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Oestrogen Receptors Enhance Dopamine Neurone Survival in Rat Midbrain

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Abstract

Previous findings in our laboratory and elsewhere have shown that ovariectomy of rats in adulthood attenuates cocaine-stimulated locomotor behaviour. Ovarian hormones enhance both cocaine-stimulated behaviour and increase dopamine overflow after psychomotor stimulants. The present study aimed to determine whether ovarian hormones have these effects in part by maintaining dopamine neurone number in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) and to investigate the roles of specific oestrogen receptors (ERs) in the maintenance of mesencephalic dopamine neurones. To accomplish this goal, we used unbiased stereological techniques to estimate the number of tyrosine hydroxylase-immunoreactive (TH-IR) cell bodies in midbrain regions of intact, ovariectomised and hormone-replaced female rats and mice. Animals received active or sham gonadectomy on postnatal day 60 and received vehicle, 17 β -oestradiol (E₂) or selective ER agonists propyl-pyrazole-triol (PPT, ER α) or diarylpropionitrile (DPN, ER β) for 1 month post-surgery. In both rats and mice, ovariectomy reduced the number of TH-IR cells in the SNpc and VTA. Replacement with E₂, PPT or DPN prevented or attenuated the loss observed with ovariectomy in both rats and mice. An additional study using ER knockout mice revealed that adult female mice lacking ER α had fewer TH-IR cells in midbrain regions than wild-type mice, whereas mice lacking ER β had TH-IR cell counts comparable to wild-type. These findings suggest that, although both ER subtypes play a role in the maintenance of TH-IR cell number in the SNpc and VTA, ER α may play a more significant role.

Keywords

dopamine; oestrogens

Midbrain dopaminergic neurones that arise in the substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA) project to forebrain regions that regulate motivated behaviour, movement and cognitive function (1). Dysregulation of these pathways is implicated in a number of pathological disorders such as drug addiction and Parkinson's disease (PD). Women are likely to progress more rapidly in drug taking and are more likely to experience relapse than men (2). Women also show a lower incidence of PD than men (3,4). Sex differences in dopaminergic function represent a potential mediator for these differences.

Adult female rats have been shown to exhibit greater locomotor responses to psychostimulant drugs such as cocaine or amphetamine than males (5,6). Some studies show

that female rats self-administer psychostimulant drugs more than males and show enhanced place preference to these drugs (7,8). Female rats also show greater electrically-stimulated striatal dopamine uptake and release than males and exaggerated dopaminergic responses to psychostimulants (9–11).

Oestrogen influences on dopaminergic function may mediate these sex differences in behaviour. In rodents, ovariectomy attenuates locomotor, self-administration and dopaminergic responses to psychostimulants, which can be restored with oestrogen replacement (5,6,11–13). Ovariectomy of female rats results in a decrease in dopamine-specific proteins, including dopamine receptors and dopamine transporters. Oestrogen replacement can restore these functional responses (14,15). In summary, oestrogen enhances many aspects of dopaminergic function, from presynaptic regulation of release to postsynaptic receptor sensitivity.

Studies in humans and animals provide evidence that oestrogen not only augments dopaminergic function, but also plays a role in dopamine cell survival. Oestrogen replacement therapy in postmenopausal women reduces the risk of PD and reduces PD-related symptoms in postmenopausal early onset patients (16,17). Gonadectomy of female non-human primates results in a loss of dopamine neurones, which is prevented by immediate replacement but not reversed if replacement is delayed (18). Oestrogen also can protect the nigrostriatal pathway from neurotoxin-induced damage. Adult female rats are less susceptible to the behavioural deficits and dopamine content loss after treatment with the neurotoxin 6-hydroxydopamine (19). Dopamine neurone damage after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is increased in ovariectomised rodents in comparison to intact rodents, and replacement with 17β -oestradiol (E_2) reverses this increased susceptibility (20–23).

The effects of oestrogen on normal dopaminergic function are mediated in part through binding to the oestrogen receptors (ERs), $ER\alpha$ and $ER\beta$, which are ligand-dependent transcription factors (24). *In vivo* studies involving ER-selective agonists and transgenic mice reveal that both subtypes may contribute to the trophic effects of oestrogen on dopamine neurones. The $ER\alpha$ -selective agonist, propyl-pyrazole-triol (PPT), and E_2 protect striatal dopamine after treatment with the dopaminergic neurotoxin MPTP in mice, whereas the $ER\beta$ selective agonist diarylpropionitrile (DPN) has more modest effects (25). $ER\alpha$ knockout (α ERKO) mice are more vulnerable to the MPTP toxicity than $ER\beta$ knockout (β ERKO) mice, suggesting that $ER\alpha$ plays a significant role in the protection of dopaminergic pathways (26). By contrast, studies in rat reveal that maintenance of dopamine D_2 receptor and dopamine transporter binding is $ER\beta$ -dependent (14,27). Studies in β ERKO mice reveal a reduction of neurones in the SNpc, although the specific cell type was not estimated (28). These studies suggest that both ER subtypes may contribute to the maintenance of dopaminergic function.

Despite the evidence that oestrogen regulates normal dopaminergic function and protects against dopamine neurone damage after neurotoxin administration, little is known about the role of oestrogen in the normal maintenance of dopamine neurone number and whether oestrogen regulation of neurone number plays a role in the sex differences observed in psychostimulant-induced behaviour. The present study aimed to investigate how the loss of oestrogen affects tyrosine hydroxylase-immunoreactive (TH-IR) cell number in midbrain regions in rat and mouse and to determine which ER subtype contributes to the trophic effects of oestrogen on dopamine neurones. We used quantitative stereology to estimate TH-IR cell number in ovariectomised rats and mice replaced with E_2 , PPT or DPN, and estimated cell number in adult female α ERKO and β ERKO mice.

Materials and methods

Animals, surgery, hormone replacement and housing

Sprague-Dawley rats and C57Bl6/J mice provided by Charles River Laboratory (Raleigh, NC, USA) were used in all studies. In Experiment 1, female Sprague-Dawley rats ovariectomised or sham ovariectomised on postnatal day (PN) 60 ± 5 were purchased from Charles River Laboratories. We selected this age for gonadectomy based on previous studies in our laboratory suggesting that sex differences in cocaine-stimulated behaviour and dopamine uptake and release in rats are established by early adulthood (5,9,10,29). Animals were segregated by surgical condition and housed in plastic cages under a 12 : 12 h light/dark cycle with *ad lib* access to food and water. In a parallel study, female C57Bl/6 mice ovariectomised or sham ovariectomised at PN 60 ± 5 (Charles River Laboratories) were separated by surgical condition and housed in plastic cages under a 12 : 12 h light/dark cycle with *ad lib* access to food and water. Rats and mice were transcardially perfused with 10% neutral buffered formalin on PN 90, 30 days after surgery.

In Experiment 2, bilateral ovariectomy and sham surgery were performed at Duke (so that replacement could be started immediately) on adult rats (PN 60) under ketamine and xylazine (60 : 8 mg/kg) anaesthesia. For ovariectomy, two one-inch incisions at the level of the kidney and just off the midline of the dorsal surface were made, followed by two cuts through the muscle layer of the abdominal cavity exposing the ovaries. Each ovary was ligated and removed. The skin incision was closed with wound clips and treated with antiseptic. All animals received acetaminophen (500 mg/kg) before surgery and in the drinking water for 48 h after surgery. During surgery, a small incision was made at the nape of the neck above the right shoulder and an osmotic minipump was implanted (Alzet, Model 2004; Alza, Palo Alto, CA, USA, 0.25 μ l/h). Minipumps contained vehicle (50% dimethylsulphoxide/normal saline), E₂ (6 μ g/day) and ER β -selective agonist DPN (8 mg/kg/day) (30–32). Because studies showed that minipump delivery of the ER α agonist PPT did not provide adequate replacement even when the concentration was raised to the limit of solubility, PPT (2 mg/kg/day) was delivered by s.c. injection in sesame oil. PPT replacement animals were implanted with a minipump containing vehicle to make them surgically comparable to the minipump-replaced animals. A subset of controls received vehicle pumps and a daily s.c. injection of sesame oil vehicle. After surgery, animals were housed under previously described conditions until PN 90, when they were perfused 30 days after surgery. In a parallel study, female mice were sham or actively ovariectomised at PN 60 by the supplier (Charles River Laboratories) and shipped the next morning. Animals were given daily s.c. injections of sesame oil vehicle, E₂ (3 μ g), PPT (2 mg/kg) or DPN (8 mg/kg) starting the day after delivery. Animals in both studies were housed in plastic cages on ventilated racks under a 12 : 12 h light/dark cycle. Food and water were provided *ad lib*. Both rats and mice in the hormone replacement study were housed until PN 90, when they were perfused 30 days after surgery. Animals in Experiment 1 were received in the laboratory 1 week later than animals in Experiment 2 because the latter were shipped a week in advance of surgery (on PN 53). All animals were housed at Duke for 30 days between surgery and euthanasia. A final, smaller cohort of sham and ovariectomised rats were treated identically to animals described in Experiments 1 and 2 and were processed to contribute to the data showing the TH-IR and TH- immunonegative (-IN) cell counts detailed in Table 1.

In Experiment 3, female α ERKO and β ERKO mice and their C57Bl6/J controls were a generous gift from Dr Ken Korach at the National Institute of Environmental Health Sciences (NIEHS) (Research Triangle Park, NC, USA) (33–35). Animals were perfused at PN 85 with 10% neutral buffered formalin by NIEHS personnel. Animal care and housing were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH

publication 865-23) and approved by the Institutional Animal Care and Use Committee at Duke and NIEHS.

Tissue preparation and immunohistochemistry

Animals were deeply anaesthetised and transcardially perfused with 10% neutral buffered formalin. After perfusion, the brains were extracted and post-fixed overnight in 10% formalin. Brains were then equilibrated in a 30% sucrose cryoprotectant solution and stored at 4 °C. Serial coronal sections (30 µm) were cut on a cryostat and thaw-mounted onto slides. For rats, every third section was collected, and for mice, every second section was collected. Sections were allowed to dry overnight at 37 °C. Heat-mediated antigen retrieval was performed to increase immunoreactivity of the tissue for TH (36). Sections were pressure cooked (Deni electric pressure cooker; Keystone Manufacturing Company, Buffalo, NY, USA) at high pressure for 1 min and 30 s in citrate buffer (pH 6.0) (37). This length of time allowed for optimal staining without compromising cell morphology. Sections were rinsed in phosphate-buffered saline and incubated in 0.3% hydrogen peroxide–methanol for 30 min to quench endogenous peroxidase. Sections were then rinsed and blocked in 0.5% bovine serum albumin + 0.3% Triton X-100 for 15 min at room temperature. After blocking, sections were incubated in primary antibody diluted in blocking buffer (dilution 1 : 10000, Immunostar, Inc., Hudson, WI, USA) overnight at 4 °C. The specificity of this antibody has been verified by the supplier, which demonstrated a lack of cross reactivity with all other phenotypic proteins for catecholaminergic neurones (dihydropteridine reductase, dopamine β hydroxylase, phenylethanolamine-N-methyl-transferase) and the presence of expression in cells transfected with protein. Other laboratories have also verified specificity by western blotting (38). The next day, sections were rinsed and incubated in a biotinylated horse antimouse secondary antibody (dilution 1 : 1000; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The sections were then rinsed and incubated in avidin-biotin complex for 1 h at room temperature. The sections were rinsed and stained with diaminobenzidine (DAB) (Vector Laboratories). Sections were rinsed, dehydrated through graded alcohols, mounted and coverslipped. To estimate the number of cells that were TH-IN or lacking the DAB stain, sections in hormone-replaced animals were counterstained with 0.5% cresyl violet after DAB staining and coverslipped. Photomicrographs showing the stained material at low and high magnification in rat and mouse are presented in Fig. 1.

Unbiased stereology

Unbiased stereological estimation of the total number of TH-IR and TH-IN cell bodies in the SNpc and VTA was performed using the optical fractionator method (39). In rats, every third section was collected through the extent of the midbrain, and every sixth was section analysed for cell counting in a rostral–caudal fashion, resulting in a total of six to eight sections sampled for both the right and left sides of the brain. For mice, every second section was collected through the extent of the midbrain and every fourth was analysed for stereology, resulting in six or seven sections per animal. A computerised counting system containing a Nikon Optiphot-2 microscope (Nikon, Tokyo, Japan), a camera (Dage MTI, Michigan City, IN, USA) and motorised stage (Ludl Electronic Products, Hawthorne, NY, USA) was used to estimate the total number of cells. Each region of interest was projected onto a monitor, traced at low ($\times 4$) magnification and a sampling grid was superimposed on the traced region using StereoInvestigator software (MicroBrightField, Williston, VT, USA). After shrinkage, the final thickness of the sections used averaged 12 µm. Therefore, a 40×40 µm counting frame with a dissector height of 8 µm was used. Each counting frame was randomly spaced 80 µm apart and guard zones of 2 µm from the top and bottom of the section were used. Individual cell bodies were visualised with a $\times 100$ oil immersion lens (numerical aperture = 1.3). Cells that were stained for TH and at least 10 µm in diameter were counted as TH-IR. Cells that were stained with cresyl violet but not TH and were at

least 10 μm in diameter were counted as TH-IR. Sufficient cells were counted to achieve a coefficient of error that was ≤ 0.10 . The stereologer was blinded to all surgical and treatment groups for each experiment.

Cell size

To verify that neither surgery nor endocrine treatment influenced cell size, cell size was counted on a subset of animals from the replacement study. To estimate cell size, seven to ten representative TH-IR cells from the SNpc and VTA were randomly selected by sampling one section from the same location (rostral–caudal and lateral–medial) for each animal. All measured cells and sections were matched in their rostral–caudal location across animals. A $40 \times 40 \mu\text{m}$ counting frame was used for both rats and mice, with a sampling grid spacing of $200 \times 200 \mu\text{m}$ for rats and $120 \times 120 \mu\text{m}$ for mice. Because cell shape varies from spherical to ovoid, the ‘diameter’ was measured along the longest axis of TH-IR cells contained within the counting frame. The mean value of cell diameters from each animal was used to represent cell size from that animal. The mean $\pm$ SEM were computed for each experimental group (mean cell size from each animal constituted $n = 1$). The mean cell size in the SNpc and VTA were measured for all experimental groups for which the matching section was available ($n = 5$ per group for rats and $n = 3$ per group for mice).

Uterine weights

Uterine weights were collected to verify successful ovariectomy and replacement by ER α active compounds. After fixation of tissues by transcardial perfusion, uteri were removed. All fat and connective tissues associated with the uteri were removed prior to weighing.

Statistical analysis

All statistical analyses were performed using one-way ANOVA (NCSS, Kaysville, UT, USA) with $P < 0.05$ considered statistically significant. Cell number was analysed by one-way ANOVA with surgical treatment (sham, ovariectomised, E₂, DPT or DPN replaced) as the between subjects factor. Tissue weights were analysed by one-way ANOVA with endocrine state (sham, ovariectomised, E₂, DPT or DPN replaced) as the between subjects factor. Post-hoc analysis was performed using Fisher’s least significant difference tests to determine group differences. There was no difference between values obtained from minipump-implanted controls and s.c. injected controls, and so all values were combined.

Results

Effect of ovariectomy on TH-IR cell number in the midbrain

To determine whether the loss of ovarian hormones affects TH-IR cell number in midbrain regions, rats and mice were sham ovariectomised or ovariectomised at PN 60, and cells were counted 1 month post-surgery. Figure 2 shows representative high and low magnification images of SNpc from a representative sham and ovariectomised rat section that was stained for TH and counter-stained with cresyl violet. Ovariectomy at PN 60 decreased TH-IR cell number in the SNpc and VTA of female rats (Fig. 3) and mice (Fig. 4) at PN 90 relative to the sham surgery controls. In rats, ANOVA revealed a significant effect of ovariectomy in the SNpc ($F_{1,12} = 10.3$, $P < 0.01$) and VTA ($F_{1,12} = 5.8$, $P < 0.05$). In mice, ANOVA also revealed a significant effect of ovariectomy in the SNpc ($F_{1,10} = 19.8$, $P < 0.01$) and VTA ($F_{1,10} = 6.8$, $P < 0.05$).

Role of oestrogen and ER-selective agonists in the midbrain on TH-IR cell number

The effect of oestrogenic compounds on TH-IR cell number in midbrain regions was assessed in rats and mice ovariectomised at PN 60 and replaced with E₂, the ER α agonist

PPT or the ER β agonist DPN, respectively until PN90. Uterine weights were collected from rats and mice post-perfusion to determine the effectiveness of hormone replacements over the 1-month period (Fig. 5). In rats, ANOVA revealed a significant effect of treatment for uterine weight ($F_{4,44} = 25.5$, $P < 0.0001$). Post-hoc analysis revealed that the uterine weights of the sham-ovariectomised animals were greater than all other treatment groups. Treatment with E₂ and the ER α -selective agonist PPT significantly increased uterine weight relative to the vehicle-treated ovariectomised animals but not to the level of the sham controls.

Replacement with ER β agonist DPN had no uterotrophic effects, and uterine weight in DPN-treated animals was comparable to the ovariectomised animals. In mice, ANOVA revealed a significant effect of treatment ($F_{4,32} = 14.3$, $P < 0.0001$). Post-hoc analysis revealed that ovariectomy resulted in a decrease in uterine weight relative to the sham controls. E₂ and PPT (ER α) replacement increased uterine weight relative to the ovariectomised controls. However, E₂-replaced uteri were greater than the sham controls. DPN (ER β)-replaced uteri were not different from the ovariectomised rats.

Cell counts revealed that ER α and ER β -selective agonists maintained TH-IR cell bodies in the SNpc and VTA. In the rat midbrain (Fig. 6), ANOVA indicated a significant effect of treatment in the SNpc ($F_{4,43} = 6.6$, $P < 0.001$) and VTA ($F_{4,42} = 4.0$, $P < 0.05$). In the SNpc, post-hoc analysis showed ovariectomy decreased cell number relative to the sham controls. Cell counts in the E₂, PPT (ER α) and DPN (ER β)-replaced animals were comparable to the sham controls and different from the ovariectomised animals. In the VTA, a similar effect of ovariectomy and hormone replacement was observed with one exception. Post-hoc tests showed that sham ovariectomised and E₂ and PPT (ER α)-replaced groups were different from the ovariectomised animals. DPN (ER β)-replaced animals were not significantly different from ovariectomised rats.

ER agonists had similar effects in ovariectomised mice. Cell counts in the SNpc and VTA of hormone-replaced mice showed that TH-IR cell number was reduced after ovariectomy (Fig. 7). ANOVA indicated a significant effect of treatment in both the SNpc ($F_{4,32} = 3.7$, $P < 0.05$) and VTA ($F_{4,32} = 5.2$, $P < 0.01$). Post-hoc tests showed that ovariectomy decreased TH-IR cell number in the SNpc and VTA. In the SNpc, cell number in E₂, PPT and DPN replaced groups was greater than the ovariectomised animals. However, these counts were slightly lower than the sham controls. In the VTA, ovariectomy decreased TH-IR cell number. All replacement groups had cell counts comparable to the sham controls and were greater than the ovariectomised animals.

TH-IR cell number in transgenic mice

TH-IR cell number in the SNpc and VTA was estimated in adult wild-type, α ERKO and β ERKO mice to further elucidate the role of each ER subtype (Fig. 8). ANOVA revealed a significant effect of genotype in the SNpc ($F_{2,15} = 16.9$, $P < 0.001$) and VTA ($F_{2,15} = 11.3$, $P < 0.01$). Post-hoc tests showed that TH-IR cell number was reduced only in the α ERKO mice relative to wild-type.

Oestrogen does not regulate TH expression

Studies have shown that TH expression is regulated by oestrogen in the SNpc of neonatal mice and in the rat LC (40,41). Therefore, one possible explanation for the loss of TH-IR cells observed with ovariectomy could be that loss of oestrogen results in an inability to detect some TH-IR cells in the SNpc and VTA. To verify that the reduction in cell number observed with ovariectomy was not a result of the loss of TH expression in midbrain cells, we estimated cell number in the SNpc and VTA of sham and ovariectomised rats in sections counterstained with cresyl violet to determine the number of TH-IR cell bodies in each region. This analysis was conducted on a group of animals that included the sham and

ovariectomised animals from the data presented in Fig. 5 and additional animals that received identical sham and active ovariectomy, and these were processed identically for the purpose of conducting counts. For TH-IR cell number, ANOVA indicated a significant effect of ovariectomy in the SNpc ($F_{1,26} = 5.9$, $P < 0.05$) and VTA ($F_{1,26} = 4.9$, $P < 0.05$) (Table 1). Ovariectomy did not affect TH-IN cell number in the SNpc and VTA. For the total number of cells (TH-IR + TH-IN), ANOVA indicated a main effect of ovariectomy in the SNpc ($F_{1,25} = 17.5$, $P < 0.01$) and VTA ($F_{1,25} = 5.0$, $P < 0.05$). Post-hoc analysis revealed that TH-IR cell number and mean total cell number in both the SNpc and VTA decreased after ovariectomy. The percentage of TH-IR cells is similar in both groups suggesting that despite cell loss with ovariectomy, the percentage of TH-IR cells remains the same in rat.

Cresyl violet cell counts were also performed in the sham ovariectomised and ovariectomised mice from the hormone replacement study presented in Fig. 6 (Table 2). ANOVA revealed a significant effect of treatment in the SNpc ($F_{1,16} = 26.7$, $P < 0.001$) and VTA ($F_{1,16} = 52.2$, $P < 0.001$) for TH-IR cell number. No significant differences in TH-IN cell number in the SNpc and VTA between treatment groups were observed, which is consistent with the data presented for the sham and ovariectomised rats. For total cell number, ANOVA indicated that there was a main effect of treatment in the SNpc ($F_{1,16} = 9.0$, $P < 0.001$) and VTA ($F_{1,16} = 33.5$, $P < 0.0001$). For mice, the number of TH-IR and total cells was significantly lower after ovariectomy in the SNpc and VTA. As observed for rat, the percentage of TH-IR cells is similar in both groups, suggesting that, despite cell loss with ovariectomy, the percentage of TH-IR cells remains the same in mouse.

In Table 3, cresyl violet cell counts for the ERKO mice are presented. ANOVA indicated a significant effect of genotype in the SNpc ($F_{2,15} = 16.9$, $P < 0.001$) and VTA ($F_{2,15} = 11.3$, $P < 0.01$) as presented in Fig. 7. No significant difference in TH-IN cell number in the SNpc and VTA of any genotype was observed. Total cell number was significantly reduced only in the α ERKO mice in both the SNpc ($F_{2,14} = 25.0$, $P < 0.0001$) and VTA ($F_{2,15} = 12.7$, $P < 0.01$). No differences in the percentage of TH-IR cells were observed in either region.

Oestrogen does not regulate cell size

Ovariectomy and/or hormone replacement could theoretically influence cell size so much that TH-IR cells no longer met the size criteria we used to positively assign a cell as dopaminergic. To verify that this was not the case, we measured cell diameter in seven to ten cells at the same rostral/caudal and medial/lateral location to obtain an average cell size per animal, and computed means for five rats from each treatment group and for three mice from each treatment group. Preliminary analysis indicated that cell size did not vary with species, and so all the data were analysed by three-way ANOVA (species $\times$ treatment $\times$ region) with region as a repeated variable. These data are shown in Table 4. ANOVA indicated a significant effect of region ($F_{1,79} = 5.08$, $P < 0.027$) with cell in SNpc being larger than VTA. Cell size did not vary significantly by species or surgical/endocrine state. All the cell diameters were significantly above the size threshold for identification as dopaminergic (10 μ m).

Discussion

In the present study, we investigated the effect of oestrogen and the role of each ER subtype in the maintenance of TH-IR cell number in the rodent SNpc and VTA. The findings obtained show that ovariectomy reduces TH-IR cell number in the SNpc and VTA of adult female rodents. Replacement with E_2 , PPT (ER α -selective agonist) or DPN (ER β -selective agonist) in ovariectomised rodents reduced or prevented cell loss, suggesting that oestrogen is required for the maintenance of TH-IR cells and that both ER subtypes contribute to the trophic effects of oestrogen in the midbrain. However, studies in transgenic mice suggest

that dopaminergic neurone maintenance is more dependent upon ER α activation. This is first study to demonstrate the role of each ER subtype in the maintenance of midbrain TH-IR cell number.

There is an extensive literature suggesting that oestradiol is required for cell survival and neuroprotection of various cell types in the brain (42), although the effects on monoamine neurones have received little attention. One study in nonhuman primates showed that both short- and long-term ovariectomy reduces the number of TH-IR cell bodies in the SNpc and that immediate but not delayed oestrogen replacement prevents cell loss (18). Studies in rodents have yielded more inconsistent results. Oestrogen protects against neurotoxin (6-hydroxydopamine or MPTP)-induced dopamine and dopamine cell loss (21–23,25). However, ovariectomy alone has been reported to have no effect on TH-IR number (43–47). Comparisons of TH-IR number in SNpc and VTA of males and females have reported slightly greater numbers in gonadally intact males in some studies but not in others (45,46,48).

The present study showed that ovariectomy during early adulthood in both Sprague-Dawley rats and C57BL/6J mice results in a decrease in TH-IR cell number in the SNpc and VTA and that oestradiol replacement prevents this loss. Furthermore, both pharmacologic replacement with ER α and ER β agonists and estimation of TH-IR in α ERKO and β ERKO mice suggest a role for both oestrogen receptors in this process.

Several differences in experimental procedure could contribute to the divergent findings about the effects of ovariectomy on TH-IR in the SNpc and VTA, including the strain of animals, duration of oestrogen deprivation and the method used to estimate TH-IR number. The studies cited above, which reported negative findings, employed slightly shorter periods of oestrogen deprivation (from 10–21 days). If dopamine neurones die gradually, this could result in changes being detectable only after 1 month or more of oestrogen deprivation. Furthermore, shorter periods of oestrogen deprivation could reflect changes in TH expression, whereas longer periods of deprivation reflect cell loss, as reported in nonhuman primates (18). In addition, slightly different methods were employed to estimate TH neurone number, and it is possible that slightly different boundaries were applied to identify the SNpc and VTA. Strain differences could also contribute to differences from laboratory to laboratory in the observed effects of ovariectomy on dopaminergic cell number. Strain differences in dopaminergic neurone number have been reported in mice, although not in rats, and sex differences in many parameters have been shown to be strain specific (49,50).

The decrease observed in dopaminergic cell number could result from a decrease in TH expression in individual cells to levels below the limit of detection by the antibody. There are studies suggesting that oestrogen regulates TH expression, but only if ER α is present. Oestrogen regulation of TH expression in the midbrain has only been observed early in development (neonatal mouse SNpc) (40). Oestrogen regulation of TH has been observed in adults in other brain regions, especially the locus coeruleus, and, in one study, in the VTA but not in SNpc after prolonged administration such as that delivered in the present study (51–53). Furthermore, immunohistochemical and *in situ* hybridisation studies reveal that there is little or no ER α in the adult rodent SNpc (54–56). If TH is regulated by oestrogen, we would expect to see fewer TH-IR cells but more TH-IN cells in the midbrain of ovariectomised rodents. However, cell counts in cresyl violet counterstained sections revealed no differences in TH-IN cell number, but only in TH-IR cell number. This is consistent with the findings of the nonhuman primate study. We also found that approximately 85–90% of cells in the SNpc and VTA are TH-IR, which is consistent with studies that employed similar cell quantification methods in the SNpc (57). A recent study demonstrated that TH mRNA is decreased in the SNpc of α ERKO mice: the findings

obtained in the present study suggest that the diminished TH mRNA expression in that study likely reflects a loss of cells in the SNpc rather than direct regulation of TH transcription (58). The results from the present study with unbiased stereology suggest that the loss of TH-IR cells after ovariectomy reflects a loss of cell numbers, and not a loss of TH expression.

The ER α agonist PPT maintained neurone number in the SNpc and VTA. TH-IR cell number was also decreased in midbrain regions of α ERKO mice relative to wild-type. This finding is concordant with the literature suggesting that many neuroprotective effects of oestrogen are ER α -mediated and that ER α provides protection against neurotoxin-induced dopaminergic neurone degeneration (42). In primary cultures derived from mouse and rat, E₂ protects cells from 1-methyl-4-phenylpyridinium ion (MPP⁺)-induced degeneration (59). In a mouse MPTP model of PD, PPT mimicked the effects of E₂ in the protection of striatal dopamine content (15,25). α ERKO mice are more sensitive to the effects of MPTP than wild-type or β ERKO mice because striatal dopamine content is depleted at lower doses than in the other genotypes (26). However, one important caveat for the present findings is that it is impossible to distinguish between activational and organisational effects of oestrogen action in the α ERKO mice because they have been deprived of oestrogen for their entire lifespan. However, the consistency between the knockout findings and the effects of ER-selective agents drugs support a role for ER α in normal maintenance of dopaminergic neurones and suggest that activational effects at least contribute to the observed findings.

Although the present findings also support a role for ER β in maintaining dopaminergic neurones, the results were not as consistent. The ER β -selective agonist DPN maintained TH-IR cell number in the SNpc and VTA of both rats and mice. However, TH-IR cell number was not decreased in midbrain regions of β ERKO mice relative to the wild-type animals. The latter finding suggests that ER β may have a secondary role in the trophic effects of oestrogen on dopaminergic neurones. Pre-treatment with DPN failed to prevent depletion of striatal dopamine in a mouse PD model (25). In addition, treatment of primary midbrain cultures with DPN failed to rescue TH-IR cells from MPTP-induced degeneration (59). Nevertheless, other studies support a role for ER β in dopaminergic function. Aged β ERKO mice have smaller and fewer neurones in the SNpc compared to wild-type mice (60). Furthermore, a number of dopamine-specific proteins are regulated by ER β . Dopamine D₂ receptor and dopamine transporter specific binding are both reduced with ovariectomy and restored by DPN (14,26). A possible explanation for the differences in the role of ER β in the replacement and transgenic animal experiments may be that the dose of DPN was high enough to cause some ER α activation. Although having approximately 70-fold greater affinity for ER β , DPN can also activate ER α , which has been shown to occur at high doses (61). We administered a dose (8 mg/kg/day) of DPN that is reported to have protective and anti-inflammatory effects in mice (31,32). A study involving long-term replacement in mice receiving this dose of DPN showed that DPN was not uterotrophic (32). In the rat, we found that DPN did not have any oestrogenic effects in the uterus. Therefore, there is no evidence that ER α activation mediated by DPN occurred in the present study. Finally, it is possible that there are species or strain differences in ER β protection of dopamine neurones. All of the negative findings originate from studies in mice. Furthermore, the brain phenotype of different ERKO mice varies. Oestrogen protects against neurotoxin (MPTP or 6-hydroxydopamine)-induced damage in the parent strain used in the present study (C57BL/6J), and in a different ER α but not ER β knockout strain derived on this background (21–23,25,26). Therefore, the present findings are consistent with the reports in the literature suggesting that ER α has a more important trophic role for midbrain dopaminergic neurones than ER β .

The efficacy of ER-selective agonists and the effects in transgenic mice lacking ER α or ER β suggest that conventional genomic receptors at least contribute to trophic effects on dopaminergic neurones. However, the mechanism by which they do so is not clear because most studies have shown that only a small percentage of cells in midbrain regions express oestrogen receptors. *In situ* hybridisation and immunohistochemical studies show that there is low to no ER α protein and mRNA expression in the midbrain, whereas there is low to moderate expression of ER β in the SNpc and VTA of both rats and mice (55,62). Studies using ^3H -oestradiol revealed that few cells contained ERs (63). Only one study suggested that 40% of neurones in the SNpc express ER β (56).

If direct genomic effects of ER α and ER β are not likely to be the result of low to absent expression of these receptors in dopaminergic neurones, indirect effects via glia or other neurones could be involved. The effects of ER β could be mediated through glial cells such as astrocytes and microglia that may trigger the release of trophic factors promoting survival and protection of dopaminergic neurones (56,64,65). Finally, nongenomic effects of oestradiol could play a prominent role in its neuroprotective effects on dopaminergic neurones, although the receptor mediating such effects remains a matter of controversy (11,13,66,67). Although the results obtained in the present study clearly indicate a role for both oestrogen receptors in maintaining dopaminergic neurones, the mechanisms by which they do so are not yet clear.

In conclusion, the findings of the present study provide evidence that oestradiol is required for maintenance of TH-IR cells in the female midbrain and that both ER α and ER β play a role in the protection of these cell populations. The results also suggest that oestrogen mediates its protective effects through ER α but may also have trophic effects through ER β as well. The modulation of TH-IR cell number by oestrogenic drugs in midbrain regions, specifically the SNpc, may provide insight into the sex differences observed in psychostimulant drug-stimulated behaviour, dopamine uptake and release and neurotoxin-induced damage in rodents. The present study also has implications for the relative protection of women from PD. Finally, the finding that DPN is partially protective indicates that this selective oestrogen receptor modulator could open doors for hormone replacement treatments because DPN lacks agonist effects in reproductive tissues.

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Fig. 1. Low and high magnification photomicrographs of tyrosine hydroxylase staining in rat and mouse. (A) Rat substantia nigra pars compacta (SNpc) at $\times 5$. (B) Mouse SNpc and ventral tegmental area at $\times 5$. (C) Rat SNpc at $\times 100$. (D) Mouse SNpc at $\times 100$. Scale bars on top images = 400 μm . Scale bars on bottom images = 10 μm (For clarity, Fig. 1. is reproduced in the companion paper: *Journal of Neuroendocrinology* 2010; 22: 238–247).

Fig. 2. Low and high magnification of tyrosine hydroxylase staining with counterstaining with cresyl violet in sham and ovariectomized (OVX) female rats to illustrate the loss of cell bodies but not cell size after ovariectomy. (A) Sham substantia nigra pars compacta (SNpc) at $\times 10$. (B) OVX SNpc at $\times 10$. (C) Sham SNpc at $\times 100$. (D) OVX SNpc at $\times 100$. Scale bars on top images = 200 μm . Scale bars on bottom images = 10 μm .

Fig. 3. Effect of ovariectomy on midbrain tyrosine hydroxylase-immunoreactive (TH-IR) cell number in rats. The total number of TH-IR cells in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) of rats sham ovariectomised (OVX) and OVX at postnatal day (PN) 60 was estimated 1 month post-surgery. Data are expressed as the mean $\pm$ SEM, (n = 6 per group). *Statistically different from sham OVX for SNpc: $P < 0.01$ and for VTA: $P < 0.05$.

Fig. 4.

Effect of ovariectomy on midbrain tyrosine hydroxylase-immunoreactive (TH-IR) cell number in mice. The total number of TH-IR cells in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) of rats sham ovariectomised (OVX) and OVX at postnatal day (PN) 60 was estimated 1 month post-surgery. Data are expressed as the mean $\pm$ SEM, (n = 5 per group). *Statistically different from sham OVX for SNpc: $P < 0.01$ and for VTA: $P < 0.05$.

Fig. 5.

Effect of 17 β -oestradiol (E₂), propyl-pyrazole-triol (PPT) or diarylpropionitrile (DPN) on uterine weight in female rats (n = 6–12) and mice (n = 5–8) compared to vehicle-treated sham ovariectomised (OVX) and OVX controls. Animals were OVX or sham OVX at postnatal day (PN) 60 and treated with vehicle, E₂, PPT or DPN for 1 month post-surgery. Data are expressed as the mean $\pm$ SEM. *Statistically different from sham OVX; **statistically different from OVX: P < 0.0001.

Fig. 6. Effect of 17 β -oestradiol (E₂), propyl-pyrazole-triol (PPT) or diarylpropionitrile (DPN) on tyrosine hydroxylase-immunoreactive (TH-IR) cell number in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) of female rats compared to vehicle-treated ovariectomised (OVX) and OVX controls. Animals were OVX or sham OVX at postnatal day (PN) 60 and treated with vehicle or oestrogenic compounds for 1 month post-surgery. Data are expressed as the mean $\pm$ SEM (n = 7–12 per group). *Statistically different from sham OVX; **statistically different from OVX, for SNpc: (P < 0.01) and for VTA: P < 0.05.

Fig. 7.

Effect of 17 β -oestradiol (E₂), propyl-pyrazole-triol (PPT) or diarylpropionitrile (DPN) on tyrosine hydroxylase-immunoreactive (TH-IR) cell number in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) of female mice compared to vehicle-treated ovariectomised (OVX) and OVX controls. Animals were OVX or sham OVX at postnatal day (PN) 60 and treated with vehicle or oestrogenic compounds for 1 month post-surgery. Data are expressed as the mean $\pm$ SEM (n = 5–8). *Statistically different from sham OVX; **statistically different from OVX, for SNpc: P < 0.01 and for VTA: P < 0.05.

Fig. 8. Total tyrosine hydroxylase-immunoreactive (TH-IR) cell number in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) of ER α knockout mice (α ERKO) and ER β knockout mice (β ERKO) mice at postnatal day (PN) 85 compared to wild-type controls (WT). Data are expressed as the mean $\pm$ SEM (n = 5 per group). *Statistically different from wild-type: P < 0.01.

Table 1

Estimated Total Number of Tyrosine Hydroxylase-Immunoreactive (TH-IR) and TH-Immunonegative (-IN) Cells in the Substantia Nigra Pars Compacta (SNpc) and Ventral Tegmental Area (VTA) of Sham Ovariectomised (Sham OVX) and Ovariectomised (OVX) Rats.

Surgical condition	TH-IR	TH-IN	Total cells	% TH-IR
SNpc				
Sham OVX	22 274 ± 1350	3868 ± 624	26 141 ± 768	84.8 ± 2.5
OVX	17 876 ± 1198*	2816 ± 326	21 227 ± 1027*	85.6 ± 1.6
VTA				
Sham OVX	19 587 ± 1368	2673 ± 599	22 260 ± 1595	84.8 ± 2.5
OVX	16 106 ± 771*	2209 ± 327	18 314 ± 749*	85.6 ± 1.6

Ovariectomy decreased TH-IR cell number in the SNpc ($P < 0.05$) and VTA ($P < 0.05$).

Significant differences were found in total cell number in the SNpc ($P < 0.01$) and VTA ($P < 0.05$). Data are expressed as the mean ± SEM.

* $P < 0.05$ or better relative to sham OVX (n = 12–13 per group).

Table 2

Estimated Total Number of Tyrosine Hydroxylase-Immunoreactive (TH-IR) and TH-Immunonegative (-IN) Cells in the Substantia Nigra Pars Compacta (SNpc) and Ventral Tegmental Area (VTA) of Sham Ovariectomised (Sham OVX) and Ovariectomised (OVX) mice.

Surgical Condition	TH-IR	TH-IN	Total cells	% TH-IR
SNpc				
Sham OVX	4353 ± 337	654 ± 96	4807 ± 403	86.1 ± 2.5
OVX	2409 ± 168*	683 ± 54	3293 ± 307*	79.4 ± 5.1
VTA				
Sham OVX	5564 ± 291	657 ± 98	5930 ± 507	89.2 ± 1.8
OVX	3193 ± 152*	631 ± 40	4116 ± 301*	84.3 ± 1.9

Ovariectomy decreased TH-IR cell number in the SNpc ($P < 0.001$) and VTA ($P < 0.001$). Significant differences were found in total cell number in the SNpc ($P < 0.001$) and VTA ($P < 0.001$). Significant differences were also observed for % TH-IR cells, $P < 0.01$ for SNpc and $P < 0.001$ for the VTA. Data are expressed as the mean ± SEM.

* $P < 0.05$ or better relative to sham ovx (n = 8 per group).

Table 3

Estimated Total Number of Tyrosine Hydroxylase-Immunoreactive (TH-IR) and TH-Immunonegative (TH-IN) Cells in the Substantia Nigra Pars Compacta (SNpc) and Ventral Tegmental Area (VTA) of Oestrogen Receptor Knockout Mice.

Surgical condition	TH-IR	TH-IN	Total cells	% TH-IR
SNpc				
Wild-type	3685 ± 328	535 ± 63	4220 ± 299	86.6 ± 2.3
αERKO	1851 ± 55*	326 ± 81	2177 ± 301*	85.2 ± 3.4
βERKO	3023 ± 207	407 ± 47	3430 ± 124	83.7 ± 4.8
VTA				
Wild-type	3981 ± 437	491 ± 80	4472 ± 389	88.1 ± 3.3
αERKO	2372 ± 142*	561 ± 175	2932 ± 86*	81.3 ± 5.4
βERKO	4138 ± 208	437 ± 21	4575 ± 200	90.4 ± 0.8

TH-IR cell number was decreased in the ERα knockout (αERKO) mice relative to the wild-type and ERβ knockout (βERKO) mice in the SNpc ($P < 0.001$ for each) and VTA ($P < 0.01$ for each). Total cell number was decreased in αERKO in SNpc ($P < 0.001$) and VTA ($P < 0.01$). Data are expressed as the mean ± SEM.

* $P < 0.05$ or better relative to wild-type.

Table 4

Estimated cell diameter of Tyrosine Hydroxylase-Immunoreactive (TH-IR) Cells in the Substantia Nigra Pars Compacta (SNpc) and Ventral Tegmental Area (VTA) of Rats and Mice that were Ovariectomised (OVX) and Hormone-Replaced (n = 5 per Group for Rats and n = 3 per Group for Mice).

Surgical Condition	Rat		Mouse	
	SNpc	VTA	SNpc	VTA
Sham OVX	22.7 ± 1.1	19.8 ± 1.0	21.4 ± 2.7	22.4 ± 1.8
OVX	21.1 ± 1.5	18.5 ± 0.3	20.3 ± 0.9	18.4 ± 1.0
Oestradiol	21.9 ± 0.7	20.8 ± 0.5	22.1 ± 1.4	19.4 ± 1.4
PPT	23.4 ± 2.4	21.1 ± 1.2	20.8 ± 1.0	23.0 ± 1.0
DPN	24.1 ± 0.9	20.9 ± 1.4	21.4 ± 0.6	21.0 ± 0.5

Data are expressed as the mean ± SEM; n = 5 per group for rat treatment groups, and n = 3 per group for mouse treatment groups. PPT, propyl-pyrazole-triol; DPN, diarylpropionitrile.