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Sex dimorphisms in the neuroprotective effects of estrogen in an animal model of Parkinson's disease

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Abstract

The incidence of certain neurological disorders, including Parkinson's disease, appears to be more prevalent in men. Studies involving estrogen treatment of ovariectomised rodents attribute this largely to the neuroprotective effects of estrogen. However, a neuroprotective role for physiological levels of circulating hormones in males and females is less clear. Using the 6-hydroxydopamine (6-OHDA) model of Parkinson's disease to lesion the nigrostriatal dopaminergic (NSDA) pathway, we have shown that in females, endogenously produced estrogen is neuroprotective, whereas in males, gonadal factors increase striatal 6-OHDA toxicity. Intriguingly, estrogen, but not dihydrotestosterone, a nonaromatizable androgen, reversed the effects of orchidectomy on lesion size, raising the novel the hypothesis that enhanced male susceptibility may be attributable to the effects of endogenous testosterone only after its aromatization to estrogen. Thus, estrogen appears to exert opposite effects in the NSDA in males and females, being neuroprotective in females, but not in males, where it may even exacerbate neurodegenerative responses, with important implications for the clinical potential of estrogen-related compounds as neuroprotective agents. Preliminary experiments support the hypothesis that sex differences in the adult NSDA may result from the organisational actions of gonadal steroids during the critical neonatal period for the masculinization of the brain. Further studies are needed to determine whether this early organisation of a sexually differentiated neural circuitry may contribute to the emergence of neurodegenerative conditions such as Parkinson's disease.

Keywords: Estrogen; Sexual dimorphism; 6-OHDA lesions; Parkinson's disease; Neurodegeneration; Neuroprotection; Substantia nigra

1. Introduction

Cumulative evidence points to significant sex differences in susceptibility to certain neurological disorders. For example, Parkinson's disease, a neurodegenerative condition involving the nigrostriatal dopaminergic (NSDA) pathway, appears to be more prevalent in men compared with women (Schrag et al., 2000; Swerdlow et al., 2001). Men also appear to be more affected by schizophrenia (Hafner et al., 1998; Szymanski et al., 1995), a condition thought to have neurodevelopmental, as well as genetic, origins (Cotter and Pariante, 2002; Lewis and Lieberman, 2000). In addition, several studies have reported sex differences in the pathophysiology and outcome of a wide range of acute neurological insults, including ischaemia (Alkayed et al., 1998; Stein, 2001), drug-induced neurotoxicity (Jonasson et al., 2000; Sancewicz-Pach et al., 1999), and neurotrauma/head injuries (Roof and Hall, 2000), with women fairing better than men. While the mechanisms responsible for sex differences in these varied conditions are not clear, a common role for sex steroid hormones is strongly implicated. Clinical studies have shown, for example, that there is a marked worsening of parkinsonian symptoms following withdrawal of hormone replacement therapy in postmenopausal women (Sandyk, 1989), as well as at times of the menstrual cycle when estrogen levels are low (Quinn and Marsden, 1986). Endogenous estrogens have also been implicated in the relative protection from stroke reported in premenopausal women (Hurn and Macrae, 2000) and the onset of Alzheimer's disease in some women (Henderson et al., 2000). The view is thus emerging that estrogen is an

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important player in limiting neurodegenerative processes in the brain.

Experimental studies involving the estrogen treatment of ovariectomised female rodents provide further support that estrogen and selective estrogen receptor (ER) modulators can attenuate neuronal damage in models of CNS injury, which mimic conditions such as Parkinson's disease and stroke (Disshon and Dluzen, 1997; Dluzen, 1997; Dubal et al., 1998; Horstink et al., 2003; Hurn and Macrae, 2000; Watanabe et al., 2001). Few studies have, however, been performed in intact females, and fewer still have made direct comparisons of the potential neuroprotective effects of estrogen in both males and females. These studies are essential, however, if we are to establish whether physiological levels of endogenous sex steroid hormones have a neuroprotective capacity at physiological levels. They are important, also, if we are to assess the clinical potential for estrogen-related compounds to act as novel agents in neurodegenerative diseases and neuronal trauma.

In the present report, we will focus on a rat model of neurodegeneration using the toxin 6-hydroxydopamine (6-OHDA) to induce a lesion in the NSDA pathway. This results in the damage or death of dopamine (DA)-producing cells within the substantia nigra (SN) and a concomitant loss of dopaminergic terminals within the striatum, similar to that seen in Parkinson's disease. In particular, our work sought first to determine whether the normal sex steroid hormone environment in intact animals provides a relative level of protection against neuronal assault in females compared with males. Second, we sought to investigate whether sex differences in the response to the neurotoxin depended solely upon male–female differences in the circulating estrogen concentrations or whether androgens could also play a role.

2. The 6-OHDA-lesioned rat model of Parkinson's disease

Parkinson's disease is the second most common neurological disorder, affecting 1:500 in the UK. It is characterised by tremor, rigidity, bradykinesia and problems of locomotion and balance (primary symptoms), as well as depression, dementia and difficulties with speech and swallowing (secondary symptoms), but the underlying causes remain elusive, and no animal model can claim to reproduce this condition accurately. Of the available models, however, the lesions produced by central injections of 6-OHDA, which target the NSDA pathway (Schwarting and Huston, 1996), bear many features in common with the human disease. These include oxidative stress, cell loss, infiltration of reactive microglia, excitotoxicity and increased iron deposition in the cell bodies of the substantia nigra pars compacta (SNc). As estrogens have the potential to interfere with many of these processes, we have adopted this model to investigate the neuroprotective influences of estrogen and androgens in male and female rats.

3. Experimental design and procedures

3.1. Animals

Age-matched male and female Sprague–Dawley rats (60±5 days of age, bred from a closed, specific pathogen-free colony) were housed in groups of five per cage under controlled lighting (lights on 8.00–20.00 h, 22±1 °C), with food and water freely available. Daily vaginal smears were obtained from the female rats, and only those displaying three regular 4-day estrous cycles were included. In the first series of experiments in the gonad-intact animals, we investigated sex differences and the influence of the estrous cycle on toxin susceptibility using graded doses of 6-OHDA. The severity of the lesion was quantified using two measures viz: the depletion of DA in the striatum and the survival of DA cells in the SN to investigate the differences at the level of both nerve terminals and perikarya. Further studies aimed to investigate whether sex differences in the neuronal assault were estrogen and/or androgen dependent. This was determined by administering female levels of estrogen and male levels of androgen to both the male and female gonadectomised (GDX) rats. These rats were then challenged with a submaximal dose of 6-OHDA, and lesion size was compared with that seen in animals that were gonad intact. Every effort was made to minimise both suffering of the animals and the numbers used. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

3.2. 6-OHDA lesions

For the sex difference study in gonad-intact animals, the females were selected at proestrus, when high physiological levels of circulating estrogen are present, to provide the greatest contrast to the male hormonal environment. For studies on estrous cycle effects, the intact females were compared at diestrus, when estrogen levels are low, and at proestrus. As described in detail elsewhere (Murray et al., 2003), unilateral lesions of the left NSDA pathway were produced under anaesthesia by injection of 6-OHDA hydrobromide (dissolved in 4 μl of 0.9% saline containing 0.1% w/v ascorbic acid; Sigma, UK) or vehicle control into the medial forebrain bundle (MFB) according to the following coordinates: 2.2 mm anterior to bregma, 1.5 mm lateral (left) of bregma and 7.9 mm ventral to the dura (Fig. 1). The animals were allowed to recover and were monitored daily until preoperative weights were restored or exceeded, and the females resumed normal cycling activity.

3.3. Hormone manipulations

Under halothane anaesthesia, bilateral gonadectomies or sham operations were performed in males and females followed by subcutaneous implantation of a pellet (Innovative Research of America, USA) containing either 0.5 mg of

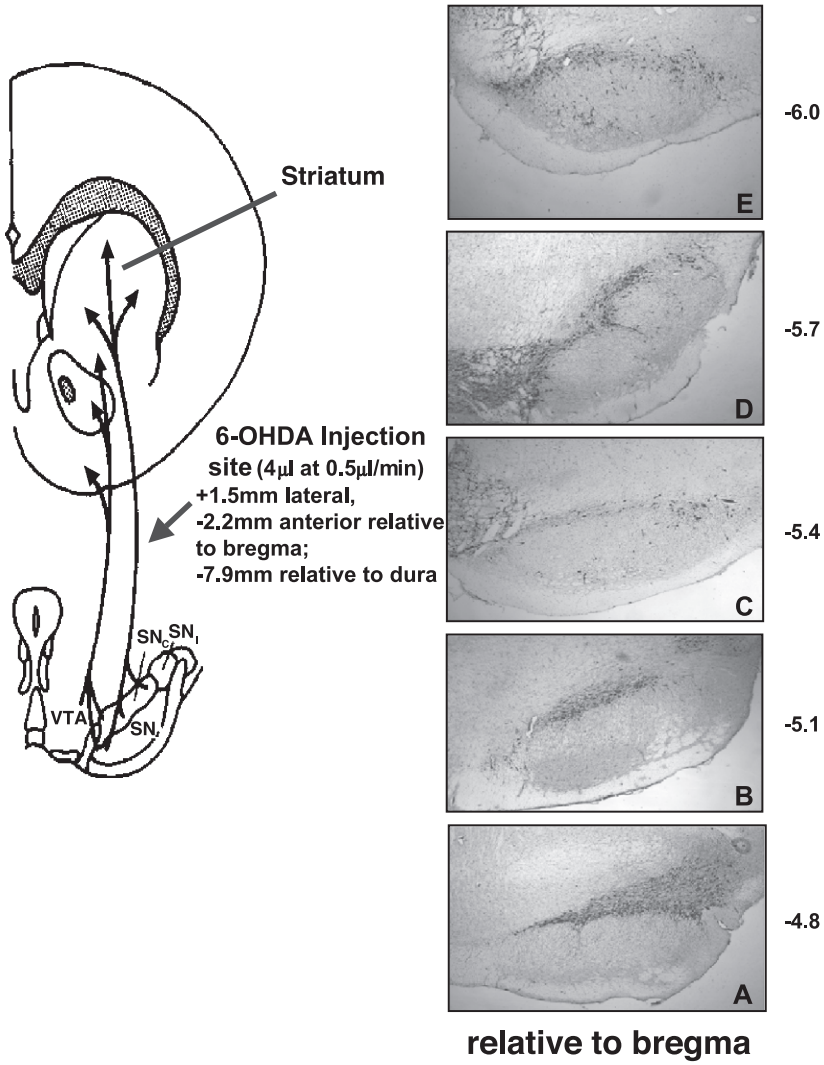

Fig. 1. Unilateral 6-OHDA model of Parkinson's disease. Schematic representation of placement of the lesions in the left MFB (left panel) and tyrosine hydroxylase immunocytochemical measurements through five levels of the SN (right panel).

17 β-estradiol (E_2—designed to replace continuous circulating hormone levels to that seen in the morning of proestrous for a 21-day period) or 1.5 mg of 5 α-dihydrotestosterone (DHT—designed to replace circulating androgen levels to that seen in normal adult male rats for a 21-day period) or vehicle control (Murray et al., 2003). Approximately 1 week after surgery, all animals received a unilateral injection of a submaximal dose of 6-OHDA (1 μg/4 μl) into the MFB, and tissue was collected as described below.

3.4. Tissue preparation and analysis

Two weeks after lesioning, when the cycling females were again in proestrus, the animals were decapitated. Brains were rapidly removed and cut coronally at the level of the infundibular stem (Bregma−4.16) to form a forebrain block containing the striatum and a hindbrain block incorporating the SN.

3.4.1. Substantia nigra

The hindbrain blocks were immediately placed in 4% paraformaldehyde and cryoprotected in 20% sucrose in PBS and were snap frozen in isopentane, as described in detail elsewhere (Murray et al., 2003). Using a polyclonal anti-TH antibody (Chemicon, UK), immunocytochemistry was carried out on free-floating sections and visualised using an ABC Vector Stain kit (Vector Laboratories, UK) as described. The number of TH+ cells in the control (right, unlesioned) and lesioned (left) SNc were counted at five levels (A–E, sections located −4.8, −5.1, −5.4, −5.7 and −6.0 mm with respect to bregma, Fig. 1), according to the method of Carman et al. (1991), using an image analysis software package (Image Proplus, Media Cybernetics). The total number of TH+ cells in 4/5 sections was counted (both lesioned and unlesioned sides) at each of the five levels. The mean cell count at each level for each animal within a group was pooled to provide the group mean (n=6).

3.4.2. Striatum

Left (lesioned) and right (unlesioned) striata were scooped out of the forebrain blocks and stored separately at −80 °C until analysis for DA and its metabolites using high-performance liquid chromatography coupled with electrochemical detection (HPLC-EC, Biotech Instruments, U.K.; Murray and Gillies, 1997; Murray et al., 2003). In studies involving striatal DA transporter site quantification, forebrains were flash frozen in cold isopentane and stored at −80 °C (Datla et al., 2003). Specific binding of the highly selective [^{125}I]RTI-121 compound (NEN, UK) was assessed in 20-μm sections after exposure to precalibrated FUJI BAS imaging plates read on a phosphoimager.

3.5. Data analysis

Preliminary studies confirmed that placement of 6-OHDA or vehicle injections on the left side had no significant effect on the DA populations on the right side. Thus, the extent of the toxin-induced lesion was calculated as the DA content or TH+ cell numbers in the lesioned side expressed as a percentage of that in the contralateral side, a convention widely used elsewhere (Schwarting and Huston, 1996). Results are presented as the mean±S.E.M. for each treatment group. The groups were analysed for statistical differences using two-way analysis of variance to determine the effect of dose and sex on the extent of the lesion. For the hormone treatment studies, multiple comparisons were made using the Student–Neuman–Keuls post hoc procedure. In all cases, a P value of <.05 was considered to be statistically significant.

4. Sex differences in susceptibility of the NSDA pathway in gonad-intact rats

While DA content in the control rodent striatal tissue extracts are identical in males and females (Murray et al., 2003; Wagner et al., 1993), Fig. 2 shows that the 1 μg dose of 6-OHDA produced a significantly greater depletion in striatal DA in males (48.7%) compared with females (27.6%). At the higher doses of neurotoxin, which produced a graded increase in lesion size, sex differences were lost, suggesting a loss of relative resistance in the female brain as the degree of neuronal insult increases. Smaller lesions in the striatum of female mice compared with males have also been reported after peripheral injection of the DA neurotoxins, methamphetamine and 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) (Miller et al., 1998; Yu and Wagner, 1994). While the marked gender differences in liver microsomal enzyme activity and drug metabolism could contribute to these differences (Becker, 1990), one study suggests that there are no sex differences in the striatal levels of the active metabolite (MPP+) of the peripherally administered toxin (Miller et al., 1998). Nonetheless, our study, employing a central administration of 6-OHDA and

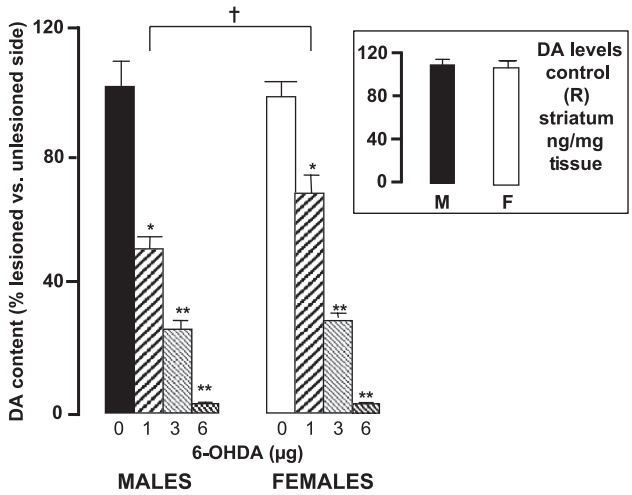

Fig. 2. Dose- and sex-dependent reduction of DA content in the striatum of adult male and female rats in response to graded doses of 6-OHDA injected into the left MFB. Absolute DA levels were similar in the right striata of males and females that were either untreated (insert) or had received an injection of vehicle or 6-OHDA into the left striata (data not shown; see Murray et al., 2003). Results are therefore presented as the percentage of DA levels remaining in the lesioned compared with the nonlesioned (right) side. Values represent mean±S.E.M. (n=6). *P<.05, **P<.01 for the effect of 6-OHDA vs. vehicle. †P<.05 for male vs. female comparisons. (Adapted from Murray et al., 2003).

another involving direct striatal infusion of MPP+ (Disshon and Dluzen, 2000), provides direct evidence for a sex difference in toxin susceptibility directly at the level of the NSDA neurone.

A greater susceptibility to 6-OHDA-induced lesions in males is seen also at the level of the cell body for both the 1- and 3-μg dose (Fig. 3). This is perhaps surprising, considering the finding that males have significantly more TH+ cells in the control unlesioned striata compared with females (Fig. 3, insert). Together, these observations might suggest the existence of a heterogeneous population of DA cells, with females possessing a relatively greater proportion of toxin-resistant cells and/or males having a greater prevalence of toxin-susceptible cells. Analysis across different levels of the SN provides further evidence for a sex difference in patterns of cell loss and vulnerability (Fig. 4). Thus, in response to 3 μg of 6-OHDA, males showed a similar extent of loss across all five regions at the time-point studied, whereas a gradient of decreasing survival was seen in females from level A, where survival rate was double that of the male, through E, where survival rate was similar in males and females (Murray et al., 2003). Notably, the level that exhibited the greatest cell survival in females was closest to the point of injection, suggesting that differences in survival were not simply due to a concentration gradient consequent on the diffusion of toxin after injection into the MFB. It would thus appear that in females, cells in the medial SN may be more resistant to degeneration than those found more caudally. Similar regional differences in cell loss have been reported in rodent studies (Carman et al., 1991), as well as in parkinsonian patients

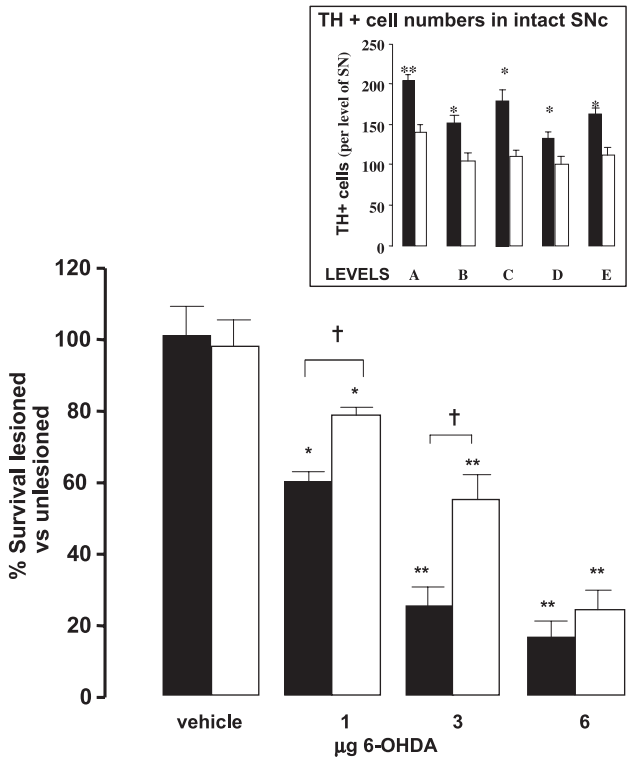

Fig. 3. Dose- and sex-dependent effects of graded doses of 6-OHDA injections into the left MFB on TH+ cell numbers in adult male (■) and female (▭) rats. Results are expressed as percent survival of cells in the lesioned SNc compared with the unlesioned side. Identical effects were seen at Levels A–E (Murray et al., 2003), and the data shown here represent average values for all SNc levels. Values represent mean ± S.E.M. ($n=5$ or 6). * $P<.05$, * * $P<.01$ for the effect of 6-OHDA vs. controls for males or females. $^{\dagger}P<.05$ for male vs. female comparisons. Insert: While cell counts reveal no significant effects of injection (6-OHDA or vehicle) into the left MFB on the absolute cell counts in the unlesioned (right) SNc, the absolute cell number for Levels A–E were significantly greater in males compared with females ($n=24$). * $P<.05$, * * $P<.01$ for males vs. females. (Adapted from Murray et al., 2003).

(Bannon and Whitty, 1997; Damier et al., 1999). It remains to be seen whether the heterogeneity of TH+ cell types could account for the sexually dimorphic prevalence of Parkinson's disease in the human population.

Although sex differences in the magnitude of 6-OHDA-induced lesions were seen at the level of the cell body for both the 1 µg and 3 µg dose (Fig. 3), this was apparent at the level of the nerve terminals only at the 1 µg dose. The reasons for these discrepancies are not clear, but they may implicate sex differences in the complex compensatory mechanisms that develop in the degenerating NSDA pathway. Although not yet clearly defined, such mechanisms are thought to explain an absence of motor defects with partial lesions, as seen in animal models and patients suffering from the early stages of Parkinson's disease (Anglade et al., 1995; Bezard et al., 2003; Finkelstein et al., 2000; Song and Haber, 2000; Zigmond and Hastings, 1998). They include changes in DA turnover, neuronal activity and expression of the dopamine transporter (DAT), as well as nondopaminergic mechanisms, which could be activated to different degrees in males and females. In support of this, it would appear that many of these potential compensatory mechanisms are already operating at a different level in normal males and females. For example, it has been proposed that similar tissue (Fig. 2) and extracellular DA levels in the male and female rat striata (Robinson et al., 1990; Walker et al., 2000) are maintained by a different balance of uptake and release processes and a greater DA terminal density in females. These differences translate into functional sex differences, as seen by exaggerated behavioural responses to cocaine (locomotor activity), amphetamine (stereotypy, rotational response) or apomorphine (stereotypy) in female rats compared with males (reviewed in Becker, 1999). It is possible that they may also contribute to the mechanisms that underlie sex differences in response to neurodegenerative stimuli within the NSDA pathway.

5. Influence of gonadal factors and sex steroid hormones in adult rats

Many measures of dopaminergic activity in the striatum change over the estrous cycle, suggesting modulation by gonadal steroid hormones (Becker, 1999). These include the behavioural responses listed above and extracellular striatal DA release, all of which are greater at proestrus than at diestrus. We have recently shown that 6-OHDA toxicity is also highly dependent on the endogenous gonadal hormonal status at the time of injection of the toxin. Specifically, we have shown that injection of 6-OHDA in the morning of proestrus, when estrogen levels are high (Fig. 5, insert) and the expected afternoon rise in progesterone has not occurred, results in a significantly smaller NSDA lesion compared with injection of toxin at diestrus, when both estrogen and

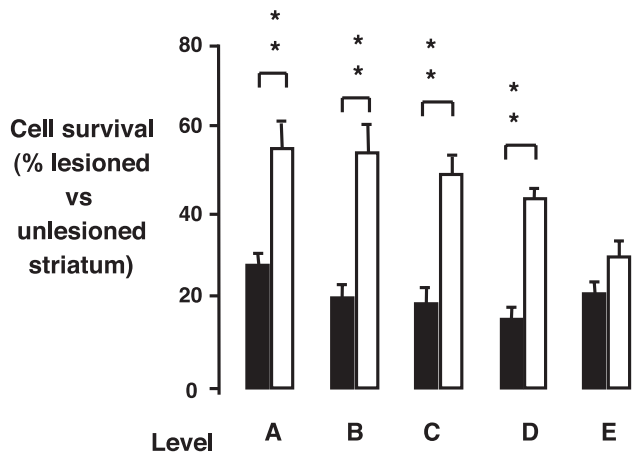

Fig. 4. Region-specific loss of TH+ cells within the SN of adult male (■) and female (▭) rats in response to the 3-µg dose of 6-OHDA. Results are expressed as the percentage survival of cells in the lesioned striatum compared with the unlesioned, contralateral side. The percentage cell survival was significantly (* * $P<.001$) greater in the female SN at Level A compared with the male, whereas in more caudal levels of the SN, females experienced a much smaller percent cell survival, which was not significantly different from that seen in the male. (Adapted from Murray et al., 2003).

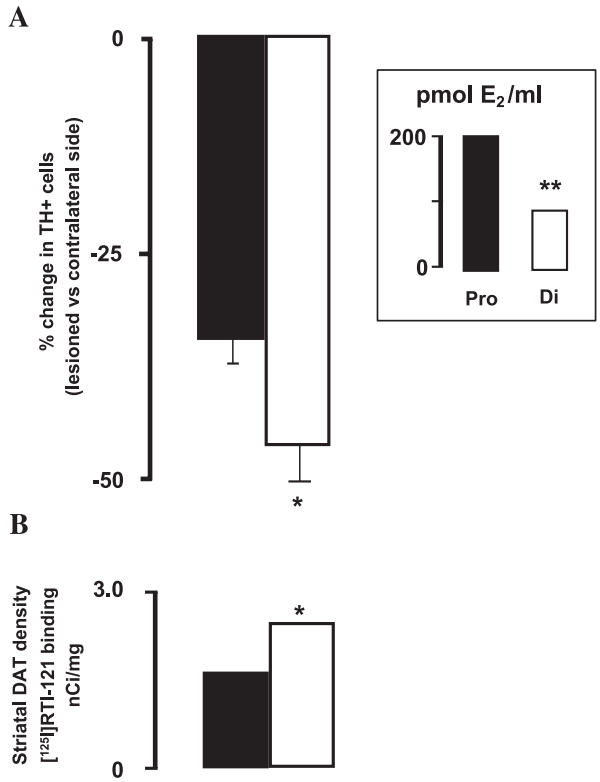

Fig. 5. Effect of estrous cycle. (A) Percentage cell loss in the lesioned compared with the unlesioned SNc 1 week after unilateral injection of 3 μg 6-OHDA is greater at diestrus (□)compared with proestrus (■). (B) This correlates with a greater density of [^{125}I]RTI-121 binding sites in the striatum at dioestrus compared with proestrus. * $P<.05$, * * $P<.02$. Insert: Circulating levels of estrogen at diestrus and proestrus. (Adapted from Datla et al., 2003).

progesterone levels are low (Fig. 5A). These findings support the view that estrogen is neuroprotective and are in agreement with the report that methamphetamine striatal toxicity also varies over the estrous cycle (Yu et al., 2000). To exert its neurotoxic effects, 6-OHDA has to enter the cell via the DAT, selectively expressed in DA neurones, where its metabolism generates damaging hydroxyl radicals and it inhibits mitochondrial complex I and cellular respiration. The greater expression of DAT at diestrus (Fig. 5B) might thus contribute to the greater toxicity of 6-OHDA at this point in the estrous cycle. Rat studies have also shown that after ovariectomy striatal DAT is up-regulated (Attali et al., 1997) and estrogen replacement attenuates DA uptake in vivo (Thompson, 1999), supporting the view that a direct suppressive role of estrogen at transporter sites may contribute to the neuroprotective ability of this hormone in females.

To determine more precisely the roles of gonadal hormones on estrous cycle and sex differences in the unilateral 6-OHDA lesion model of Parkinson's disease, we have made comparisons of responses to partial lesions produced by 1 μg neurotoxin in intact (sham-operated), GDX and hormone-replaced animals. In the control, unlesioned striata, these hormonal manipulations had no effect on DA content in males or females (Murray et al., 2003).

5.1. Females

Striatal DA depletion in response to unilateral injections of 1 μg 6-OHDA lesions increased from around 20% in intact female rats to 50% in animals that had previously been ovariectomised (Fig. 6), and this effect was reversed by the replacement of estrogen to proestrus levels. We conclude, therefore, that the neuroprotective effects of gonadal factors in the female are due primarily to physiological levels of circulating estrogen. In contrast, some studies in mice have failed to observe an effect of ovariectomy on MPTP-induced lesion (Yu and Wagner, 1994). However, others, using ovariectomised mice or rats, agree with our finding that estrogen has a neuroprotective capacity in females (Dluzen, 1997; Dluzen et al., 1996a).

5.2. Males

In marked contrast to our results in the female, gonadectomy of the male reduced striatal DA loss caused by the 1 μg dose of 6-OHDA from around 50% to 25%

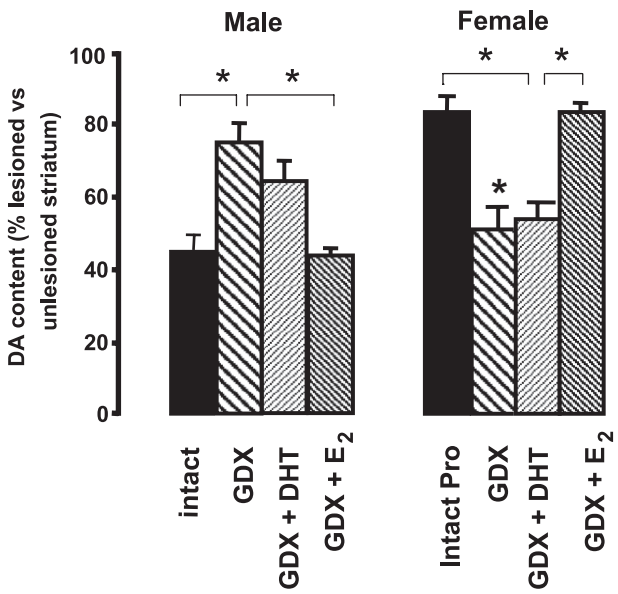

Fig. 6. Effect of manipulation of the adult hormonal environment on striatal DA content in response to unilateral injections of 1 μg of 6-OHDA into the left MFB. As altered hormonal status did not affect DA content in the control, unlesioned striata (Murray et al., 2003), results represent the percentage striatal DA remaining in the lesioned striatum compared with the contralateral, unlesioned side. Sham-GDX animals are represented by the solid bars (■). Following orchidectomy (▨), the levels of DA in the male striatum were significantly greater than in their sham-GDX counterparts. This effect was unaffected by treatment with dihrdrotestosterone (DHT) (▨) but was reversed by treating the males with physiological levels of 17 β-estradiol E_2 (▨). Following ovariectomy (▨), the levels of DA in the female stratum were significantly reduced compared with their GDX counterparts. This effect was unaffected by treatment with DHT (▨) but was reversed by treating the females with physiological levels of E_2 (▨). * $P<.05$ for percent DA content in GDX and GDX-DHT treated animals compared with sham-operated animals, * $P<.05$.

(Fig. 6). Thus, by their ability to oppose neuroprotective capacity in males but to enhance it in females, gonadal factors are likely to be the principal determinants of sex differences in neurodegenerative responses to neurotoxins. Studies in male mice, however, found that the striatal toxicity of methamphetamine (Yu and Wagner, 1994) and MPTP (Dluzen, 1996) was unaffected by gonadectomy. In contrast, another study found that testosterone treatment of orchidectomised male CD-1 mice decreased L-DOPA-evoked striatal DA release in vitro compared with a no-testosterone group (Dluzen et al., 1994), supporting our view that testosterone may increase the degree of neurodegeneration elicited by NSDA toxins. Apart from obvious differences in species and toxin, it should be considered that differences in the metabolizing enzymes between intact and GDX animals may well influence the central toxicity of these peripherally administered drugs.

Perhaps, surprisingly, we found that the effects of gonadectomy, to reduce lesion size in the male, were reversed by treatment with estrogen but not DHT (an androgen that cannot be converted to estrogen by endogenous aromatases). These findings therefore raise the possibility that the greater susceptibility of the NSDA pathway in males to neurotoxin may be engendered by endogenous testosterone only after its conversion to estrogen by aromatase enzymes, which are known to be present in the striatum. In contrast to these deleterious effects of estrogen in males, a protective effect of estrogen on MPTP-induced striatal DA depletion has been claimed in GDX (Dluzen et al., 1996b) and ageing (Grandbois et al., 2000) male as well as female mice. Equally, however, there are reports that estrogen can protect against methamphetamine toxicity in female but not male mice (Gao and Dluzen, 2001), yet, tamoxifen, a tissue-specific ER antagonist, appears to be neuroprotective (Dluzen et al., 2001). Many questions thus surround the physiological importance of estrogen in the chemically injured male brain. A systematic approach is therefore needed to resolve the differences in strain, species and toxin, as well as the bioavailability of various ligands (Disshon and Dluzen, 2000; Maggs et al., 1992) and their relative binding to the alpha and beta forms of the ER (Harris et al., 2002) in male and female brains. A further complexity may be added by the suggestion that there are two functionally distinct populations of DA neurones whose basal discharge rates react in opposition upon exposure to estrogen (Chiodo and Caggiula, 1983). Moreover, there is evidence to suggest that exogenously administered estrogen, while being protective for one population of cells, may actually be toxic to another (Scallet, 1999; Zsarnovszky et al., 2000). It is thus feasible that sex differences in the influence of estrogen on neurodegenerative responses may be attributable to differences in the relative distribution of specific subpopulations, which yet need to be clearly defined.

6. Organisational influences of gonadal factors and steroid hormones in the neonate

Our work described above has demonstrated that in the absence of circulating gonadal steroids, there are sex differences in response to estrogen as far as toxin-induced degeneration of mesencephalic dopaminergic neurones is concerned. Gonadectomised animals also exhibit functional sex differences in these populations, as reflected, for example, in basal- and amphetamine-stimulated striatal DA release (Becker, 1999). Together, these findings suggest an inherent sexual dimorphism in the organisation of this brain region, irrespective of the activational influences of the adult sex hormone environment. Moreover, studies using a tyrosine hydroxylase promoter-Lac-Z transgenic mouse model to monitor TH gene transcription in vivo suggest that there is a sex difference in the hormonal regulation of TH gene activity, at least in the locus coeruleus (Thanky et al., 2002). Thus, in a manner reminiscent of the effects of gonadectomy and estrogen treatment in our 6-OHDA Parkinson's disease model, gonadectomy reduced transgene expression in females but increased it in males, and estrogen reversed these effects in both sexes. While the reasons for the sexually differentiated activational effects of estrogen in the brain are unknown, we propose that they have their origins in the early neonatal period, when sex differences in the endogenous hormonal environment are thought to underlie sexual differentiation of the brain. This hypothesis is well substantiated in the hypothalamus, which is the most extensively studied region with respect to sexually dimorphic organisation, which contains DA nuclei that exhibit sex differences (Simerly, 1989). It states that the transient perinatal rise of testosterone production by the male testes permanently masculinizes an essentially 'neutral' or bipotential brain, but this can occur only after the circulating testosterone has been converted to estrogen by aromatase enzymes in the brain. At this stage of development, it is thought that the brain is protected from fetal and maternal estrogens. Therefore, to examine whether similar factors operate differentially on the organisation of the NSDA pathway, we investigated the effects of neonatal gonadectomy +/− treatment with DHT or estrogen on adult DA neurones, using treatment regimes outlined in Table 1 and described in detail elsewhere (Simonian et al., 1998). Sham-operated animals treated with vehicle acted as controls.

We found no effects of these hormonal manipulations during the first few days of life on the TH+ cell numbers in the adult SNc compared with sham-operated and vehicle-treated controls. However, unlike adult gonadectomy, which had no significant effect on DA levels in control, unlesioned striata (Fig. 6 legend), neonatal gonadectomy significantly reduced striatal DA content (Table 1). This effect was reversed by neonatal treatment with DHT but not estrogen. These data therefore support the view that the mesencephalic DA pathways are subject to the perinatal organisational actions of sex hormones and raise the novel hypothesis that

Table 1
Effect of gonadal steroid hormone manipulations in newborn male rats on adult striatal levels of DA and DOPAC

Neonatal treatment	DA (pg/mg tissue)	DOPAC (pg/mg tissue)	DOPAC/DA×100
Sham operation+V (n=5)	350±8.5[a]	96.75±2.4[a]	27.7±0.7[a]
GDX+V (n=9)	235.9±30.3[b]	145.7±20.6[b]	76.15±17.1[b]
GDX+EB (n=4)	192.3±46.7[b]	172.7±26.9[b]	111.8±31.8[b]
GDX+DHT (n=6)	415.2±23.7[a]	102±7.7[a]	25±2.4[a]

Under halothane anaesthesia, male rats were GDX on Postnatal Day 1 and given daily subcutaneous injections of vehicle (V, 50 μl sesame oil) or vehicle containing estradiol benzoate (EB; 1 μg/50 μl oil; n=5) or dihydrotestosterone (DHT; 50 μg/50 μl oil; n=5) until Postnatal Day 5. Each of these treatment regimens has been described previously for the manipulation of gonadal steroid levels in the neonatal rat (Crowley and Kalra, 1994; Shughrue and Dorsa, 1994). Values with different superscript letters in each column are significantly different.

the masculinisation of the DA pathways may operate via androgen receptor-mediated mechanisms rather than through the ER. While we do not yet know what implications this may have for neurotoxic responses to 6-OHDA, a preliminary report by Dluzen and Anderson (2003) suggests that neonatal gonadectomy does not substantially alter the neuroprotective capacity of estrogen against methamphetamine-induced striatal DA toxicity in adult mice. Interestingly, however, sex differences in adult striatal DA depletion often reported in this mouse model (Wagner et al., 1993; Yu et al., 2000) were abolished by gonadectomy on postnatal day 20 (Yu et al., 2002). Moreover, the ability of estrogen to protect against methamphetamine toxicity in adult mice that were ovariectomised at 6 weeks of age appears to be lost when ovariectomy is performed at 4 weeks of age (Yu et al., 2002). Together, these data suggest that the neural substrate that underlies sex differences in the sensitivity to methamphetamine DA neurotoxicity, at least in C57BL/6J mice, may develop as late as 4–6 weeks postpartum and that the organisational effects of gonadal steroids may well influence sex differences in neuroprotective capacity in the adult brain. It would thus appear that the developmental window when sex steroid hormones can establish sex differences in NSDA neurones may extend beyond the classical period for sexual differentiation of the brain, which is thought to begin in late gestation and extend into the second week of life.

7. Summary and conclusions

Our studies have provided direct evidence that sex differences in the activational effects of gonadal steroids on NSDA neurones are responsible, at least in part, for the gender-related differences in susceptibility to the neurotoxin. Degeneration of the NSDA pathway is thought to be the principal pathophysiological feature in Parkinson's disease, which occurs with a greater prevalence in men. Our data thus support the view that the prevailing hormonal environment may contribute to disease susceptibility by virtue of protective effects in females, but deleterious effects in males. Furthermore, our findings strongly suggest that there are sex differences in the mechanisms whereby the NSDA neurones respond to injury and estrogen.

In agreement with numerous other reports, our data indicate that estrogen is the principal neuroprotective gonadal hormone in females. Findings in males are, however, much more variable. They range from indications that estrogen may be neuroprotective against MPTP-induced lesions (Dluzen et al., 1996b) or have no effect against methamphetamine (Gao and Dluzen, 2001) to our data suggesting that estrogen may even worsen the capacity of the NSDA pathway to respond to injury. Together, these observations caution that the reported clinically beneficial effects of estrogen in females may not be universally adopted as a line of treatment in males. This has important implications for the development of novel neuroprotective therapeutic strategies for Parkinson's disease, especially as estrogen ranks among 12 compounds that have recently been identified as attractive candidates for further clinical trials in Parkinson's disease (Ravina et al., 2003).

The experiments in which the neonatal hormonal environment was manipulated provide preliminary evidence that masculinisation of the mesencephalic DA neurones may involve early influences of sex steroid hormones. The finding that neonatal testosterone treatment influences striatal DA-dependent pacing behaviours in rats (Gans and Erskine, 2003), which are related to reproductive success (Becker, 1999), also provides functional evidence that perinatal testosterone programs these DA populations. Further studies are needed to determine whether this early organisation of a sexually differentiated neural circuitry may contribute to the emergence of neurodegenerative conditions such as Parkinson's disease.

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