Differential Protective Properties of Estradiol and Tamoxifen against Methamphetamine-Induced Nigrostriatal Dopaminergic Toxicity in Mice

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Abstract
Mechanisms implicated in protective potential of estrogens are poorly understood. Tamoxifen, a selective estrogen receptor modulator (SERM), presents a neuroprotective effect against methamphetamine (MA)- and methoxy-phenyltetrahydropyridine (MPTP)-induced toxicity when used alone but abolishes estrogen’s positive effects when combined with this hormone. In order to understand tamoxifen’s protective properties, the present study compared it to estradiol on several markers of dopaminergic neurons to achieve a relatively comprehensive comparison between these two agents. Estradiol benzoate (E) or tamoxifen were used at different concentrations (E: 1, 10 or 40 \(\mu\)g; tamoxifen: 12.5, 125 or 500 \(\mu\)g) 24 h prior to a MA injection in ovariectomized CD-1 mice. The effects of the lesion and treatments were studied on striatal dopamine (DA) concentrations, dopamine and monoamine vesicular transporters (DAT and VMAT2), and preproenkephalin (PPE) mRNA levels. Both treatments, at all concentrations, prevented the MA-induced decrease of striatal DA concentrations and VMAT2 binding. Only E was able to prevent loss of DAT binding in the lateral striatum and to attenuate the MA-induced increase in striatal PPE mRNA levels (at 1 or 40 \(\mu\)g). Therefore, in this paradigm, E and tamoxifen differentially modulated MA-induced neuronal damages. While both treatments prevented the DA decrease, E protected more efficiently other dopaminergic parameters suggesting that overall E is more effective than tamoxifen as a neuroprotectant of the nigrostriatal dopaminergic system.

Introduction
Clinical studies have demonstrated a greater prevalence of Parkinson’s disease (PD) in men than in women [1, 2]. This gender difference may, in part, be attributable to estrogen since epidemiological studies have demonstrated a beneficial role of estrogens in PD [3, 4]. Laboratory studies have also shown the protective potential of estrogens on brain against methamphetamine (MA)
[5–9] and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [10, 11], two substances that induce damages similar to those encountered in PD [12]. Although these positive effects are well documented, concerns have been raised regarding the clinical use of estrogens. As a specific example for this is the clinical study suggesting that estrogens increases PD risk in women with hysterectomy but not in women with natural menopause [13]. More generally, the Women’s Health Initiative (WHI) study recently showed that use of conjugated equine estrogen (Premarin) with and without simultaneous medroxyprogesterone acetate lack treatment benefit and increase risk of stroke and other adverse outcomes in a large group of post-menopausal women [14–17]. Hence, it becomes important to compare the effect of estradiol to other estrogenic compounds in order to find better and safer hormonal treatments for neuroprotection.

To avoid peripheral problems associated with estrogens, different molecules such as SERMs (selective estrogen receptor modulators) have been developed. Ideally, these molecules, while presenting all the beneficial effects of estrogens on many organs, would be devoid of adverse effects on the reproductive organs. One of these molecules, tamoxifen, is used clinically for treatment and prevention of breast cancer [18]. However, tamoxifen acts as an estrogenic agonist on the uterine tissue [19]. Tamoxifen presents the beneficial effects of estrogen in monal treatments for neuroprotection.

One recent study has shown that tamoxifen is effective in reducing dopamine loss in PD [12]. However, the precise mechanism by which tamoxifen acts as a neuroprotective agent is not fully understood. Therefore, we included doses above and below these concentrations to examine the potential range of effectiveness. The dose of E and tamoxifen were selected based upon previous work showing that the middle concentrations (10 μg for E and 125 μg for tamoxifen) were effective in altering striatal DA function [8, 27].

**Methods**

**Animals and Treatments**

Female CD-1 mice (2–3 months) were purchased from Charles River Laboratories (Wilmington, Mass., USA). Mice were housed individually in plastic cages with free access to food and water, while maintained at room temperature of approximately 22°C under a 12 h light-dark cycle (lights on at 06:00 h). All mice were ovarioctomized (OVX) prior to use in these experiments. All conditions were maintained according to NIH regulations and approved by the Institutional Animal Care and Use Committee (IACUC) at Northeastern Ohio Universities College of Medicine (NEUCOM). All efforts were made to minimize the number of animals used and their suffering.

Mice were distributed in groups of 5–9 animals. Mice were treated with E or tamoxifen at different concentrations (1, 10 or 40 μg of E and 12.5, 125 or 500 μg of tamoxifen per animal, subcutaneous injection in the dorsal neck region) 24 h prior to an injection of MA (40 mg/kg, i.p.), while a control group received an injection of the vehicle only (sesame oil). Tamoxifen, E and MA were purchased from Sigma (St. Louis, Mo., USA). These doses of E and tamoxifen were selected based upon previous work showing that the middle concentrations (10 μg for E and 125 μg for tamoxifen) were effective in altering striatal DA function [8, 27]. Therefore, we included doses above and below these concentrations to examine the potential range of effectiveness. The dose of MA was selected based upon its capacity to produce a relatively moderate lesion of the striatal DA system [8].

**Brain Tissue Preparation**

One week after MA, all mice were euthanized by rapid decapitation. Brains were removed and bisected, using a unilateral stria-
tum to assay DA concentrations. The contralateral hemisphere was frozen in liquid nitrogen. The striatum and the substantia nigra (SN) were cut on a cryostat in 12-μm slices. These brain slices were kept at –80°C until the experiments.

**Striatal Dopamine Assay**

The unilateral striatum was dissected, weighed and placed in 0.1 M HClO₄ at 4°C. The samples were sonicated and centrifuged. The supernatants were used to measure DA concentrations by HPLC with electrochemical detection, as previously performed [9].

**DAT and VMAT2 Autoradiography**

Before radioligand binding autoradiography, brain slices were rehydrated in baths containing the same buffer as for the binding. DAT and VMAT2-specific binding were realized using the ligands 3β-[125I]-iodophenyltropane-2β-carboxylic acid isopropyl ester ([125I]-RTI-121) and [3H]dihydrotetrabenazine ([3H]-TBZ-OH) respectively, as previously described [9, 11, 28]. DAT- and VMAT2-specific binding were performed on slices containing the striatum (bregma 1.54 to –0.94 mm) and SN (only for DAT binding) (bregma –2.80 mm to –3.28 mm) [29]. Two to three slices per animal were used in this protocol. Specific binding to DAT was measured using 20 pmol of [125I]-RTI-121 (2,200 Ci/mmol), in the presence of 2 nM of mazindol to evaluate non-specific binding, while specific binding to VMAT2 used 20 nM of [3H]-TBZ-OH (20 Ci/mmol) and 10 μM of cold TBZ-OH for non-specific binding. Slices were apposed to Kodak film, 40 h for DAT and 4 weeks for VMAT2. Autoradiograms were analyzed using the software NIH Image 1.68. The specific binding measured was considered to reflect changes of density of the transporters. For the VMAT2 binding we used a saturating concentration of the radioligand [28, 30]. For technical reasons we did not use a saturating concentration of [125I]-RTI-121 but based on our previous experiments we believe that in the present experiments, changes of specific binding using one concentration of the radioligand estimate changes of density of the DAT well. Estradiol treatments as well as male/female differences and estrous cycle variations change density and leave affinity of DAT unchanged [31, 32]. Furthermore, we previously reported in neuroprotection experiments a significant positive correlation between DAT-specific binding and DA concentrations [23, 24]. Hence, the changes of DAT-specific binding that reflect changes of density are believed here to measure different densities of DA terminals in the striatum due to lesion and neuroprotection.

**PPE mRNA in situ Hybridization**

PPE mRNA levels in the striatum (bregma 1.54–1.34 mm) [29] were measured by in situ hybridization as described by Calon et al. [33]. An oligonucleotide complementary to bases 304–350 of the mouse PPE sequence (GenBank accession No. M13227) was labeled at the 3’ end by terminal transferase with a [35S]-d-adenosine triphosphate (1,250 Ci/mmol, NEN Mandel, Boston, Mass., USA) using a 3’-terminal deoxyxynucleotidytransferase enzyme kit (Amersham Pharmacia Biotech, Baie d’Urfé, Québec, Canada). Non-specific hybridization was assessed using a sense oligonucleotide probe.

All solutions and buffers were prepared with distilled water treated with diethylpyrocarbonate (0.01%) to inhibit RNase activity and autoclaved. The sections were dried at 4°C under vacuum with dessicant. The sections were fixed 5 min in 3% paraformaldehyde prepared in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature and after rinsed twice for 5 min in sodium phosphate buffer, pH 7.4, at room temperature. The sections were rinsed in 2× SSC (standard NaCl/Na citrate) for 10 min at room temperature, 3 min at room temperature in 0.1 M triethanolamine (pH 8.0) and afterwards incubated in a fresh solution of 0.25% acetic anhydride in triethanolamine 0.1 M for 10 min at room temperature. The slides were then rinsed briefly twice in 2× SSC. The slide-mounted sections were subsequently dehydrated in a series of ascending concentrations of ethanol (50, 70, 95 and twice 100%, 1 min each), air-dried and stored at room temperature 2–3 h under vacuum with dessicant.

For in situ hybridization, [35S]-labeled oligonucleotide probes were added in hybridization buffer to reach a concentration of 5×10⁶ cpm/ml. Hybridization buffer is composed of 50% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 0.25 mg/ml yeast tRNA, 0.5 mg/ml denatured salmon sperm DNA, and 4× SSC. 100 μl of hybridization buffer containing labeled probe was added to each brain section. The sections were then covered with glass coverslips and incubated at 37°C for 15 h in a humid chamber. After incubation, the coverslips were floated off in 2× SSC at room temperature and the slide-mounted sections were washed for 1 h in 2× SSC at room temperature followed by one wash for 1 h in 1× SSC. The slides were then washed successively in three washes in 0.5× SSC 30 min each at 37°C, room temperature and 50°C respectively. The slides were subsequently air-dried and dehydrated in a series of ascending concentrations of ethanol (50, 70, 95 and twice 100%, 1 min each). The slide-mounted tissue sections were then exposed to Kodak BioMax film for 5 days at room temperature. The autoradiograms were analyzed using the software package NIH Image 1.68.

**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**

Administration of 40 mg/kg MA led to a moderate depletion of DA of 59% as compared to the control group (fig. 1). When pretreated with E, MA-treated mice had striatal DA concentrations significantly higher than those of the MA-treated group, for all three doses of E tested. DA levels for these animals were equivalent to those of the control group. Tamoxifen administration also prevented the MA-induced DA depletion. All three doses administered (12.5, 125 or 500 μg) maintained DA concentrations at the control levels.

Examples of autoradiograms of specific binding to DAT as determined by the specific binding of [125I]-RTI-
121 are shown in figure 2; it was decreased following MA injection (fig. 3). This diminution in binding was statistically significant in the lateral (fig. 3A), but not in the medial striatum (fig. 3B). All three doses of E prevented the decrease in striatal DAT binding within the lateral striatum. The decrease in DAT-specific binding was prevented in the tamoxifen-treated mice receiving 12.5 and 500 µg of tamoxifen, 24 h prior to MA injection (40 mg/kg), while control animals received injection of vehicle. Overall ANOVA values were $F_{4,7} = 3.878$, $p = 0.002$ for DA, while subsequent pairwise comparisons were $\ast\ast\ast p < 0.0001$ vs. Control; $\dagger p < 0.05$, $\dagger\dagger p < 0.005$ and $\dagger\dagger\dagger p < 0.0005$ vs. MA. Values are the mean (pg/mg tissue) ± SEM of 5–8 mice/group.

**Discussion**

The results presented herein support the neuroprotective role of estradiol on DA concentrations against MA toxicity, in agreement with previous reports [5, 7–9]. Moreover, our results with tamoxifen also support a neuroprotective role of this SERM on DA content, as previously proposed [6]. While both E and tamoxifen at the lowest concentrations tested were effective to prevent MA-induced DA depletion, differences between E and tamoxifen were observed for other markers studied. Therefore, several markers seem necessary to get an accurate state of neuronal damage, since DA concentrations could be normal (equivalent to controls) while other markers could still be decreased.

DAT represents an important dopaminergic marker responding discriminatively to these treatments. While MA administration led to a decrease in DAT-specific binding in the lateral striatum, all E doses used prevented this decrease. On the other hand, only the two extreme doses of tamoxifen protected against MA-induced DAT decrease in the lateral striatum. Such regional (lateral to medial) differential responses to a lesion and treatments have been observed previously [9]. DAT-specific binding
was not significantly decreased in the SN following MA. This result is in agreement with previous work showing that damages are more extensive to the terminals in the striatum than to the cell bodies in the SN [34]. Although the small decrease in DAT binding was not significant in the SN, a positive and significant correlation between this binding in the striatum and binding in the SN was observed.

Studies with the MA model mainly investigated interactions between neuropeptide systems and mesolimbic DA pathways [35]. On the other hand, in the MPTP model, PPE expression is referred to as a presymptomatic marker of neurodegeneration [26]. In situ hybridization of PPE mRNA revealed that MA-induced damages also increased PPE mRNA levels, which is in agreement with results on rat striatum following MA [35]. This is the first report of PPE mRNA regulation after MA damage in

Fig. 2. Examples of autoradiograms of specific binding to DAT (A, D) and VMAT2 (B) transporters as well as PPE mRNA (C) in the striatum (A–C) and SN (D) of CD-1 OVX mice treated with MA and 1 µg 17β-estradiol-3-benzoate (E) or 12.5 µg tamoxifen (TAM) as compared to vehicle-treated mice. NS = Non-specific binding.
mice and modulation of this damage by E. MA administration increased PPE mRNA levels by 130 and 120% in the lateral and medial striatum, respectively, and remained elevated in the tamoxifen-treated mice receiving E (1 and 40 µg) as compared with vehicle controls and a diminished increase of ~80%. These results suggest that the effects of E at 1 and 40 µg (~80% increases) can be distinguished from tamoxifen (~150% increases) upon this presymptomatic marker of neurodegeneration.

In accordance with the results obtained for DA concentrations, VMAT2 also responded similarly to E and tamoxifen. Indeed, the MA-induced decrease in VMAT2-specific binding was prevented by both treatments and at all concentrations investigated. There seemed to be no regional differences between the lateral and medial striatum in response to the lesion and/or treatments, in con-
The difference between these two striatal dopaminergic transporters could be attributed to differential sensitivity to the toxin or differential regulating mechanisms, since it has been described that DAT is more easily regulated than VMAT2 [36, 37]. A differential dose-response was also observed between E and tamoxifen. While all three E doses presented the same protective profile (with the exception of the 10 µg dose for PPE mRNA levels), the middle dose of tamoxifen (125 µg) was less effective than the other two tamoxifen doses to protect DAT-specific binding in the lateral striatum. Also, while tamoxifen failed to protect half of the markers tested against MA as examined in this report, the 125-µg dose was the least effective. At present we have no clear explanation for these dose-related differences, however, protection obtained at the highest concentration (500 µg) on DAT-specific binding overruled the possibility of switching from an agonist to antagonist role at higher doses.

The present results reveal that although the protective capacity of E and tamoxifen seemed equivalent on DA content, dissimilarities appeared on other dopaminergic markers. This differential protective role between these two molecules was also observed on MA toxicity in intact male mice [6]. In this study, pretreatment with E in intact male mice accentuated MA toxicity, while a pretreatment with tamoxifen resulted in less mortality. In this way, while the present results indicate that tamoxifen is a less potent modulator of MA-induced neurotoxicity than E on a number of markers of striatal neurodegeneration, this SERM are less toxic in response to MA within male mice.

**Fig. 4.** Effects of 17β-estradiol-3-benzoate (E) and tamoxifen on the dopamine transporter (DAT) binding in the SN of CD-1 OVX mice treated with MA as compared to saline-treated OVX animals (control). Overall ANOVA values were $F_{5,77} = 0.813, p = 0.580$.

**Fig. 5.** Effects of 17β-estradiol-3-benzoate (E) and tamoxifen treatments on preproenkephalin (PPE) mRNA levels in striatum of CD-1 OVX mice treated with MA as compared to saline-treated OVX animals (control). Overall ANOVA values were $F_{5,77} = 2.615, p = 0.021$ and $F_{5,77} = 2.493, p = 0.027$ for (A) lateral and (B) medial PPE mRNA levels respectively, while subsequent pairwise comparisons were * $p < 0.05$ and ** $p < 0.005$ vs. Control. Values of relative optical density are the mean (values in percentage of control) ± SEM of 6–9 mice/group.
These differential responses might be due to different mechanisms of action. Estrogens act on the brain through genomic and non-genomic mechanisms. The genomic mechanism involves activation of their specific estrogen receptors (ER), ERα and ERβ. E can induce transcription or inactivation of specific genes, via binding of the complex hormone-ER on estrogen response elements or AP-1 sites [38]. Although both molecules are able to bind to estrogen receptors, tamoxifen appears to be more anti-estrogenic when bound to ERβ, while estradiol functions as an agonist on both subtypes [39]. Moreover, tamoxifen inhibits transcription of genes regulated by the classical estrogen response elements [40], while being an agonist through AP-1 element [41].

Non-genomic mechanisms could also be implicated in the differential responses of E and tamoxifen. While E is known to have many effects on intracellular components, not many in vivo studies on the brain are available for tamoxifen. Therefore, this differential potential could be explained by the capacity of E to activate other cellular components, such as signaling pathways, growth factors or antiapoptotic molecules implicated in neuronal survival. Evidence suggests that E can activate the MAPK and Akt pathways, leading to neuroprotective actions [42, 43]. Akt is a kinase implicated in the phosphorylation and inactivation of pro-apoptotic factors, such as BAD [44, 45]. E can also regulate Bcl-2, an anti-apoptotic factor [46]. Moreover, E interacts with IGF-1, leading to neuroprotection [47, 48]. Results from an in vitro study demonstrated that tamoxifen had no effect on IGF-1 mRNA in primate cortical neurons, while E increased these levels [49]. The ability of tamoxifen to interact with these components remains to be determined. However, tamoxifen has been identified as a protein kinase C (PKC) inhibitor, therefore negatively regulating the PKC signaling cascade [50, 51]. This inhibitory effect could explain the differences in neuroprotection obtained since the PKC cascade not only involves many intracellular components, but also leads to differential transcription through CREB.

Other non-genomic mechanisms possibly implicated include antioxidative properties, since MA toxicity is associated with increased reactive oxygen species production and induction of apoptosis through mitochondrial damages [22]. While no data are available for tamoxifen, E is known to act on antiapoptotic molecules [46], which could explain why E is more potent at inhibiting the degenerating neuronal effects of MA. Moreover, antioxidative properties of E have also been linked to the phenolic ring present on its structure [52]. The fact that tamoxifen does not have a phenolic ring in its molecule could explain a differential antioxidative capacity. However, neuroprotection by an antioxidative mechanism usually occurs at pharmacological doses, while the doses used herein are considered to be in the physiological range [18].

In summary, we demonstrated that although beneficial effects of tamoxifen on DA concentrations were observed, measurements of several dopaminergic markers better define and achieve a more comprehensive determination regarding protective capacities of any molecule. Overall, our results suggest that more parameters of dopaminergic function are protected by E, suggesting that E is more effective than tamoxifen to exert neuroprotection upon the striatal dopaminergic system. Specific ER agonists or molecules based on SERMs and E differences/similarities would be helpful to understand these differential neuroprotective properties.

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