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Androgens exacerbate motor asymmetry in male rats with unilateral 6-hydroxydopamine lesion

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by dopamine neuron loss in the nigrostriatal pathway that shows greater incidence in men than women. The mechanisms underlying this gender bias remain elusive, although one possibility is that androgens may increase dopamine neuronal vulnerability to oxidative stress. Motor impairment can be modeled in rats receiving a unilateral injection of 6-hydroxydopamine (6-OHDA), a neurotoxin producing nigrostriatal degeneration. To investigate the role of androgens in PD, we compared young (2 months) and aged (24 months) male rats receiving gonadectomy (GDX) and their corresponding intact controls. One month after GDX, rats were unilaterally injected with 6-OHDA, and their motor impairment and asymmetry were assessed 2 weeks later using the cylinder test and the amphetamine-induced rotation test. Plasma samples were also collected to assess the concentration of testosterone and advanced oxidation protein products, a product of oxidative stress. GDX decreased lesion-induced asymmetry along with oxidative stress and increased amphetamine-induced rotations. These results show that GDX improves motor behaviors by decreasing motor asymmetry in 6-OHDA-treated rats, an effect that may be ascribed to increased release of striatal dopamine and decreased oxidative stress. Collectively, the data support the hypothesis that androgens may underlie the gender bias observed in PD.

Keywords

Testosterone; Oxidative Stress; Aging; Gonadectomy; Substantia Nigra; Motor Function

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Introduction

Male gender and age are risk factors for Parkinson's disease (PD) (Gillies et al., 2004; Hanrott et al., 2006; Hofman et al., 1989; Lotharius and Brundin, 2002; Mayeux et al., 1995; Schrag et al., 2000; Van Den Eeden et al., 2003), as well as for developing parkinsonism symptoms following focal ischemic lesions of the striatum (Demirkiran et al., 2001; Fenelon and Houeto, 1997; Friedman et al., 1986; Horner et al., 1997; Kulisevsky et al., 1996; Murrow et al., 1990; Thanvi et al., 2005; Tolosa and Santamaria, 1984; van Zagten et al., 1998; Zijlmans et al., 2004).

Clinical characteristics of PD include motor impairments, such as tremor, rigidity, asymmetry, and bradykinesia (Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Forno, 1996; Mouradian, 2002). The primary pathological feature of PD is the loss of dopamine neurons within the substantia nigra pars compacta of the midbrain (Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Dietz et al., 1981; Forno, 1996; Hanrott et al., 2006; Lotharius and Brundin, 2002; Shastri, 2003), and several studies point to oxidative stress as a key player in the progressive degeneration of dopamine neurons (Gille et al., 2004; Hanrott et al., 2006; Hirsch et al., 1997; Jenner, 2003; Kanthasamy et al., 2003; Lotharius and Brundin, 2002).

Oxidative stress increases with age (Beal, 1998), and aged brains have reduced antioxidant capacity, decreased free radical scavenging enzymes, and enhanced sensitivity to free radical damage (Mo et al., 1995; Stark and Pakkenberg, 2004). Oxidative stress has been shown to initiate the apoptotic pathway in animal models of PD (Heikkila and Cohen, 1971; Kumar et al., 1995; Oishi et al., 1993; Przedborski et al., 1992). Interestingly, testosterone, the major male androgen hormone, can increase cellular apoptosis in dopaminergic neurons (Cunningham et al., 2009; Estrada et al., 2006).

Androgens have both neuroprotective (Hammond et al., 2001; Nguyen et al., 2005; Ramsden et al., 2003) and toxic effects, (Gavrielides et al., 2006) depending on the biological system studied and the concentration of hormone used (Hammond et al., 2001; Ramsden et al., 2003). The neuroprotective properties of testosterone may be ascribed to its conversion to estradiol, which has neuroprotective actions *in vitro* on dopamine neurons (Bains et al., 2007; D'Astous et al., 2004; Morissette et al., 2007). Testosterone can also be reduced to dihydrotestosterone (DHT) through the enzyme, 5 α -reductase (Edinger and Frye, 2005; Luttge, 1979; Roselli and Resko, 1993). Androgens, testosterone and DHT, have been shown to protect against kainic acid neurotoxicity (Ramsden et al., 2003), oxidative stress, and nutrient-induced toxicities (Hammond et al., 2001). Furthermore, androgens can activate different intracellular pathways, such as the ERK/MAPK, which are known to play a role in neuroprotection (Nguyen et al., 2005; Ramsden et al., 2003). On the other hand, androgens can also initiate apoptosis, which is intricately involved in neurotoxicity (Estrada et al., 2006). Interestingly, loss of dopamine receptors have been found in the midbrain of animal models treated with androgens (Kindlundh et al., 2001), and we previously showed that androgens can induce oxidative stress in dopamine neurons in culture (Cunningham et al., 2009). Therefore, these observations suggest that testosterone participates in the progressive neurodegeneration process observed in PD.

The role of androgens in conferring susceptibility to PD in men is currently unclear. Although androgen levels decrease with age in both men and women (Hollander et al., 2003), it is worth pointing out that they continue to remain higher in males (Gavrilova and Lindau, 2009). Androgens may explain the increased vulnerability of dopaminergic neurons to oxidative stress insult in males, which is among the factors that are thought to underlie the aging and PD (Hanrott et al., 2006; Harman, 1981; Hirsch et al., 1997; Lotharius and

Brundin, 2002). Therefore, the purpose of our study was to determine if androgens further increase dysfunction in substantia nigral dopamine neurons previously subjected to oxidative stress, such as occurs with age or ischemia. To test this hypothesis, we used gonadally intact (INTACT) and gonadectomized (GDX) aged (24 months) and young (2 months) male rats receiving a unilateral injection of the oxidative stressor 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle (MFB).

6-OHDA, which is selectively up-taken by dopamine neurons via the dopamine transporter (DAT), induces an oxidative stress insult leading to apoptotic cell death (Kumar et al., 1995; Mazzio et al., 2004; Soto-Otero et al., 2000) and an almost complete lesion of the nigrostriatal pathway (Deumens et al., 2002). As aged rats show a more extensive loss of nigrostriatal neurons in response to 6-OHDA than young rats (Cass et al., 2002; Marshall et al., 1983), we used a lower concentration of 6-OHDA (2 ug) to assess dopamine-dependent behavioral differences between INTACT and GDX male rats.

Methods

Animals

Gonadally intact 2 (young) and 24 (aged) month old male F344/BN F1 hybrid rats were purchased from the National Institute of Aging (through Harlan Laboratories, Indianapolis, IN). Since our study focused on the effects of hormones on neurotoxic insult to dopamine neurons, we used the F1 hybrid male rats because of their low tendency for developing tumors, especially testicular tumors, which can alter hormone levels and consequently confound data interpretation (Holmes, 2004; Spangler et al., 1994). Animals were housed in a temperature-controlled room (23°C) in standard Plexiglas cages (25 × 20 × 18 cm), and had *ad libitum* access to food and water. Lighting was maintained on a 12:12 light/dark cycle, with lights off at 18:00h. Animals were habituated to housing conditions one week prior to experimentation and divided into three different experimental groups (n=6 per group) according to the following variables: 1) Hormone concentrations, with two levels: gonadectomy (GDX) and gonadally intact (INTACT); 2) Age with two levels: Young and Aged; 3) Lesion with three levels: 2 ug 6-OHDA, 4 ug 6-OHDA, and SHAM lesion. Animal care and experimental procedures were performed following the National Institutes of Health's guidelines for animal care and use, Animal Welfare Act, and US Department of Health and Human Service "Guide for the Care and Use of Laboratory Animals". The protocol was approved by the University of Texas Health Science Center at San Antonio Animal Care and Use Committee and supervised by animal care personnel and veterinary staff.

Gonadectomy

One month prior to the 6-OHDA lesion, gonadectomy by a midline scrotal incision was conducted under 2% isoflurane anesthesia (Vedco, Inc, St. Joseph, MO) in half the animals, while the rest remained gonadally intact. In gonadectomized males, the spermatic cord was tied off with sterile surgical suture proximal to the testes and the cord was cut distal to the thread. The testes were removed and the incision closed with wound clips (Harding and McGinnis, 2003).

Unilateral 6-OHDA Lesion of the Medial Forebrain Bundle (MFB)

Administration of 6-hydroxydopamine hydrochloride (6-OHDA) into the MFB is a well-established animal model of oxidative stress-induced degeneration of dopamine neurons (Blum et al., 2001; Deumens et al., 2002; Kirik et al., 1998). Rats received an injection of the noradrenaline transporter inhibitor, desipramine (25 mg/kg i.p.) thirty minutes before surgery. For 6-OHDA treatment, rats were anesthetized with 2% isoflurane and positioned

into the stereotaxic frame (Kopf Instruments, Tujunga, CA). For lesion operated rats, 6-OHDA (2 ug/ul or 4 ug/ul) (Sigma Chemicals, St. Louis, MO) dissolved in 0.1% ascorbate saline was injected into the left MFB (AP -4.3, mediolateral +1.6, DV -8.3, relative to Bregma and midline in mm) at a flow rate of 0.5 ul/min using a 10 ul Hamilton microsyringe with a 32 gauge needle (Figure 1A). Sham operated rats received 0.1% ascorbate saline alone. Animals were allowed to recover for two weeks following surgery (for details see (Cenci and Lundblad, 2007; Morgese et al., 2009). The location of the lesion was assessed histologically post-mortem.

Behavioral Tests

The majority of substantia nigra pars compacta cell loss occurs two weeks after the 6-OHDA lesion (Sauer and Oertel, 1994). At this point, the following behavioral tests were carried out between 12:00 and 16:00 under dim red lighting and videotaped.

A. Cylinder Test and Motor Assessment—The cylinder test was used to examine spontaneous motor behaviors, motor impairment, and asymmetry. Animals, without a habituation period, were placed in a clear glass cylinder (6.5 cm diameter, 29 cm height) for ten minutes. The frequency of left or right forelimb placement against the glass wall during rearing was quantified and the frequency of spontaneous ipsi- or contra-lateral 360° rotations was also examined (Schallert and Tillerson, 1999). Total motor behavior was calculated as total ipsi- and contra-lateral forelimb use + total number of ipsi- and contra-lateral rotations. Motor impairment and behavioral asymmetry were assayed by examining the motor function of the contralateral (right) forelimb and the ipsilateral (left) rotational behavior, respectively (Deumens et al., 2002; Schallert and Tillerson, 1999). These behaviors were quantified using the following equations: 1) percent of contralateral forelimb use = (right forelimb use/total forelimb use) * 100 and 2) percent of ipsilateral rotations = (number of left 360° ipsilateral rotations/total 360° ipsi- and contra-lateral rotations) * 100.

B. Amphetamine-Induced Ipsilateral Rotations—We used amphetamine-induced ipsilateral rotations as an indirect marker for striatal dopamine release when comparing GDX to INTACT rats (Dluzen and Ramirez, 1989; Shemisa et al., 2006). Animals were injected with *d*-amphetamine (2.5 mg/kg, i.p.) (Sigma Chemicals, St. Louis, MO) and drug-induced ipsilateral rotations were observed for 10 minutes, starting 30 minutes after injection. As previously reported (Ungerstedt, 1971), unlesioned rats do not show ipsilateral rotations in response to amphetamine challenge.

Bioassays

Change in Body Weight—Animals were weighed prior to the 6-OHDA lesion and amphetamine injections. Percent change in body weight was calculated using the following equation: (weight at amphetamine injection – weight at 6-OHDA lesion)/(weight at amphetamine injection + weight at 6-OHDA lesion) * 100. This measure was used as an overall indicator of animal health.

Plasma Collection—Upon completion of behaviour testing, animals were lightly anesthetized with isoflurane and sacrificed by decapitation. Trunk blood (5–7 ml) was collected into chilled centrifuge tubes containing 50 ul heparin sodium (American Pharmaceutical Partners, Inc, Los Angeles, CA). The blood was centrifuged for 10 minutes (4 g) at 4° C. After centrifugation, plasma was promptly separated, placed in microcentrifuge tubes (Fisher, Pittsburgh, PA), and stored at -80° C until testosterone and oxidative stress analysis.

Testosterone measurements—Plasma testosterone levels were determined by Testosterone ELISA, using a rabbit anti-testosterone antibody (BioVendor), according to manufacturer's instructions. The intra-assay coefficient of variation was 7.9% and the inter-assay coefficient of variation was 7.3%. The sensitivity of this assay is 0.022 ng/ml. Specificity of this assay is as follows: testosterone (100%), 5 -DHT (5.2%), androstenedione (1.4%), androstanediol (0.8%), progesterone (0.5%), and androsterone (0.1%).

Oxidative Stress—Plasma advanced oxidation products (AOPP) are proteins, predominantly albumin, damaged by oxidative stress (Kalousova et al., 2005). AOPP was quantified by a colorimetric assay, according to manufacturer's instructions (Cell Biolabs). AOPP content was determined by comparison to a chloramine standard curve.

Immunohistochemistry

Brains were removed and fixed in 10% Formalin. After fixation, the brain was placed in a stainless steel rat brain slicer matrix and sliced in 1.0 mm coronal sections. Sections containing the substantia nigra were cut into 8 μ m slices with a microtome and paraffin-embedded. Tissue was mounted on glass slides, deparaffinized in xylene for 10 minutes, and rehydrated in 100%, 95% and 70% ethanol for 10 minutes each. Slides were heated at 100 C in sodium citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) for 30 min. After 1-hour incubation in 10% goat serum in SuperBlock T20 buffer (ThermoFisher), slides were incubated with anti-tyrosine hydroxylase (TH⁺) primary rabbit antibody (#AB152, Millipore, 1:50 dilution) at 4°C overnight. Slides were subsequently washed 3 times with PBS and incubated for 3 hours with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (#87-9963, Invitrogen, 1:500 dilution) at room temperature in the dark. Slides were washed 3 times with PBS and developed with 3, 3'-Diaminobenzidine (DAB) (Invitrogen), according to manufacturer's instructions (Li et al.). Slides were dehydrated by 10-minute incubations in 70%, 95%, and 100% ethanol followed by xylene, mounted with Permount (ThermoFisher), and imaged with a 4X objective on an Olympus BX51 upright light microscope equipped for epifluorescence and connected to a digital Olympus DP70 camera. TH⁺ staining was quantified using the NIH Image J program. Images were converted to 8 bit and inverted. Corrected Integrated Density for each region of interest was calculated by the following equation: Integrated Density of region of interest – (area of region of interest * mean gray value of the background) (Gavet and Pines, 2010).

Statistical Analysis

All data were analyzed using the StatView 5.0 software and presented as mean $\pm$ SEM. Measures were collected according to a 2 $\times$ 2 $\times$ 3 (GDX and INTACT $\times$ Young and Old $\times$ 6-OHDA-2 μ g, 6-OHDA-4 μ g, and Sham) design and analyzed by ANOVA followed by the Fisher's Protected Least Significance Difference (PLSD) test for post hoc comparisons when appropriate. The Pearson's correlation coefficient was calculated to determine the degree of linear relationship of total motor activity, amphetamine-induced rotations, and AOPP levels to testosterone, followed by a Fisher's r to z transformation to determine probability level. P values $\leq$ 0.05 were designated as significant, and p $\leq$ 0.10 indicated a non-significant trend.

Results

Figure 1 shows tyrosine hydroxylase (TH⁺) staining of dopamine neurons in the substantia nigra pars compacta. As expected, 6-OHDA caused a significant loss of TH⁺ neurons, as indicated by decreased TH⁺ staining, in the ipsilateral hemisphere compared to the contralateral hemisphere ($F = 21.870$; $p \leq 0.05$). No changes in TH⁺ staining between the ipsilateral and contralateral hemispheres were observed in sham-operated controls (Figure 1B). ANOVA applied to percent change in body weight revealed a significant main effect

for the factors Hormone ($F = 16.686$; $p \leq 0.05$) and Age ($F = 6.957$; $p \leq 0.05$). 6-OHDA did not alter body weight. GDX young rats gained significantly less body weight than INTACT young rats, regardless of lesion ($p \leq 0.05$) (Figure 1C).

Total spontaneous (ipsi- and contra-lateral) motor activity was not altered by 6-OHDA. ANOVA applied to total motor behaviors revealed a significant main effect for the factor Age ($F = 14.378$; $p \leq 0.05$) and a trend for the factor Hormone ($F = 2.809$; $p \leq 0.10$). Young male rats had significantly higher motor activity than aged male rats ($p \leq 0.05$). Among young sham-operated rats, GDX tended to decrease motor behaviors compared to INTACT ($p \leq 0.10$) in Figure 2A.

ANOVA applied to plasma testosterone levels revealed a significant main effect for the factors Hormone ($F = 22.607$; $p \leq 0.05$) and Age ($F = 7.542$; $p \leq 0.05$), and an interaction between the factors Hormone and Age ($F = 7.542$; $p \leq 0.05$). Plasma testosterone levels were not altered by 6-OHDA treatment. Young rats had significantly higher plasma testosterone levels than aged animals ($p \leq 0.05$), and GDX significantly decreased testosterone levels ($p \leq 0.05$) in Figure 2B. Testosterone was positively correlated with total motor activity in both experimental groups (Figure 2C; Pearson $r = 0.616$; $p \leq 0.05$).

The unilateral 6-OHDA lesion produced motor impairment and asymmetry in all experimental groups (Figure 3). ANOVA revealed a significant main effect of the factor Lesion on the percent of contralateral forelimb use ($F = 89.178$; $p \leq 0.05$). 6-OHDA treated males used their contralateral paw significantly less than sham-operated males. Neither Hormone nor Age altered lesion-induced motor impairment in 6-OHDA-treated males, as assessed by measurements of contralateral forelimb use (Figure 3A). Both 2 ug and 4 ug 6-OHDA concentrations produced similar motor impairments in young rats (Figure 3B).

ANOVA applied to the percent of ipsilateral rotations revealed a significant main effect for the factor Lesion ($F = 5.533$; $p \leq 0.05$) and an interaction between the factors Hormone and Lesion ($F = 4.615$; $p \leq 0.05$). 6-OHDA significantly increased ipsilateral rotations in INTACT males compared to Sham-operated males, regardless of age ($p \leq 0.05$). In young males receiving 2 ug of 6-OHDA, GDX significantly improved motor asymmetry by decreasing ipsilateral rotations compared to INTACT males ($p \leq 0.05$) in Figure 3C. ANOVA applied to the effects of 2 ug 6-OHDA and 4 ug 6-OHDA on ipsilateral rotations found a significant interaction between Lesion and Hormone ($F = 4.267$; $p < 0.05$). The GDX-dependent improvement of motor asymmetry in young animals was lost when higher concentrations of 6-OHDA (4 ug) were used, resulting in a significant increase of ipsilateral rotations in GDX 4 ug 6-OHDA treated males compared to GDX 2 ug 6-OHDA treated males ($p \leq 0.05$) (Figure 3D).

In animals receiving 2 ug 6-OHDA, ANOVA applied to amphetamine-induced rotations revealed a significant main effect for the factor Hormone ($F = 13.692$; $p \leq 0.05$). GDX significantly increased amphetamine-induced rotational behaviors compared to INTACT rats, regardless of age ($p \leq 0.05$) (Figure 4A). ANOVA applied to the effects of 2 ug 6-OHDA and 4 ug 6-OHDA on amphetamine-induced rotations found a significant main effect for the factor Hormone ($F = 10.321$; $p \leq 0.05$). GDX significantly increased amphetamine-induced rotational behaviors compared to INTACT rats ($p \leq 0.05$). In young rats, the higher concentration of 6-OHDA (4 ug) had no effect in the GDX group. No differences were found between GDX and INTACT 4 ug 6-OHDA treated rats (Figure 4B). The increase in amphetamine-induced rotations was negatively correlated with plasma testosterone levels (data not shown; Pearson $r = -0.05$; $p \leq 0.05$).

ANOVA indicated a significant main effect for the factors Age ($F = 5.602$; $p \leq 0.05$) and Hormone ($F = 5.034$; $p \leq 0.05$) on AOPP levels. A significant interaction between Age and

Lesion ($F = 6.231$; $p \leq 0.05$) was found. Aged males had significantly higher levels of AOPP than young males, indicative of increased global oxidative stress ($p \leq 0.05$). 6-OHDA lesion significantly increased AOPP levels in young males compared to sham-operated males ($p \leq 0.05$). GDX significantly decreased AOPP levels in young 6-OHDA males compared to INTACT ($p \leq 0.05$) (Figure 5).

Discussion

In this study, we showed that GDX decreased 6-OHDA-induced asymmetry and oxidative stress and increased amphetamine-induced rotations, indicative of increased striatal dopamine transmission. Our results show that GDX improves motor asymmetry in 6-OHDA-treated rats, an effect that may be ascribed to increased dopamine release in the striatum and decreased oxidative stress.

6-OHDA did not alter total motor behavior, body weight, and plasma testosterone levels, indicating that the overall health of the animals was not affected. However, young GDX rats gained significantly less body weight than INTACT young rats (Woodward, 1993). Consistent with previous reports, intact aged males showed less spontaneous motor activity and lower testosterone levels than INTACT young males. Total motor behavior was positively correlated with plasma testosterone levels, suggesting that male hormones can increase motor activity. Although previous reports found that testosterone reduces motor activity (Beatty et al., 1982; Dluzen et al., 1986; Savageau and Beatty, 1981), testosterone replacement has been shown to reverse the hypomotility observed in GDX males (Adler et al., 1999; Hollander et al., 2003; Tamas et al., 2006).

Motor impairment and asymmetry is characterized by low contralateral forelimb use and high ipsilateral rotations, and the degree of dopamine depletion is negatively correlated with contralateral forelimb use (Tillerson et al., 2001). In this study, rats receiving 6-OHDA used their non-impaired ipsilateral forelimb almost exclusively. Neither age, hormone status, nor 6-OHDA concentration altered the frequency of non-impaired forelimb use, a behavioral phenotype occurring after a unilateral 6-OHDA lesion causing a depletion of dopamine neuron greater than 95% (Schallert and Tillerson, 1999).

Interestingly, GDX was able to improve motor asymmetry in rats. These results differ from previous studies that found no behavioral differences between gonadally intact and gonadectomized males (Tamas et al., 2006). The observed effect of GDX can be attributable to the lower 6-OHDA concentration (2 ug) used in our experiments compared to those carried out by Tamas (2006). Indeed, when our young rats were exposed to a higher concentration of 6-OHDA (4 ug), we also did not observe any behavioral differences between the Intact and GDX groups. A possible mechanism underlying the gonadectomy-mediated improvement of motor asymmetry is a testosterone-induced decrease of dopamine release in the striatum. In keeping with this hypothesis, half of the TH⁺ neurons in the substantia nigra express androgen receptors (Heritage et al., 1981; Kritzer, 1997; Kritzer and Creutz, 2008). Further, recent reports found that gonadectomy increases TH⁺ staining in the substantia nigra, as well as dopaminergic innervation of the striatum, two effects that can be reversed by testosterone administration (Creutz and Kritzer, 2004; Johnson et al., 2010; Kritzer, 2000; Kritzer et al., 2007).

When the nigrostriatal pathway is unilaterally damaged, animals exhibit a circling behavior contralateral to the hemisphere that has the greatest dopamine activity (intact side) (Glick et al., 1977; Moore and Kelly, 1978; Ungerstedt, 1974). In unilaterally denervated animals, amphetamine can only induce dopamine release in the intact hemisphere, resulting in rotations ipsilateral to the damaged hemisphere (Ungerstedt, 1971). Previous studies carried

out in males failed to report any changes in amphetamine-induced rotations in response to gonadectomy (Camp et al., 1986). By contrast, our study indicates that gonadectomy can increase amphetamine-induced rotations in males, only if the animals are treated with a low dose of 6-OHDA. At the higher 6-OHDA concentrations (4 ug), no significant differences were found between GDX and INTACT males, as previously reported. These behavioral differences between GDX and INTACT male rats may be ascribed to a higher sensitivity of dopamine neurons to testosterone in the latter group.

Gonadectomy has been shown to increase striatal dopamine release in males (Dluzen and Ramirez, 1989; Shemisa et al., 2006), and to protect striatal dopamine content from 6-OHDA insult (Gillies et al., 2004; Murray et al., 2003). Further, testosterone has been shown to produce a dose-dependent increase in the degree of striatal dopamine depletion in response to methamphetamine (Lewis and Dluzen, 2008). Similarly, testosterone replacement in mice receiving MPTP, which also induces dopamine cell loss in the substantia nigra, blocked the gonadectomy's protective effect on striatal dopamine (Dluzen, 1996; Ekue et al., 2002). These studies suggest that gonadal hormones modulate the activity of the nigrostriatal pathway. As amphetamine-induced rotational behavior reflects striatal dopamine release in the intact hemisphere (contralateral to the 6-OHDA lesion) (Glick et al., 1977; Moore and Kelly, 1978; Ungerstedt, 1974), and in our study, gonadectomy increased amphetamine-induced rotations, we infer that gonadectomy also increased dopamine release from nigrostriatal neurons in the undamaged hemisphere and the few remaining nigrostriatal neurons in the damaged hemisphere. Amphetamine-induced rotations were negatively correlated with plasma testosterone, indicating that striatal dopamine release is reduced by androgens. These effects of gonadectomy on dopamine release may modulate the protective effects of gonadectomy on motor asymmetry.

Another mechanism that could mediate androgens effects on motor asymmetry in 6-OHDA-treated male rats is oxidative stress. Oxidative stress has been shown to initiate apoptosis in animal models of PD (Heikkila and Cohen, 1971; Kumar et al., 1995; Oishi et al., 1993; Przedborski et al., 1992). In addition, previous studies showed that androgens can increase apoptosis in dopamine neurons (Cunningham et al., 2009; Estrada et al., 2006) and are positively correlated with oxidative stress levels (Cunningham et al., 2009). In 6-OHDA-treated rats, cellular markers of oxidative stress in the brain increase within 24 hours of the lesion and return to baseline levels within seven days (Henze et al., 2005; Smith and Cass, 2007). Therefore, rather than investigating possible causative agents of oxidative stress, we decided to measure products of oxidative stress, such as AOPP levels. Consistent with prior studies, we found that aged sham-operated males had higher levels of AOPP than young sham males (Aksenova et al., 1998; Calabrese et al., 2004; Mo et al., 1995; Stark and Pakkenberg, 2004). Interestingly, gonadectomy in young 6-OHDA-treated males significantly reduced oxidative stress, while no effects were observed in aged males.

Our data show that aged rats display a less pronounced response to gonadectomy than young rats. One possible explanation for these results could be that aged male rats are less responsive to gonadectomy, since testosterone levels are significantly lower in aged (24 months) male rats compared to young (2 months) male rats. Another explanation could be the physiological state of the aged brain, which is more sensitive to free radical damage and has decreased antioxidant capacity compared to a young brain (Mo et al., 1995; Stark and Pakkenberg, 2004). In agreement with these observations, aged rats show more extensive brain damage in response to 6-OHDA than young animals (Cass et al., 2002; Marshall et al., 1983). Furthermore, as the capacity of neurons to compensate for oxidative stress imbalances diminishes over time, so that even a minor stress could lead to irreversible damage (Polidori et al., 2007), we cannot rule out that the lack of a robust response to gonadectomy in aged rats might be due to the oxidative stress that these animals underwent

prior to the 6-OHDA lesion as part of their aging process and/or their low levels of testosterone.

While the precise mechanism by which androgens could contribute to the increased risk for PD seen in males is unclear, our data suggest that testosterone may further the progression of PD in males through the promotion of oxidative stress and subsequent striatal-nigral dopamine dysfunction, both of which are characteristics of the progressive neurodegeneration process observed in PD (Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Dietz et al., 1981; Forno, 1996; Gille et al., 2004; Hanrott et al., 2006; Hirsch et al., 1997; Jenner, 2003; Kanthasamy et al., 2003; Lotharius and Brundin, 2002; Shastri, 2003). Although androgen levels decrease with age in both men and women (Hollander et al., 2003), they continue to remain higher in males (Gavrilova and Lindau, 2009) and thus may contribute to a heightened state of dopamine neuronal vulnerability in males and render the male neurons more vulnerable to the pathogenic mechanisms of PD. Similar behavioral studies in females have not been conducted. Yet, studies examining androgen's effects on striatal dopamine content in MPTP and 6-OHDA treated females found that androgens either decreased striatal dopamine content or had no effect (Gillies et al., 2004; Lewis and Dluzen, 2008; Tomas-Camardiel et al., 2002). Therefore, the decreased susceptibility of PD observed in women may be mediated by a stronger role for estrogen acting as a neuroprotectant, since studies have shown that estradiol has neuroprotective actions on dopamine neurons (Bains et al., 2007; D'Astous et al., 2004; Gillies et al., 2004; Morissette et al., 2007).

In conclusion, these results suggest that gonadectomy and the consequent reduction of circulating androgen levels, decreases dopamine-dependent motor asymmetry in 6-OHDA-treated male rats by promoting striatal dopamine release and decreasing oxidative stress. The data implicate that testosterone plays a role in the exacerbation of motor asymmetry and oxidative stress generation caused by 6-OHDA, thus supporting the hypothesis that androgens may underlie the gender bias observed in PD.

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Highlights

Male sex & age are risk factors for dopamine cell loss in Parkinson's disease (PD).

Androgens, major male sex hormone, may influence the vulnerability of dopamine cells.

Symptoms of PD were modeled in GDX and gonadally intact male rats with 6-OHDA lesion.

GDX decreased 6-OHDA induced motor asymmetry and oxidative stress generation.

The data implicate that androgens underlie the gender bias observed in PD.

Figure 1.

6-OHDA lesion and effects on body weight. Sagittal section of the rat brain, stained with cresyl violet (modified from (Paxinos and Watson, 1998)). The dashed line indicates the course of the guide cannula and the dot at the end of this line indicates the site for the 6-OHDA lesion of medial forebrain bundle. A medial forebrain lesion induces dopamine neuronal loss in the substantia nigra pars compacta (A). The arrow points to the lack of TH⁺ staining of dopamine neurons in the substantia nigra pars compacta in brain sections of 6-OHDA-treated compared to SHAM-operated rats (B). 6-OHDA did not significantly alter body weight compared to SHAM. Young GDX males had significantly less weight gain than young INTACT males, regardless of 6-OHDA (Fisher's PLSD, * = $p \leq 0.05$) (C). STR = Striatum, MFB = Medial Forebrain Bundle, SN = Substantia Nigra

Figure 2.

Motor activity and plasma testosterone levels. Total motor activity (total forelimb use + total rotation frequency) was reduced in aged rats compared to young rats (Fisher's PLSD, $p \leq 0.05$). Young GDX males showed a trend for decreased motor activity compared to young INTACT males (*: $p \leq 0.10$) (A). Testosterone levels were significantly decreased with age (t-test, * = $p \leq 0.05$). GDX significantly reduced testosterone levels compared to INTACT in all groups (#: $p \leq 0.05$) (B). Total motor activity was positively correlated with plasma testosterone levels (Pearson, $r = 0.616$; $p \leq 0.05$) (C).

Figure 3.

Motor impairment and asymmetry. Contralateral forelimb use was significantly decreased in 6-OHDA rats compared to Sham-operated (Fisher's PLSD, *: $p \leq 0.05$), but contralateral forelimb use was not altered by GDX or age in 2 ug 6-OHDA lesion males (A). No differences in contralateral forelimb use were found between 2 ug and 4 ug 6-OHDA (B). GDX significantly decreased percent of ipsilateral rotations compared to INTACT in young 2 ug 6-OHDA males (# = $p \leq 0.05$). Aged and young 6-OHDA-treated INTACT males showed increased ipsilateral rotations compared to SHAM males (*: $p \leq 0.05$) (C). In young lesioned males, 4 ug 6-OHDA significantly increased ipsilateral rotations in GDX males compared to 2 ug 6-OHDA (* = $p \leq 0.05$). GDX significantly decreased percent of ipsilateral rotations compared to INTACT in young 2 ug 6-OHDA males (# = $p \leq 0.05$). No differences were found between 4 ug 6-OHDA young GDX males and 4 ug 6-OHDA young INTACT males (D).

Figure 4.

Amphetamine induced rotations. 2 ug 6-OHDA-treated GDX males showed significantly higher amphetamine-induced ipsilateral to lesion rotations compared to INTACT males, regardless of age (Fisher's PLSD, $* = p \leq 0.05$) (A). 2 ug 6-OHDA-treated GDX males showed significantly higher amphetamine-induced ipsilateral to lesion rotations compared to INTACT males ($* = p \leq 0.05$). Increased 6-OHDA (4 ug) did not further increase rotations in the GDX group. No differences between GDX and INTACT 4 ug 6-OHDA-treated rats (B).

Figure 5.

Advanced oxidation protein products (AOPP), a product of oxidative stress. In young males, AOPP was significantly increased by 6-OHDA lesion compared to SHAM-operated males (Fisher's PLSD, + = $p \leq 0.05$). Aged males had significantly higher AOPP levels compared to young males (# = $p \leq 0.05$), but no differences in AOPP levels were found between aged GDX males and aged INTACT males. Young GDX 6-OHDA treated males had significantly lower AOPP levels than young INTACT 6-OHDA treated males (* = $p \leq 0.05$), while GDX did not alter AOPP levels in young SHAM-operated males.