Experimental data from a variety of *in vitro* and *in*

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vivo models indicate estrogen may prevent the cell damage and necrosis observed following excitotoxic, metabolic and oxidative insult.¹²⁻¹⁵ Recently, Dluzen and his co-workers^{2,3} found estrogen protects against neurotoxicant-induced damage to the nigral-striatal pathway—the pathway affected in idiopathic Parkinson's disease.

Although Dluzen and colleagues^{2,3} convincingly demonstrated that estradiol (E_2) replacement lessened the severity of the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in ovariectomized (OVX) mice, neurotoxicity was estimated either by direct measurement of dopamine (DA) and its metabolites or by measurement of endpoints linked to DA function. There is, however, sufficient evidence suggesting that estrogen also plays a regulatory role in the nigral-striatal pathway,^{16,17} and the contribution of this regulation to the observed decreased severity in DA-linked endpoints cannot be estimated without an independent measure of the dopaminergic damage caused by MPTP. Consequently, astrogliosis, as assessed by an increase in the astrocyte-localized protein, glial fibrillary acidic protein (GFAP), was used as a quantitative and independent biochemical marker of neural injury in the striatum. Our previous work has established the viability of this marker in evaluating neural damage induced by many different neurotoxicants, and we have specifically established the dose- and time-related increases in GFAP that follow exposure to MPTP.¹⁸ The effect of gender in the production of the putative active metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP⁺) was also examined, as males and females may metabolize and distribute compounds differently and this can account for sex differences in a toxicant's effect.¹⁹ While gender affected the degree of neurotoxicity induced by MPTP as well as methamphetamine (METH), a second well-characterized striatal dopaminergic neurotoxicant, it did not affect striatal levels of MPP⁺. We also demonstrated that E_2 replacement in OVX mice protects against MPTP-induced striatal damage as evidenced by a reduction in both striatal astrogliosis and DA depletion. Finally, the ability of E_2 supplementation to provide neuroprotection was examined as endogenous estrogen levels decline with age, but ovaries may still secrete minor amounts while OVX results in a total loss of estrogen. However, the striatal neurotoxic effects of MPTP were equivalent in 2-yr-old intact female mice whether supplemented or not with E_2 .

MATERIALS AND METHODS

Materials

All reagents were of at least analytical grade and were obtained from a variety of commercial sources. The following drugs and chemicals were obtained from the sources indicated: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Aldrich Chemical Co., Milwaukee, WI or Research Biochemicals, Inc., Natick, MA), METH, sesame oil, 17 β -estradiol (Sigma Chemical Co., St. Louis, MO), bichinchonic acid protein assay reagent and bovine serum albumin (Pierce Chemical Co., Rockford, IL), high-performance liquid chromatography (HPLC) reagents (Burdick & Johnson, Muskegon, MI). The materials used in the GFAP assay were previously described.²⁰

Animals and Dosing

All animal use procedures were according to the NIH guide for the Care and Use of Laboratory Animals and were approved by the local FDA or EPA Animal Care and Use Committee. Intact male and female cesarean delivered (CD)-1 mice, aged 4 to 5 months, were obtained from the NIDA/ARC breeding colony and were housed 4 per cage. In-

tact female C57BL/6J mice (2 yr old) or retired breeders (approx. 8 mo old) were purchased OVX from Jackson Labs (Bar Harbor, ME) and shipped within 48 hr of surgery. Within 24 hr of arrival OVX mice were briefly anesthetized with ether and implanted subcutaneously (s.c.) with a 10-mm silastic implant (0.062 mm ID \times 0.125 mm OD, sealed with silastic elastomer) containing sesame oil or 235 μ g E₂/ml sesame oil. These implants were of the same type and dosage utilized in the Dluzen *et al.*^{2,3} studies.

All mice were group-housed (4–6 per cage) under controlled conditions with water and feed provided ad libitum. Dosage regimens for MPTP and METH were based on our previously published work.^{21,22} Mice received 0.9% NaCl (SAL) or MPTP prepared as the base in SAL according to 1 of 2 regimens. CD-1 mice received Regimen A consisting of 0 or 30 mg/kg MPTP administered intraperitoneally (i.p.) once per day for 3 days, and tissue was obtained for catecholamine analysis at 5 days following dosage 3. A subset of these mice were killed at 4, 12 and 24 hr following dosage 1 or 3 and striatum collected for analysis of MPP⁺ levels. C57BL/6J intact or OVX mice received Regimen B consisting of a single s.c. injection of 12.5 mg/kg MPTP, and striatal tissue was obtained for analysis of GFAP and catecholamines at 48 hr after the injection of MPTP. Intact male and female C57BL/6J mice received METH administered at a dosage of 10 mg/kg s.c. every 2 hr \times 4 with tissue collected for analysis at 48 hr.

Brain Dissection and Tissue Preparation

At specified time points following the injection of MPTP (see above and figure legends) tissue was obtained for biochemical assay. A blunt curved forceps was used to immediately remove the whole brain from the skull. Dissections were conducted free-hand on a thermoelectric cold plate (Model TCP-2, Aldrich Chemical Co., Milwaukee, WI) using a pair of fine curved forceps (Roboz, Washington, DC). Striatum from the left side of the brain was weighed, frozen on dry ice and stored at -70°C for subsequent analysis of DA and its metabolites or MPP⁺ levels by high-performance liquid chromatography (HPLC). Striatum from the right side was weighed, homogenized with an ultrasonic probe (Model XL-2005, Heat Systems, Farmingdale, NY) in 10 vol hot ($90\text{--}95^{\circ}\text{C}$) 1% sodium dodecylsulfate (SDS) and stored frozen at -70°C before determination of GFAP by immunoassay. Weight of the uterus was obtained in OVX mice at the time of sacrifice as an indicator of the efficacy of E₂ replacement.⁵

Protein Assay

SDS homogenates were assayed for total protein by the method of Smith *et al.*²³ Bovine serum albumin was used as the standard.

GFAP Immunoassay

GFAP was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA)²⁰ with the modifications specified in O'Callaghan & Miller.²² Microtiter plate wells were coated with a rabbit polyclonal antibody to GFAP for 1 hr, and nonspecific binding was then blocked with nonfat dry milk. Dilution of the SDS homogenates with sample buffer and their addition to the wells of the microtiter plates was accomplished using a Tecan robotic sample processor (Model 5052, Tecan, U.S., RTP, NC), in dual-tip mode, running on RSP-Integrator/Immuno-AMI software (Tecan, U.S., RTP, NC). After several more blocking and washing steps, a mouse monoclonal antibody to GFAP

was added followed by the addition of an enzyme-linked antibody directed against mouse immunoglobulin G (IgG). The colored reaction product was obtained by subsequent addition of enzyme substrate and quantified by spectrometry at 405 nm using a microplate reader (UV Max running on a Soft Max program, Molecular Devices, Menlo Park, CA).

Catecholamine and MPP⁺ Analysis

Concentrations of DA and its metabolites DOPAC and HVA were quantified by HPLC with electrochemical detection (ECD). Samples were homogenized in ice-cold 0.2 N perchloric acid containing a known amount of the internal standard 3,4-dihydroxybenzylamine (DHBA). After centrifugation at $10,000 \times g$ for 10 min, an aliquot of the supernatant (20–40 μ l) was injected via a refrigerated (4°C) automatic sample injector (WISP 710B, Waters Division, Millipore Corp., Marlborough, MA) onto a C18 reverse-phase column (Waters Radial Pak, Millipore Corp., Marlborough, MA) equipped with a precolumn filter. Detection was achieved with a M 460 amperometric ECD (Waters Division, Millipore Corp., Marlborough, MA) and elution peaks were acquired and integrated using Baseline 810 software (Dynamic Solutions Division, Millipore Corp., Marlborough, MA).

Concentrations of MPP⁺ in striatum were determined by HPLC.²⁴ Preparation of samples was the same as those to be analyzed for DA content, but samples were assayed using a Waters Associates 510A pump (Milford, MA), a Rheodyne 7125 injector (Rheodyne, Cotati, CA), a Bioanalytical Systems MF-6017 Biophase ODS 5 micron column (250 \times 4.6 mm) and a Waters 474 fluorescence detector. Chromatograms were recorded and integrated on a Perkin-Elmer LCI-100 integrator (Perkin-Elmer, Norwalk, CT). Excitation and emission wavelengths of 290 and 370 nm, respectively, were used to detect MPP⁺ with a retention time of 4.2 min.

Statistics

Data analysis was conducted using the Statistical Analysis System.²⁵ An analysis of variance was used to evaluate individual variables followed by Duncan's Multiple Range Test for mean comparisons. The α level used to determine significance was 0.05 in all cases.

RESULTS

Gender and Dopaminergic Neurotoxicity of MPTP and METH

There were clear gender differences in the response to both MPTP (FIG. 1) and METH (FIG. 3). MPTP caused a pronounced loss of DA in the striatum of both male and female CD-1 mice (FIG. 1). However, it is quite apparent that the depletion in males was substantially larger (69%) than in females (45%). This significant gender difference was not due to a difference in the production of the proximal toxicant MPP⁺ (FIG. 2). Both sexes produced significant, but equivalent, amounts of MPP⁺ in striatum within 4 hr of injection of MPTP, and both sexes produced more of the pyridinium ion following the third injection (FIG. 2, bottom panel) relative to the first injection (FIG. 2, bottom panel). MPP⁺ was virtually nondetectable in striatum within 24 hr of the first or third injection of MPTP, and again there were no gender differences.

A significant gender difference was also apparent in the DA depletion observed in

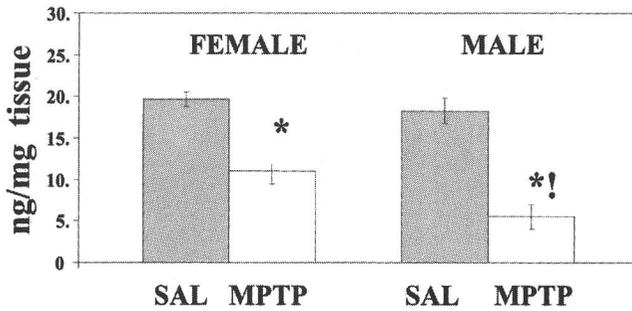


FIGURE 1. Effects of MPTP on striatal DA levels in intact male and female CD-mice. Striatal samples were obtained 5 days post the third daily injection of MPTP (30 mg/kg, i.p. as the base). Each value represents the mean \pm SEM of at least 5 mice. An *asterisk* (*) indicates a significant decrease of at least $p < 0.05$ when compared to the SAL group. An *exclamation point* (!) indicates the males given MPTP have lower DA levels than females ($p < 0.05$). (Adapted from Freyaldenhoven *et al.*)³⁶

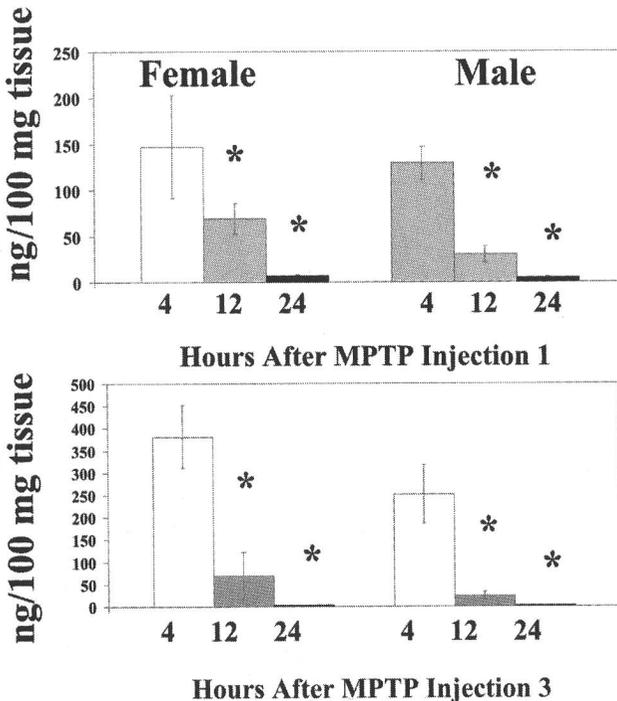


FIGURE 2. Effects of gender on striatal MPP⁺ production in CD-1 mice. Striatal samples were collected at 4, 12, or 24 hr following 1 or 3 daily i.p. injections of MPTP (30 mg/kg as the base). Each value represents the mean \pm SEM of 4 mice. An *asterisk* (*) indicates a significant decrease of at least $p < 0.05$ in amount of striatal MPP⁺ produced relative to the 4-hr time point. No difference between males and females were apparent at any time point. (Adapted from Freyaldenhoven *et al.*)³⁶

the C57BL/6J mice following a neurotoxic regimen of METH (FIG. 3). Both sexes showed a significant depletion of DA in striatum, but the effect was significantly larger in the males (92%) relative to the females (68%). This demonstration of the increased vulnerability of the male to a second neurotoxicant utilizing a different mouse strain extends the generality of the gender effect observed with MPTP.

Effect of OVX and Estrogen Replacement and MPTP on Uterine Weight

OVX mice have atrophy of the uterus, and as would be expected the OVX mice receiving E₂ replacement have a significant increase in the weight of the uterine tissue

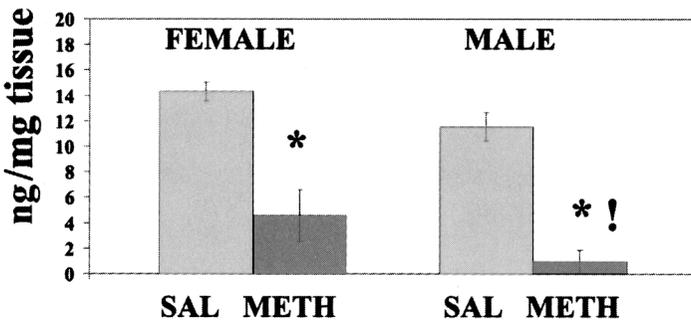


FIGURE 3. Effects of METH on striatal DA levels in intact male and female C57BL/6J mice. Striatal samples were obtained at 72 hr post the fourth injection of METH (10 mg/kg as the base, s.c. every 2 hr × 4). Each value represents the mean ± SEM for at least 5 mice. An asterisk (*) indicates a significant decrease of at least $p < 0.05$ when compared to the SAL group. An exclamation point (!) indicates the males given METH have lower DA levels than females ($p < 0.05$).

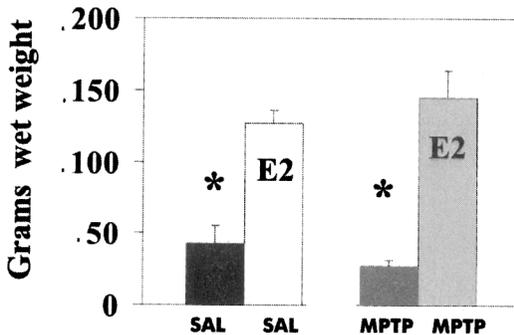


FIGURE 4. Effects of estrogen (E2) replacement and MPTP in OVX C57BL/6J mice. Uterine weights were collected at 48 hr post a single s.c. injection of MPTP (12.5 mg/kg as the base). Each value represents the mean ± SEM for at least 6 mice. An asterisk (*) indicates a significant increase in uterine weight of at least $p < 0.05$ when compared to mice not given estrogen replacement. A single injection of MPTP (12.5 mg/kg, s.c.) did not alter the uterotrophic effects of estrogen.

(FIG. 4). Mice receiving MPTP displayed slightly more hypertrophy in response to E_2 than those receiving only saline (118 vs 84 mg), but this difference was not significant.

Effect of OVX and E_2 Replacement on the Striatal Neurotoxicity of MPTP

OVX C57BL/6J mice receiving no E_2 replacement had a significant depletion of striatal DA as well as the metabolites DOPAC and HVA (left side of the upper, middle and bottom panel of FIG. 5, respectively). In contrast, mice with E_2 implants showed significantly less depletion of DA and DOPAC (33 and 28% less, respectively). Even more striking was the total lack of HVA depletion exhibited (FIG. 5, bottom panel) in mice with E_2 replacement; a 49% depletion was observed in the nonreplaced group. The GFAP data obtained from these same animals (FIG. 6) serve as an independent measure of neural injury and confirm that the differences in DA and its metabolites observed in animals receiving replacement of E_2 reflect protection against the striatal damage caused by MPTP. As expected (see Ref. 18) treatment with a 12.5 mg/kg dosage of MPTP resulted in a significant elevation (169%) in this astrocyte-localized protein at 48 hr post injection. Mice given E_2 replacement showed only a 67% increase in GFAP, which represents a significant reduction (102%) relative to the nonreplaced animals.

Effect of E_2 Supplementation and MPTP on Uterine Weight in 2-Yr-Old Mice

Two-yr-old intact female C57BL/6J mice receiving E_2 supplementation showed a pronounced increase in uterine weight (FIG. 7), suggesting endogenous estrogen production was reduced in animals of this age. As was found with the OVX mice, the treatment with MPTP did not interfere with the uterotrophic actions of estrogen.

Effect of E_2 Supplementation on the Striatal Neurotoxicity of MPTP

Treatment of 2-yr-old intact C57BL/6J mice with MPTP produced damage to the dopaminergic elements of the striatum (FIG. 8) as evidenced by a large depletion (54%) in DA as well as an elevation (73%) in GFAP. As we have previously reported,²⁶ there is an age-related increase in the basal levels of GFAP (compare the SAL groups in FIGS. 6 and 8). Although, E_2 supplementation did slightly modulate the DA depletion (10% less) as well as the GFAP elevation (28% less), these differences were not significant.

DISCUSSION

Gender and its associated hormonal differences have long been known to affect both the biochemistry and function of the nigral-striatal pathway. Our data and that of Dluzen suggest this gender difference extends to neurotoxicant-induced injury to this pathway.^{2,3} The greater neurotoxicity in male relative to female mice as evidenced by more pronounced depletions in striatal DA and its metabolites suggests hormonal differences may play a role in MPTP-induced injury. The lack of a gender difference in the striatal levels of MPP⁺, the proximal neurotoxicant, produced following a given dosage of MPTP lend greater credence to this idea.

The failure to see neuroprotection in the 2-yr-old female mice receiving E_2 supple-

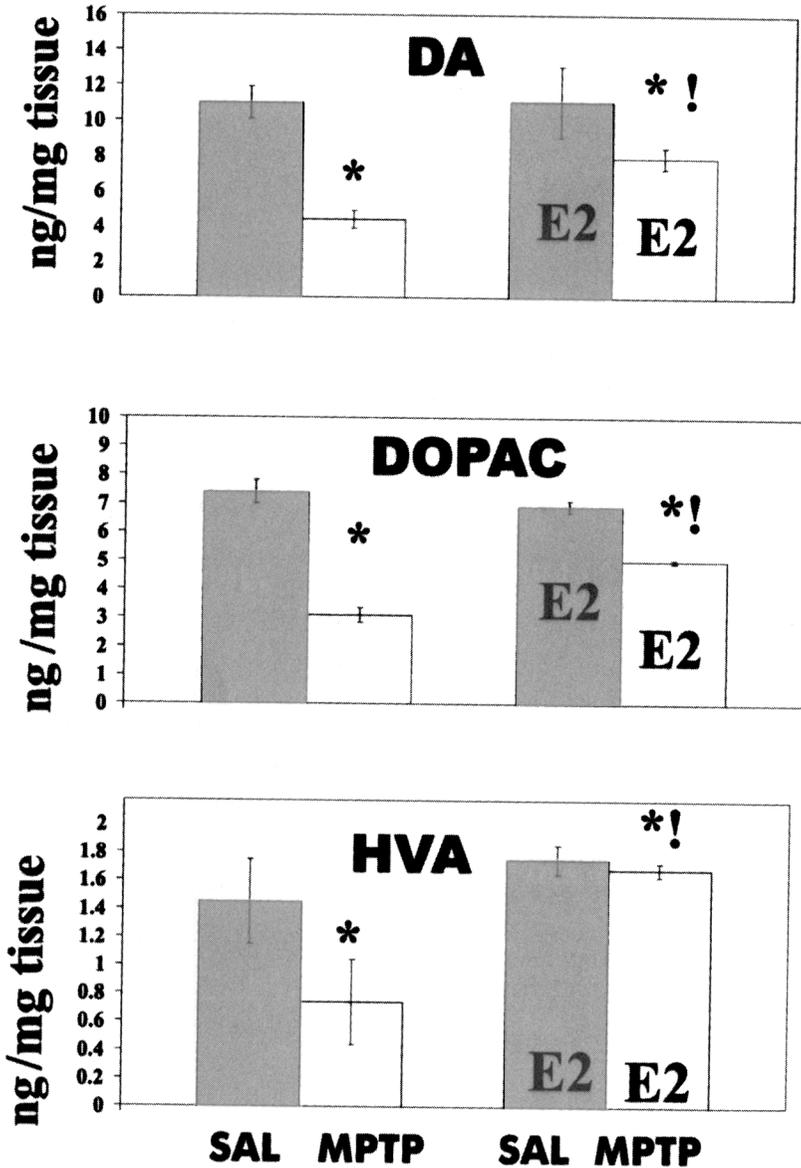


FIGURE 5. Effect of estradiol (E₂) replacement on striatal DA, DOPAC and HVA depletions induced by MPTP in OVX C57BL/6J mice. Striatal samples were collected at 48 hr post a single s.c. injection of MPTP (12.5 mg/kg as the base). Each value represents the mean \pm SEM of at least 6 mice. An asterisk (*) indicates a significant decrease of at least $p < 0.05$ relative to SAL. An exclamation point (!) indicates mice receiving E₂ replacement showed less decrement in DA, DOPAC and HVA than mice not given E₂ replacement.

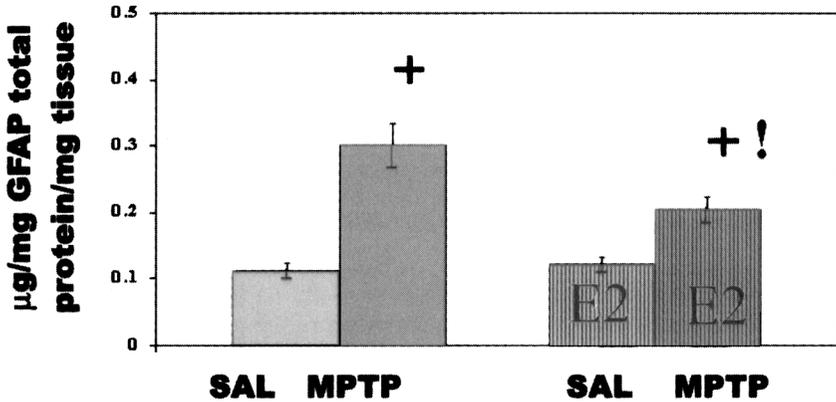


FIGURE 6. Effect of estradiol (E₂) replacement on the striatal GFAP elevation induced by MPTP in OVX C57BL/6J mice. Striatal tissue was collected at 48 hr post a single s.c. injection of MPTP (12.5 mg/kg as the base). Each value represents the mean ± SEM of at least 6 mice. A *plus sign* (+) indicates a significant increase of at least $p < 0.05$ relative to SAL. An *exclamation point* (!) indicates mice receiving E₂ replacement showed less of an MPTP-induced elevation in GFAP than mice not given E₂ replacement.

mentation is puzzling, but the differences in reproductive status and age at the time of neurotoxicant exposure between these and the OVX groups may be responsible. Despite the age of the intact mice their ovaries may have produced a level of endogenous estrogen sufficient to mask any protective effect of E₂ supplementation. Unfortunately, serum levels of estrogen were not monitored in these mice, but a comparison of uter-

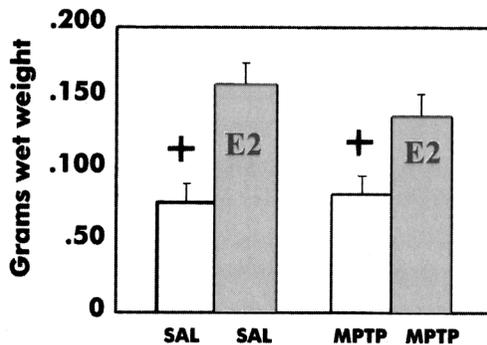


FIGURE 7. Effects of estradiol (E₂) supplementation and MPTP in intact 2-yr-old C57BL/6J female mice. Uterine weights were collected at 48 hr post a single s.c. injection of MPTP (12.5 mg/kg as the base). Each value represents the mean ± SEM for at least 6 mice. A *plus sign* (+) indicates a significant increase in uterine weight of at least $p < 0.05$ when compared to mice not given E₂ replacement. A single injection of MPTP (12.5 mg/kg, s.c.) did not alter the uterotrophic effects of E₂.

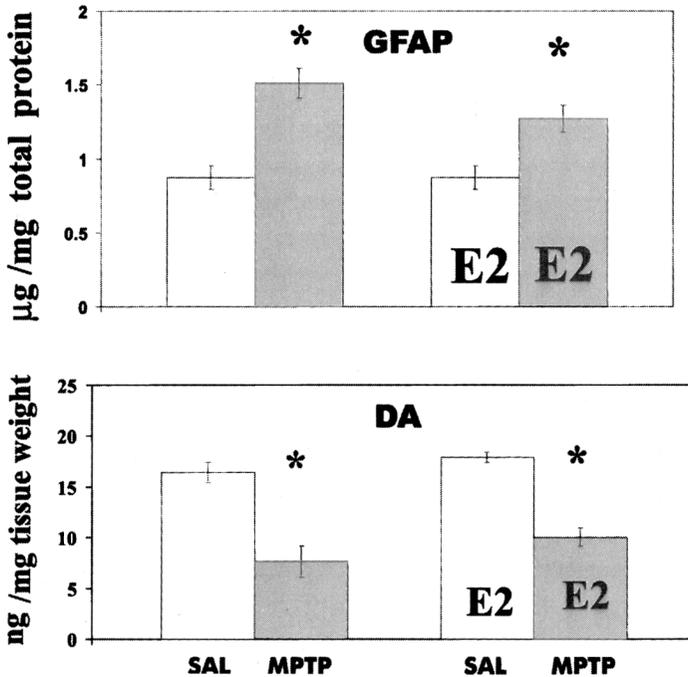


FIGURE 8. Effect of estradiol (E₂) supplementation on the striatal neurotoxicity induced by MPTP in 2-yr-old intact C57BL/6J female mice. Striatal samples were collected at 48 hr post a single s.c. injection of MPTP (12.5 mg/kg as the base). Each value represents the mean \pm SEM of at least 6 mice. An asterisk (*) indicates a significant (at least $p < 0.05$) increase in GFAP (*top panel*) or decrease in DA (*bottom panel*) relative to SAL. E₂ supplementation did not significantly alter the degree of DA depletion or GFAP elevation.

ine weights (compare FIGS. 4 and 7) in the nonsupplemented 2-yr-old mice and OVX mice not receiving E₂ replacement suggests sufficient endogenous hormone was present to maintain partial hypertrophy of the uterus in the intact mice. It was also clear that estrogen production was reduced with age as supplementation with E₂ did produce a significant further hypertrophy. An evaluation of the neuroprotective properties of a range of dosages of E₂, including a low physiological dosage, in OVX mice receiving MPTP, would aid in resolving this issue.

Although our work clearly demonstrates the neuroprotective properties of E₂ in the MPTP model of dopaminergic neurotoxicity, it provides no information on the mechanism(s) responsible. Estrogen influences multiple systems, and many of these actions are classic genomic effects involving binding to the estrogen receptor (ER) and subsequent activation of specific genes.²⁷ Hypertrophy of uterine tissue with estrogen replacement in an OVX animal is but one example of such a receptor-mediated action. The reported lack of genomic ERs in the nigral-striatal²⁸ (but see Ref. 16 for a discussion of this issue) would appear to rule out a direct receptor-mediated action of estrogen in our neuroprotective finding, but it is now known that the ER exists as both an α and β subtype.²⁹ Most pertinent to our work is the reported expression of the mRNA

for ER- β in substantial nigra.³⁰ Thus, the elimination of a receptor-mediated mechanism for our E₂ findings may be premature. Future neurotoxicity studies examining the neuroprotective actions of estrogen in mice null for either or both of the ER subtypes may prove as useful in elucidating neuroprotective mechanisms as it has in explaining protective effects in the vasculature.^{7,8}

Although many actions of estrogen require transcriptional activation of genes, there are numerous reports of short-latency actions in brain, heart and vasculature that appear nongenomic in nature, as they are not blocked by protein synthesis inhibition.²⁷ These rapid nongenomic effects include direct action on membranes themselves, membrane receptor sites and ion channels or cyclic AMP response elements.^{8,27,31} The direct and immediate effects of estrogen on neuronal DA release and firing rate^{16,32} are unlikely to be the mediators of estrogen's protection against MPTP-induced neural damage, as manipulation of such parameters does not appear to alter the neurotoxicity of this agent. A more likely mediator is a reduction of oxidative processes by estrogen;^{12,13,33} the neurotoxicity of both MPTP and METH is ameliorated by manipulations that involve reduction of oxidative stress (see Ref. 34 for a discussion). The putative role of oxidative processes in the neural damage associated with MPTP and METH as well as the reported protection against oxidative stress-induced neuronal cell death provided by estrogen and certain estradiol derivatives provide another plausible area for future mechanistic investigations. The extensive characterization of the dopaminergic neurotoxicity associated with MPTP and METH exposure in the mouse provide useful models that can be used in such investigations concerning the *in vivo* neuroprotective properties of estrogen in the nigral-striatal pathway.

In conclusion, the lack of effect of gender on production of the proximal toxicant MPP⁺ as well as the greater striatal DA depletions sustained by males given either MPTP or METH suggest some aspect of female physiology provides neuroprotection against the dopaminergic striatal damage induced by these neurotoxicants. A lessened astrogliosis and DA depletion in OVX mice exposed to MPTP and receiving E₂ replacement suggest that estrogen may be the factor protecting the striatum of intact female mice receiving dopaminergic neurotoxicants. Future studies should more explicitly explore the neuroprotective properties of estrogen in the nigral-striatal pathway. It should be noted, however, that any manipulation of estrogen can alter other hormones including progesterone (P), and this hormone also has demonstrated neuroprotective properties.³⁵ Whether P is neuroprotective in various experimental models of dopaminergic neurotoxicity is unknown. Finally, the failure of E₂ supplementation to ameliorate the neurotoxicity of MPTP in intact 2-yr-old female mice suggests the neuroprotective properties of endogenous estrogen may occur at quite low, but apparently still physiologically active, levels.

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