Castration Induces Parkinson Disease Pathologies in Young Male Mice via Inducible Nitric-oxide Synthase* 

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Background: Developing a simple irreversible animal model to study nigrostriatal pathologies is important for Parkinson disease (PD).

Results: Castration induces glial activation and death of dopaminergic neurons in wild type, but not iNOS−/−, young male mice.

Conclusion: Castration induces nigrostriatal pathologies via iNOS.

Significance: Castrated male mice may be used as a simple, toxin-free, nontransgenic, and irreversible animal model for PD.

Although Parkinson disease (PD) is a progressive neurodegenerative disorder, available animal models do not exhibit irreversible neurodegeneration, and this is a major obstacle in finding out an effective drug against this disease. Here we delineate a new reversible model to study PD pathogenesis. The model is based on simple castration of young male mice. Levels of inducible nitric-oxide synthase (iNOS), glial markers (glial fibrillary acidic protein and CD11b), and α-synuclein were higher in nigra of castrated male mice than normal male mice. On the other hand, after castration, the level of glial-derived neurotrophic factor (GDNF) markedly decreased in the nigra of male mice. Accordingly, castration also induced the loss of tyrosine hydroxylase-positive neurons in the nigra and decrease in tyrosine hydroxylase-positive fibers and neurotransmitters in the striatum. Reversal of nigrostriatal pathologies in castrated male mice by subcutaneous implantation of 5α-dihydrotestosterone pellets validates an important role of male sex hormone in castration-induced nigrostriatal pathology. Interestingly, castration was unable to cause glial activation, decrease nigral GDNF, augment the death of nigral dopaminergic neurons, induce the loss of striatal fibers, and impair neurotransmitters in iNOS−/− male mice. Furthermore, we demonstrate that iNOS-derived NO is responsible for decreased expression of GDNF in activated astrocytes. Together, our results suggest that castration induces nigrostriatal pathologies via iNOS-mediated decrease in GDNF. These results are important because castrated young male mice may be used as a simple, toxin-free, and nontransgenic animal model to study PD-related nigrostriatal pathologies, paving the way for easy drug screening against PD.

Parkinson disease (PD)2 is a progressive neurodegenerative disease with unclear etiology. PD may appear at any age, but it is uncommon in people younger than 30. The usual age of onset is between 50 and 70. The actual cause of PD is not known. It is believed that a complex interaction between environmental and genetic factors plays the major role in causing the disease (1–3). PD is characterized by a severe shortage of dopamine (DA), an important neurotransmitter. It is this deficiency that causes the symptoms of PD. The deficiency of DA is caused by the gradual loss of dopaminergic neurons that being present in the substantia nigra pars compacta (SNpc) synthesize DA from tyrosine via tyrosine hydroxylase. Once patients are diagnosed with PD, there is no drug available to halt its progression.

One of the major roadblocks for discovering drugs against PD is the unavailability of a true chronic persistent animal model for PD (3). One neurotoxin, MPTP, has been being used since the 1980s to model PD. However, for most MPTP models, the loss of dopamine is rapid and reversible. The extent of nigrostriatal damage also decreases in chronic MPTP models with time (4, 5). Because familial PD is associated with mutations of different genes, such as α-synuclein, parkin, PINK1, and DJ1 (6), efforts have been made to generate genetically engineered mice that express these mutated genes (7). Transgenic animals that overexpress α-syn or mutated human α-syn A53T have been used to study the role of this protein in dopaminergic degeneration. Although some transgenic mice expressing mutated α-syn display motor neuron pathology, these mice do not exhibit death of dopaminergic neurons (8). Many mutations in the gene encoding parkin cause a significant portion of early onset familial PD (8). Most of these mutations likely cause a loss of function in parkin, an E3 ubiquitin ligase, probably leading to proteasomal dysfunction. However, mice with mutated parkin exhibit progressive sensorimotor dysfunction without any DA cell loss (9). DJ1 mutations cause decreased resistance to oxidative stress in cells, flies, and mice (9). DJ1 KO mice, however, have little phenotype and do not develop DA cell loss. Furthermore, almost all of these transgenic mice exhibit any pathology at a much later age (>10 months), making studies more expensive and difficult.

Therefore, developing a chronic irreversible animal model to study the pathogenesis of PD is of paramount importance, and here we describe a simple, toxin-free, and nontransgenic mouse model for PD. Castration induces nigral glial activation, decrease in nigral GDNF, demise of tyrosine hydroxylase (TH)-

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2 The abbreviations used are: PD, Parkinson disease; DA, dopamine; DHT, 5α-dihydrotestosterone; DOPAC, 3,4-dihydroxyphenylacetic acid; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HVA, homovanillic acid; iNOS, inducible NOS; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SNpc, substantia nigra pars compacta; α-syn, α-synuclein; TH, tyrosine hydroxylase.
positive neurons in the nigra, loss of TH fibers and neurotransmitters in the striatum, and impairment of locomotor activities in young male mice. However, castration is unable to induce these nigrostriatal pathologies in male inducible nitric-oxide synthase (iNOS)-null mice. Furthermore, we demonstrate that the expression of GDNF is suppressed by NO. Taken together, these results suggest that castration induces PD-like pathologies in male mice via iNOS-mediated suppression of GDNF.

**MATERIALS AND METHODS**

**Reagents**—Fetal bovine serum (FBS), Hanks’ balanced salt solution, trypsin, and DMEM/F-12 were from Mediatech. 5α-Dihydrotestosterone (DHT) 30-day release pellets and placebo pellets were obtained from Innovative Research of America (Sarasota, FL). Animal maintenance and experiments were in accordance with National Institute of Health guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Rush University of Medical Center. Primary antibodies, their sources, and concentrations used are listed in Table 1. Alexa Fluor antibodies used in immunostaining were obtained from Jackson Immunoresearch, and IR dye-labeled reagents used for immunoblotting were from LifeCor Biosciences.

**Isolation of Primary Mouse Astroglia**—Astroglia were isolated from brains of 7–9-day-old mouse pups as we described before (10–12).

**Castration of Male C57BL/6 Mice**—Mice (4 weeks old) were anesthetized, shaved, and prepared with providone and ethanol. An incision was made in the scrotum, followed by an incision in the tunica of the first testicle. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized. A hemostat was used to clamp the blood vessels, and a figure-of-8 knot was tied above. The testis, vas deferens, and attached testicular fat pad were exteriorized.

**Assessment of Serum Testosterone Levels**—Testosterone was measured from the serum samples using Testosterone EIA kit from Enzo Life Sciences, following the manufacturer’s instructions. Samples were diluted (1:20) and run in duplicate. The detection range of this kit is 7.81–2,000 pg/ml.

**Implantation of DHT Pellets**—DHT as well as placebo pellets were implanted subcutaneously in the scapular area of the neck of castrated male mice using a trochar.

**Semiquantitative RT-PCR Analysis**—To remove any contaminating genomic DNA, total RNA was digested with DNase. Semiquantitative RT-PCR was carried out as described earlier (10–12) using a RT-PCR kit from Clontech. Briefly, 1 μg of total RNA was reverse-transcribed using oligo(dT)12–18 as primer and MMLV reverse transcriptase (Clontech). The resulting cDNA was appropriately diluted, and diluted cDNA was amplified. Amplified products were electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide staining. Primers were: iNOS sense, 5′-CCCT-TCCGAAGTTTCTGGCAGACG-3′ and antisense, 5′-GGCT-GTCAAGAGGCTGTCGTTGG-3′; IL-1β sense, 5′-TTGGGACTTTTGTACAAGG-3′ and antisense, 5′-GGTATGATGTA-ACCAGTTGAG-3′; TNF-α sense, 5′-TCTCTGTCGACTGAAGCTG-3′ and antisense, 5′-GAAATCAGTGCCTGG-3′; CD11b sense, 5′-GGGTGAGTCCTCTACGGGAACCAGG-3′ and antisense, 5′-GGCCTACTTACAGCGAGCCTAAC-3′; GFAP sense, 5′-GCGGTGTCATGTCGGTGCT-3′ and antisense, 5′-TGCTCTACAGCCGAGCT-3′; GAPDH sense, 5′-GGATGGTTGATCTGCTGGTTG-3′ and antisense, 5′-GGGATTTGACTTGGTGCTTG-3′.

**Real-time PCR Analysis**—PCR analysis was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) as described earlier (10–12). The mRNA expressions of respective genes were normalized to the level of GAPDH mRNA. Data were processed by the ABI Sequence Detection System 1.6 software and assessed by analysis of variance.

**Immunohistochemistry and Quantitative Morphology**—Mice were perfused and their brains fixed, embedded, and processed for TH staining as described previously (13–15). Total numbers of TH-stained neurons in the SNpc were counted stereologically with STEREO INVESTIGATOR software (MicroBrightfield, Williston, VT) by using optical fractionators (13–16). Quantitation of striatal TH immunostaining was performed as described (13, 14, 17). Optical density measurements were obtained by digital image analysis (Scion, Frederick, MD). Striatal TH optical density reflected dopaminergic fiber innervation. For immunofluorescence staining on fresh frozen sections, rat anti-mouse CD11b (1:100), goat anti-mouse GFAP (1:100), and rabbit anti-mouse iNOS (1:250) were used. The samples were mounted and observed under a Bio-Rad MRC1024ES confocal laser scanning microscope.

### Table 1

| Antibody | Manufacturer | Catalog no. | Host | Application* | Dilution/amount |
|----------|--------------|-------------|------|--------------|----------------|
| GDNF | Abcam | ab18956 | Rabbit | WB/IF | 1:1000 |
| GDNF | Millipore | AB5252P | Ship | IHC | 1:500 |
| β-Actin | Abcam | ab6276 | Mouse | WB | 1:5000 |
| iNOS | BD Biosciences | 610432 | Rabbit | WB | 1:200 |
| IL-1β | Santa Cruz | sc7884 | Rabbit | WB | 1:200 |
| GFAP | Dako | Z0334 | Rabbit | ICC/WB | 1:1500 |
| Iba-1 | Santa Cruz | sc6171 | Goat | ICC/IF | 1:200 |
| CD11b | Cedarlane | sc8941A | Goat | IHC/WB | 1:500 |
| TH | Calbiochem | 657012 | Rabbit | WB | 1:1000 |

* WB, Western blotting; IF, immunofluorescence; IHC, immunohistochemistry; ICC, immunocytochemistry.
HPLC Analysis for Measurement of Striatal DA and Its Metabolite Levels—Striatal levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were quantified as described earlier (13–17). Briefly, mice were sacrificed by cervical dislocation, and their striata were collected and immediately frozen in dry ice and stored at −80 °C until analysis. On the day of the analysis, tissues were sonicated in 0.2 M perchloric acid containing isoproterenol, and the resulting homogenates were centrifuged at 20,000 × g for 15 min at 4 °C. After pH adjustment and filtration, 10 μl of supernatant was injected onto an Eicompak SC-3ODS column (Complete Stand-Alone HPLC-ECD System EiCOMHTEC-500 from J&M Science Inc., Grand Island, NY) and analyzed following the manufacturer’s protocol.

Behavioral Analysis—Two types of behavioral experiments were conducted 30 days after castration. This included an open field experiment for locomotor activity and rotarod experiment for foot movement as described earlier (13–15, 17). The foot movement of the mice was observed on rotarod that was set to accelerate from 4 to 40 rpm over a 5-min period. Locomotor activity was monitored in Digiscan Monitor (Omnitech Electronics, Inc., Columbus, OH). This Digiscan Monitor records stereotypy and rearing, behaviors that are directly controlled by the striatum, as well as other basic locomotion parameters, such as horizontal activity, total distance traveled, number of movements, movement time, rest time, mean distance, mean time, and center time. Mice were placed inside the Digiscan Infra-red Activity Monitor for 10 min daily for 2 consecutive days for acclimatization followed by test on the 3rd day. Briefly, animals were removed directly from their cages and gently placed nose first into a specified corner of the open-field apparatus and after release, data acquisition began every 5-min interval. DIGISCAN software was used to analyze and store horizontal and vertical activity data, which were monitored automatically by infrared beams.

Statistics—All values are expressed as means ± S.E. Differences among means were assessed by one-way analysis of variance. In other cases, Student’s t test was used to compare outcomes between two groups (e.g. sham versus castrated, WT versus iNOS-null).

RESULTS

Castration Up-regulates Glial Activation in the SNpc—In an effort to explore a toxin-free nontransgenic model to study PD-related nigrostriatal pathologies, we castrated young 4–5-week-old male C57BL/6 mice. Because glial activation plays an important role in the pathogenesis of PD (13, 17–19), at first we monitored the status of glial activation in the SNpc of castrated mice. Glial activation is characterized by increase in glial marker proteins such as GFAP for astrocytes and CD11b for microglia. Therefore, we examined the effect of simple castration on the expression of GFAP and CD11b in the SNpc. One month after castration, we observed a marked increase in the mRNA expression of GFAP and CD11b in the SNpc compared with age-matched sham male mice (Fig. 1, A and B). In addition to CD11b, microglia are also known to immunoreact with Iba1. Western blotting and immunofluorescence analysis also revealed marked increase in the expression of GFAP (Fig. 1, C, D, and F) and Iba-1 (Fig. 1, C, E, and G) proteins in the SNpc of castrated mice.

Glial activation is always associated with the up-regulation of various proinflammatory molecules, including IL-1β, TNF-α, and iNOS (17, 20). Accordingly, we observed increased mRNA expression of iNOS, IL-1β, and TNF-α in the nigra of castrated mice compared with sham mice (Fig. 2, A and B). Western blot analysis of nigral homogenates also revealed marked increase in iNOS and IL-1β proteins in the nigra of castrated male mice compared with sham mice (Fig. 2, C–E). As evident from immunofluorescence analysis, increased iNOS co-localized to GFAP-positive astroglia (Fig. 2F) or CD11b-positive microglia (Fig. 2G) in the SNpc of castrated mice. α-Synuclein accumulation is another important feature of PD. Interestingly, castration also increased the level of α-syn in the SNpc of male mice as compared with normal male mice (Fig. 2H).

Castration Induces the Loss of Dopaminergic Neurons in the Nigra and TH-positive Fibers in the Striatum and Decreases Striatal Neurotransmitters in Male Mice—Male C57BL/6 mice (4–5 weeks old) were castrated, and 1 month after castration, mice were sacrificed and nigral homogenates were immunoblotted with TH. As evident from Fig. 3, A and B, the expression
of TH protein markedly decreased in the nigra of castrated male mice compared with sham mice. To further confirm this finding, brains of castrated and sham mice were fixed, embedded, and processed for TH staining. Total numbers of TH-stained neurons in the SNpc were counted stereologically with STEREO INVESTIGATOR software. Only castration was sufficient to induce the loss of TH-positive neurons on the nigra (Fig. 3, C and E) and the decrease in TH fibers in the striatum (Fig. 3, D and F). Because the levels of DA and its metabolites DOPAC and HVA decrease in the striatum of PD patients, we examined whether castration alone was capable of decreasing these neurotransmitters in male mice. As evident from Fig. 3G, castration significantly decreased the levels of DA, DOPAC, and HVA in the striatum of male C57BL/6 mice. Next, we examined whether this castration-induced decrease in striatal DA was confined to only C57BL/6 background. Decrease in striatal DA in castrated male SJL/J mice compared with sham control (Fig. 3H) suggests that castration-mediated loss of DA is not a strain-specific effect.

To further prove that this unexpected result is not an artifact of our surgical procedure, we obtained sham and castrated young male mice from Harlan Laboratories (Indianapolis, IN). Mice were castrated at the age of 4–5 weeks and shipped to us 1 week after castration. We also observed marked decrease in striatal DA in all castrated male mice (n = 10) 30 days after castration compared with sham control (data not shown). The incidence of striatal DA loss was 100%.

FIGURE 2. Castration increases the level of proinflammatory molecules and α-syn in the SNpc of male mice. A and B, male C57BL/6 mice (4–5 weeks old) were castrated, and 30 days later, the mRNA expression of iNOS, IL-1β, and TNF-α was measured by semiquantitative RT-PCR (A) and real-time PCR (B). C, protein levels of iNOS and IL-1β were monitored in the nigra by Western blotting. D and E, actin was run as loading control. Bands were scanned and expressed as relative to sham control (D, iNOS; E, IL-1β). Results are mean ± S.E. (error bars) of six mice per group. a, p < 0.001 versus sham-control. F and G, nigral sections were also double-immunolabeled for either GFAP and iNOS (F) or CD11b and iNOS (G). DAPI was used to visualize nucleus. H, nigral sections were immunostained with mouse anti-α-syn and visualized using 3,3′-diaminobenzidine staining. Nissl (0.1% Cresyl Violet solution) was used to visualize neurons. Results represent analysis of two sections, each of six mice per group.
To understand whether the effect of castration is specific for nigra or other parts of the brain are also affected, 30 days after castration, hippocampal and cortical sections were immunolabeled for NeuN. Interestingly, castration did not decrease the NeuN immunoreactivity in different parts of the hippocampus (Fig. 4, A and B). Similarly, castration also had no effect on NeuN immunostaining in the cortex (Fig. 4 C), suggesting the specificity of the effect.

**Effect of Castration on Locomotor Activities**—Next, to examine whether castration caused not only structural and neurotransmitter damage but also functional deficits, we monitored locomotor activities. On the day of testing, mice were recorded twice at an interval of 2 h. Similar to MPTP intoxication, castration alone decreased rotarod performance (Fig. 5A), horizontal activity (Fig. 5B), total distance (Fig. 5C), number of movements (Fig. 5D), movement time (Fig. 5E), rearing (Fig. 5F), stereotypy counts (Fig. 5G), and stereotypy time (Fig. 5H).

**Do Castration-induced Nigrostriatal Pathologies Persist?**—PD is both chronic and progressive. Although the MPTP mouse model, the most widely used animal model of PD, is a good one for initial drug screening and investigating the mode of action of a drug, a potential limitation of this model is that its effects are spontaneously reversed over time. Therefore, we examined whether the effects of castration on the nigrostriatum also reversed over time. Seven, 15, 30, 60, and 120 days after castration, mice were sacrificed, and their nigra and striata were collected. Nigra were used for monitoring the mRNA expression of iNOS, CD11b, and GFAP by real-time PCR, and the level of DA was measured in striata by HPLC (Fig. 6A). Within 7 days of castration, we observed significant increase in the expression of iNOS (Fig. 6B), GFAP (Fig. 6C), and CD11b (Fig. 6D), which subsequently increased and became maximum 30 days after castration (Fig. 6, B–D). Interestingly, castration-mediated expression of iNOS, GFAP, and CD11b persisted until the duration (120 days) of the study (Fig. 6, B–D). Although we found glial activation on 7 days after castration, striatal loss of DA was not significant at that time point (Fig. 6E). However, the DA level significantly decreased from 15 days onward and even 120 days after castration, we found no improvement in striatal DA level in castrated male mice compared with...
age-matched sham male mice (Fig. 6E). These results suggest that castration induces persistent glial activation and stable loss of striatal DA in male mice.

**Does Castration of Male Mice at Any Age Induce Nigrostriatal Pathology?**—To address this important question, 4–5-week-old, 8–9-week-old, and 13–14-week-old male mice were castrated, and 30 days later the status of TH and glial activation in the nigra and the level of striatal DA were monitored. As mentioned above, castration of 4–5-week-old male mice led to increased expression of GFAP, Iba-1, and iNOS in the nigra compared with sham control (Fig. 7, A and B). Consistently, we also observed decreased expression of nigral TH (Fig. 7, A and B) and loss of striatal DA (Fig. 7C) in 4–5-week-old castrated male mice. In contrast, castration of either 8–9-week-old (Fig. 7, D–F) or 13–14-week-old (Fig. 7, G–I) male mice did not up-regulate nigral glial activation (Fig. 7, D, E, G, and H) compared with respective sham controls. Similarly, castration of 8–9-week-old and 13–14-week-old male mice also did not decrease nigral TH (Fig. 7, D, E, G, and H) and modulate striatal DA (Fig. 7, F and I). These results suggest that castration of male mice at a particular age is important for the induction of nigrostriatal pathology.

At present, we do not know the correct reason for this particular age requirement. However, in an attempt to find a possible rationale for this interesting observation, we measured the level of testosterone in serum of male mice at different age groups. Decreased serum testosterone levels in 8–9-week-old, 13–14-week-old, and 16–17-week-old male mice compared with 4–5-week-old male mice (Fig. 7J) suggest that 4–5-week-old male mice are probably susceptible to castration-induced nigrostriatal pathology due to the presence of high testosterone level.
Does Testosterone Supplementation Normalize These PD-related Pathologies in Castrated Male Mice?—To find out whether the observed pathological changes are due to castration-related loss of male sex hormone, we performed this experiment. However, testosterone may be converted into estrogen by aromatase. Therefore, to avoid the complexity, castrated male mice received DHT pellets (30-day release), which were implanted subcutaneously in the scapular area of the neck of castrated male mice on the day of castration (Fig. 8A). It is interesting to see that DHT supplementation protected nigral TH (Fig. 8, B and C) and preserved striatal DA (Fig. 8D) in 4–5-week-old castrated male mice. Accordingly, DHT supplementation also improved rotarod performance (Fig. 8E), horizontal activity (Fig. 8F), total distance (Fig. 8G), movement time (Fig. 8H), number of movement (Fig. 8I), and stereotypy (Fig. 8J).

Castration Induces Stable PD Pathologies—To understand the mechanism of these unexpected results, we decided to castrate iNOS /–/– mice because iNOS is downstream of proinflammatory cytokine signaling and castration induced the expression of iNOS and proinflammatory cytokines.

Castration Is Unable to Induce Nigrostriatal Pathologies in Male iNOS /–/– Mice—To understand the mechanism of these unexpected results, we decided to castrate iNOS /–/– mice because iNOS downstream of proinflammatory cytokine signaling and castration induced the expression of iNOS and proinflammatory cytokines.
inflammatory cytokines in the nigra (Fig. 2). Similar to that observed in male C57BL/6 mice, castration also led to a decrease in TH immunoreactivity in nigra (Fig. 9A) and striatum (Fig. 9B), and loss of TH-positive neurons in the nigra (Fig. 9C) and fibers in the striatum (Fig. 9D) of wild type (C57BL/6) mice. Western blot analysis of nigral homogenates showed marked loss of TH protein in the nigra of castrated male mice compared with sham male mice (Fig. 9F and G). Accordingly, castration also decreased striatal DA in male WT mice (Fig. 9E). However, castration remained unable to induce these nigrostriatal pathologies in iNOS−/− male mice (Fig. 9E), indicating that iNOS plays a critical role in castration-induced manifestation of nigrostriatal pathologies in male mice.

Castration Does Not Decrease the Expression of GDNF in the Nigra of Male iNOS−/− Mice—How does iNOS couple to nigrostriatal pathologies? Although iNOS can affect the nigrostriatum via many different ways, we examined whether there was any cross-talk between iNOS and the nigral trophic system. GDNF is a particularly potent factor for survival and axonal growth of mesencephalic dopaminergic neurons (21) and has been shown to ameliorate motor deficits and reduce brain damage in several animal models of PD (22). We tested whether castration was capable of modulating the expression of GDNF in the SNpc of male C57BL/6 mice. One month after castration, we observed a marked decrease in the expression of GDNF mRNA (Fig. 10, A and B) and protein (Fig. 10, C and D) in the SNpc of male mice compared with sham control. Double-label immunofluorescence analysis also revealed that nigral sections of castrated male mice expressed more GFAP and less GDNF than that of sham control. These results
suggest that castration leads to a decrease in the level of GDNF in the SNpc of male mice.

Because nigral astroglia in normal male mice expressed GDNF, but not iNOS, and nigral astroglia in castrated male mice expressed iNOS, but not GDNF, we examined the role of iNOS in castration-induced loss of GDNF. As observed in C57BL/6 mice, castration of male C57BL/6J mice also led to a marked increase in GFAP and decrease in GDNF protein in the SNpc (Fig. 11, A and B). However, we did not observe any decrease in GDNF or increase in GFAP in iNOS−/− mice after castration (Fig. 11, A and B). Western blot analysis of GDNF also supports this finding (Fig. 11, C and D). Interestingly, the basal level of GDNF was found to be higher in the nigra of iNOS−/− mice than that of WT mice (Fig. 11, A and B). Together, these results suggest that castration leads to a decrease in GDNF in the nigra of male mice via up-regulation of iNOS.

Does iNOS-derived NO Suppress GDNF?—Next, we examined whether there was any direct cross-talk between NO and GDNF. At first, we performed a time course experiment for GDNF expression and NO production in IL-1β-stimulated primary mouse astrocytes. Although IL-1β initially increased the mRNA expression of GDNF after 6 h of stimulation (Fig. 10A), GDNF mRNA expression gradually vanished with time (Fig. 12A). Interestingly, this expression pattern of GDNF in activated astrocytes was inversely related to NO production (Fig. 12B). To find out whether NO is actually involved in the down-regulation of GDNF, astrocytes were stimulated with IL-1β in the presence or absence of 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-oxo-3-oxide (PTIO; a NO scavenger) and N6-(1-iminoethyl)-L-lysine (L-NIL; an inhibitor of NOS). After 24 h of stimulation, the mRNA expression of GDNF was monitored by semiquantitative RT-PCR (Fig. 12C), and the protein level (Fig. 12D) of GDNF was assayed by ELISA (Promega). Both PTIO and L-NIL abrogated the inhibitory effect of IL-1β on GDNF, indicating that IL-1β inhibits the expression of GDNF in astrocytes via NO. To prove the role of iNOS in the suppression of GDNF, primary astrocytes isolated from WT and iNOS−/− mice were stimulated with IL-1β followed by analysis of GDNF expression. Interestingly, IL-1β reduced the expression of GDNF protein in WT, but not iNOS−/−, astroglia (Fig. 12, E and F). It was also supported by...
Castration led to the loss of dopaminergic neurons in the nigra and toxins. Our conclusion is based on the following. First, castration induced PD-related nigrostriatal pathologies in male mice in the absence of any demonstrable dopamine neurodegeneration. Second, the DA level markedly decreased 30 days after castration; and even 120 days after castration, we found no improvement in striatal DA level in castrated male mice compared with age-matched normal male mice. Third, castration also led to impairment in locomotor activities, suggesting that castration caused not only structural and neurotransmitter damage but also functional deficits. Because simple castration induced these PD-related nigrostriatal and behavioral changes, our results suggest that some forms of PD (e.g. early onset) in males may be due to a sudden decrease in male sex hormone.

Why did we think of such an unconventional approach? It has been shown that testosterone deficiency is common in the older male population and has an increased prevalence in parkinsonian patients (23, 24). In another study, testosterone therapy led to significant improvement in the resting tremor and fine motor control in parkinsonian patients with testosterone deficiency (25). Testosterone treatment also improved the nonmotor symptoms of PD (26). Finally, TH is also localized to the Leydig cells at both the mRNA and protein levels (27). MPTP intoxication causes marked decrease in serum testosterone level and loss of Leydig cells (28). The loss of Leydig cells is accompanied by a marked decrease in TH protein in the interstitial and significant fall in norepinephrine levels in the testis (28). Therefore, we thought that testosterone could be intimately coupled to the dopaminergic pathway and that castration may induce nigrostriatal pathologies. However, in contrast to our finding, some articles have shown either no effect or an increase in markers of nigrostriatal function (e.g. TH-positive neurons in the SN, striatal dopamine content) following castration (29, 30). In these cases, older mice or rats were used for castration. We also did not observe glial activation or loss in nigral TH and striatal DA 1 month after castration when 8–9-week-old and 13–14-week-old mice were castrated (Fig. 5), indicating that castration induces nigrostriatal pathologies after certain time only when young male mice are castrated probably when testosterone level is maximum (Fig. 7). Our results may have implications in females as well. Although we have not examined females mice, Rocca et al. (31) have reported increased risk of parkinsonism in women who underwent oophorectomy before menopause. According to this study (31), the risk increased with younger age at oophorectomy. Together, these results suggest that sudden loss of sex hormone in young population may increase the risk of having PD later in life.

How does castration induce nigrostriatal pathologies? Although the disease mechanisms that cause PD are poorly understood, recent studies strongly support a role of inflammation in nigrostriatal degeneration in PD. It has been found that early intervention with nonsteroidal anti-inflammatory drugs slows disease incidence. Furthermore, significant microglial activation occurs in close proximity to damaged or dying dopaminergic neurons. Several studies have shown that iNOS-NO-ONOO− (peroxynitrite) plays important role in the loss of dopaminergic neurons in PD. First, the concentration of NO2− (nitrite), a metabolite of NO, increases in the CSF of patients with PD compared with a group of patients without dopaminergic neurons in the nigra and fibers in the striatum. This loss was irreversible; even at 4 months after castration, the extent of nigrostriatal damage did not decrease. Second, the DA level markedly decreased 30 days after castration; and even 120 days after castration, we found no improvement in striatal DA level in castrated male mice compared with age-matched normal male mice. Third, castration also led to impairment in locomotor activities, suggesting that castration caused not only structural and neurotransmitter damage but also functional deficits. Because simple castration induced these PD-related nigrostriatal and behavioral changes, our results suggest that some forms of PD (e.g. early onset) in males may be due to a sudden decrease in male sex hormone.

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ergic dysfunction (32). Second, it has been shown that many cells in the SNpc from postmortem PD samples express considerable amounts of iNOS, whereas those from age-matched controls do not (33). Third, iNOS is not only up-regulated in the SNpc of MPTP-treated mice, but its ablation in mutant mice significantly attenuates MPTP neurotoxicity, thus indicating that iNOS is essential in MPTP-induced SNpc dopaminergic neurodegeneration (18). Therefore, we examined the role of iNOS in castration-induced nigrostriatal pathologies in male mice. Interestingly, castration remained unable to induce the loss of nigral TH-positive neurons and striatal TH fibers and the decrease in striatal neurotransmitters in iNOS−/− mice, clearly describing an important role of iNOS in castration-mediated nigrostriatal pathologies.

NO, a short lived and diffusible free radical, plays many roles as a signaling and effector molecule in diverse biological systems; it is a neuronal messenger and is involved in vasodilation as well as in antimicrobial and antitumor activities (34, 35).

**FIGURE 12.** Down-regulation of GDNF in mouse astrocytes by nitric oxide. A and B, primary mouse astrocytes were stimulated with 20 ng/ml IL-1β under serum-free conditions for different time periods followed by monitoring the mRNA expression of IL-1β in cells by semiquantitative RT-PCR (A) and the level of nitrite in supernatants by Griess reagent (B). Results are mean ± S.D. (error bars) of three separate experiments. 

- a, p < 0.001 versus 0 h.
- c and d, cells were stimulated with IL-1β in the presence or absence of different concentrations of L-NIL and PTIO for 24 h followed by monitoring the mRNA expression of GDNF in cells (C) and the protein level of GDNF in supernatants (D).Results are mean ± S.D. of three separate experiments. 

- a, p < 0.001 versus IL-1β.
- b, p < 0.001 versus L-NIL.
- c, p < 0.001 versus PTIO.

E, primary astrocytes isolated from wild type (WT) and iNOS−/− mice were stimulated with IL-1β for 18 h followed by monitoring the level of GDNF protein in cells by Western blotting (E). F, bands were scanned using the ImageJ software. Results are mean ± S.D. of three separate experiments. 

- a, p < 0.001 versus WT-cont.

G and H, WT (G) and iNOS−/− (H) astrocytes were stimulated with IL-1β for 24 h followed double-immunolabeling for GFAP and GDNF. Results represent three independent experiments. I, possible hypothesis is shown for castration-induced development of PD-like pathologies.
the other hand, NO has also been implicated in several CNS disorders, including inflammatory, infectious, traumatic, and degenerative diseases (18, 36–38). There is considerable evidence for the transcriptional induction of iNOS (the high output isoform of NOS) in the CNS that is associated with degenerative brain injury (18, 35, 37, 38). NO is potentially toxic to neurons and oligodendrocytes that may mediate toxicity through the formation of iron-NO complexes of iron-containing enzyme systems (39), oxidation of protein sulfhydryl groups (40), nitration of proteins, and nitrosylation of nucleic acids and DNA strand breaks (41). Here, we have described a new mechanism by which iNOS-derived NO may cause nigrostriatal degeneration (summarized in Fig. 12f). Whereas castration increased the expression of iNOS in the nigra, the level of GDNF went down drastically in the nigra after castration. Interestingly, castration remained unable to decrease the expression of GDNF in nigra and striatum in iNOS−/− mice. This also paralleled with the inability of castration of inducing nigrostriatal pathologies in iNOS−/− mice, suggesting that castration causes the loss of dopaminergic neurons in the nigra via iNOS-mediated down-regulation of GDNF (Fig. 12f). GDNF is a particularly potent factor for survival and axonal growth of mesencephalic dopaminergic neurons (21). Kordower et al. (42) used a lentiviral vector to increase GDNF production in the striatum and demonstrated functional and structural recovery when initiating treatment 1 week after a systemic MPTP lesion in monkeys. Although GDNF gene therapy does not work well in PD patients, intraventricular administration of GDNF protein exhibits protection in PD patients (43). Therefore, increasing the levels of these trophic factors and/or maintaining their physiological levels in the CNS of patients with neurodegenerative disorders is/are an important area of research. However, such mechanisms are poorly understood. Here, we demonstrate that NO is a negative regulator of GDNF, suggesting that scavenging of NO may help in restoring and/or increasing the level of GDNF in the degenerative brain.

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