Parkin Protects against Neurotoxicity in the 6-Hydroxydopamine Rat Model for Parkinson’s Disease

Linda Vercammen,1 Anke Van der Perren,1 Elisabetta Vaudano,1,* Rik Gijsbers,2 Zeger Debyser,2 Chris Van den Haute,1,# and Veerle Baekelandt1,#,

1Laboratory for Neurobiology and Gene Therapy, Molecular Medicine, K.U. Leuven, Kapucijnenvoer 33 VCTB+5, B-3000 Leuven, Flanders, Belgium
2Laboratory for Molecular Virology and Gene Therapy, Molecular Medicine, K.U. Leuven and IRC KULAK, B-3000 Leuven, Flanders, Belgium

*Current address: Protein Laboratory, Institute of Molecular Pathology, Enkamp Pharmaceuticals AFS, Panum Institute, Copenhagen, Denmark.

#Shared last authors.

Available online 17 August 2006

Loss-of-function mutations in the PARK2 gene are the major cause of early onset familial Parkinson’s disease. The gene product, parkin, is an E3 ligase of the ubiquitin–proteasome pathway involved in protein degradation. Dopaminergic neuron loss may result from the toxic accumulation of parkin substrates, suggesting a key role for parkin in dopaminergic neuron survival. In this study, we have investigated the neuroprotective capacity of parkin in the 6-OHDA rat model for Parkinson’s disease. 6-OHDA induces the generation of reactive oxygen species leading to the degeneration of catecholaminergic neurons, but may also impair proteasome activity. Lentiviral vectors encoding human wild-type parkin or green fluorescent protein were stereotactically injected into the substantia nigra 2 weeks prior to a striatal 6-OHDA lesion. Histological analysis 1 and 3 weeks after lesioning showed a significant preservation of dopaminergic cell bodies and nerve terminals. Moreover, lesioned rats overexpressing parkin displayed a corresponding behavioral improvement as measured by the amphetamine-induced rotation test and the cylinder test. The improved performance in the amphetamine-induced rotation test lasted until 20 weeks after lesioning. Our results demonstrate that parkin acts as a potent neuroprotective agent in vivo against 6-OHDA toxic insults. These data support the therapeutic potential of parkin for the treatment of not only familial but also sporadic Parkinson’s disease.

Key Words: Parkinson’s disease, parkin, gene therapy, neuroprotection, lentiviral vector

INTRODUCTION

Parkinson’s disease (PD) is the second most common neurodegenerative disorder, characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta. The characteristic clinical features are bradykinesia, tremor, and rigidity [1]. Both environmental and genetically inherited factors have been implicated in the development of sporadic or familial PD, respectively. Tremendous progress has been made over the past few years in the identification of the genes linked to hereditary forms of PD [2]. Mutations in α-synuclein and LRRK2/dardarin cause autosomal-dominant forms of PD, while mutations in parkin, DJ-1, and PINK1 lead to autosomal-recessive forms of PD. Numerous mutations in the parkin gene (approved gene symbol PARK2) are involved in autosomal recessive, juvenile forms of parkinsonism (AR-JP), accounting for more than 50% of early onset PD cases [3,4]. Parkin is a 465-amino-acid-long protein of approximately 52 kDa that contains a ubiquitin-like domain in its amino-terminus and two RING finger domains in its carboxy-terminus [5]. Parkin functions as a ubiquitin–protein ligase (E3) that acts along with the ubiquitin-conjugating enzymes (E2’s) UbcH7 and UbcH8 [6,7]. Familial PD-associated mutations in parkin result in loss of function through defects in E3 ligase activity, solubility, or substrate binding [8]. Although a number of parkin substrates with diverse functions have been identified, no one is specifically expressed in dopaminergic neurons. It remains therefore not understood how the accumulation of these substrates in the absence of parkin can lead to selective degeneration of nigral dopaminergic neurons. Recent in vivo studies have demonstrated a neuroprotective effect of parkin against α-synuclein-induced toxicity...
in *Drosophila* [9,10] and in rats [11,12]. We wondered whether the therapeutic potential of parkin would also apply to toxin-induced PD models, rather than be limited to α-synucleinopathies. We therefore investigated the effects of lentiviral vector-mediated overexpression of parkin in the well-characterized 6-OHDA rat lesion model.

**RESULTS**

**Lentiviral Vectors Mediate Overexpression of Human Parkin in Cell Culture and in Rat Brain**

After construction of lentiviral vector (LV) plasmids encoding human wild-type parkin, we verified expression of the transgene in cell culture and in rat brain. Western blot analysis of extracts from transduced SH-SY5Y cells confirmed overexpression of the parkin protein (Fig. 1A). Next, we stereotactically injected LV encoding parkin into the rat substantia nigra (SN). Immunocytochemical staining revealed high expression of the transgene at the injected side compared to endogenous levels (Figs. 1B–1E). The transduced cells displayed a predominantly neuronal morphology, as described before [13], which was confirmed by double-staining for the neuronal marker NeuN (data not shown). Transduction of dopaminergic neurons was demonstrated by double staining for tyrosine hydroxylase (TH) and confocal analysis (Fig. 1F).

**Parkin Protects against 6-OHDA-Induced Dopaminergic Cell Death in the Rat Substantia Nigra**

To examine the neuroprotective capacity of parkin against 6-OHDA toxicity *in vivo*, we stereotactically injected LV encoding parkin or eGFP unilaterally into the SN of adult rats. Two weeks later, we induced a unilateral 6-OHDA lesion in the striatum ipsilateral to the vector injection. The 6-OHDA lesioning protocol used in this experiment results in a direct toxic damage to the dopaminergic axons in the striatum, followed by a progressive loss of the nigral dopaminergic neurons in the ipsilateral SN over several weeks or months [14,15]. We used saline-injected animals as controls (Table 1). One or three weeks after 6-OHDA administration, we sacrificed the animals. We performed tyrosine hydroxylase immunostaining to visualize dopaminergic neurons in the SN and their nerve terminals in the striatum. We investigated the survival of dopaminergic neurons in the substantia nigra pars compacta via two different quantitative methods. We made a first estimation of the lesion size by stereological quantification of the TH-positive nigral volume (Fig. 2A). In the eGFP-injected...
animals the size of the lesion increased from 32 ± 4% at 7 days to 77 ± 4% at 3 weeks. This was not different from rats that received a 6-OHDA lesion without a lentiviral vector injection. In the parkin-overexpressing rats the size of the lesion at 1 week was similar to that in the eGFP controls, but after 3 weeks the lesion was about 51 ± 4%, which was significantly smaller than in the eGFP-expressing rats. Next, we counted the number of dopaminergic neurons in the SN (Fig. 2B). Again, at 1 week postlesioning, there was no significant difference between the eGFP group and the parkin group. Three weeks after 6-OHDA lesioning, TH-positive cell counts in the lesioned SN were reduced to 1001 ± 173 in the eGFP-expressing animals, which was not significantly different from the control group that received just a 6-OHDA lesion (787 ± 110). In contrast, in the parkin-overexpressing rats there was significant sparing of the dopaminergic neurons (5514 ± 479) at 3 weeks postlesion. Compared to the nonlesioned side, the number of dopaminergic neurons was reduced to 10 ± 2 and 8 ± 1% in the GFP-expressing rats and the rats that received only a 6-OHDA lesion, respectively, while in the parkin group, 56 ± 5% of the dopaminergic neurons survived 3 weeks postlesion. To exclude an effect of LV transduction on TH-positive cell counts per se, we also analyzed rats that were injected with LV encoding eGFP or parkin into the SN, followed by an injection with saline instead of 6-OHDA into the striatum. The total estimated number of TH-positive neurons in the SN was not different in rats that received LV-eGFP or LV-parkin injections in the SN in the absence of a 6-OHDA lesion (10,122 ± 816 and 9000 ± 427, respectively) from the dopaminergic cell counts in the contralateral intact SN (9916 ± 248) (Fig. 2C).

### Parkin Reduces 6-OHDA-Induced Degeneration of Dopaminergic Nerve Terminals in the Striatum

Next, we analyzed the survival of dopaminergic nerve terminals in the striatum, since it has been shown that their integrity is essential for functional recovery [16]. TH immunostaining through the striatum revealed a marked reduction in dopaminergic innervation on the side ipsilateral to the 6-OHDA lesion compared to the intact hemisphere. The extent of denervation was most prominent in the central, mediolateral, and caudal parts, while relatively more fibers remained in the rostral part of the striatum (Fig. 3A). Stereological quantification of the density of TH-positive fibers in the 6-OHDA-lesioned striatum demonstrated a significant preservation of TH-positive fibers in rats overexpressing human parkin at 7 days and 3 weeks postlesion (30 ± 5 and 29 ± 4% of the intact side) compared to the LV-eGFP-treated rats (18 ± 1 and 15 ± 3%) (Fig. 3B). In contrast, we saw no difference between the eGFP-transduced rats and the 6-OHDA control group.

### Overexpression of Human Parkin Attenuates Motor Impairment After 6-OHDA Lesioning

To examine whether neuroprotection by parkin could result in behavioral improvement, we carried out three different motor tests 3 weeks postlesioning. In the amphetamine-induced rotation test rats injected with LV encoding eGFP were not different from the 6-OHDA lesion control group (Fig. 4A). In contrast, rats overexpressing parkin in the SN performed significantly fewer ipsilateral turns after administration of amphetamine. Second, we administered the cylinder test to register impairments in forelimb use (Fig. 4B). Three weeks postlesioning, all rats were severely impaired in the cylinder test. The usage of the lesioned paw was similar for the eGFP group and the 6-OHDA control group, i.e., 22 ± 10 and 19 ± 10%, respectively. The parkin group also showed impairments in this test, but the ability to use the impaired paw was significantly better (33 ± 8%). Finally, we compared the performance of the various groups in the skilled reaching test (Fig. 4C). We chose the 6-OHDA injection site contralateral to the side of the paw preferentially used in the skilled reaching test. The rats overexpressing parkin showed no significantly higher reaching success compared with the eGFP or 6-OHDA group (P = 0.15). In all tests, the performance of rats injected with eGFP-LV or parkin-LV in the SN in the absence of a 6-OHDA lesion in the striatum was not different from that of normal untreated animals (data not shown).

To evaluate the duration of the neuroprotective effect of parkin, we tested a subgroup of animals for amphetamine-induced rotational behavior at two later time points, namely 20 and 40 weeks after 6-OHDA lesioning (Fig. 5). At 20 weeks postlesion, the parkin-treated rats still performed significantly fewer turns per minute than the eGFP or 6-OHDA group (P = 0.03). However, the turning behavior of the parkin-treated rats increased gradually and was not significantly different any more.

---

**TABLE 1: Overview of the various experimental groups of rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>SN (week 0)</th>
<th>Striatum (week 2)</th>
<th>Analysis (week 3)</th>
<th>Analysis (week 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 4</td>
<td>LV-eGFP</td>
<td>6-OHDA</td>
<td>Histology</td>
<td>Behavior + histology</td>
</tr>
<tr>
<td>n = 9</td>
<td>LV-eGFP</td>
<td>6-OHDA</td>
<td>Behavior + histology</td>
<td></td>
</tr>
<tr>
<td>n = 4</td>
<td>LV-eGFP</td>
<td>Saline</td>
<td>Behavior + histology</td>
<td></td>
</tr>
<tr>
<td>n = 4</td>
<td>LV-parkin</td>
<td>6-OHDA</td>
<td>Histology</td>
<td>Behavior + histology</td>
</tr>
<tr>
<td>n = 9</td>
<td>LV-parkin</td>
<td>6-OHDA</td>
<td>Behavior + histology</td>
<td></td>
</tr>
<tr>
<td>n = 4</td>
<td>LV-parkin</td>
<td>Saline</td>
<td>Behavior + histology</td>
<td></td>
</tr>
<tr>
<td>n = 5</td>
<td>Saline</td>
<td>6-OHDA</td>
<td>Behavior + histology</td>
<td></td>
</tr>
</tbody>
</table>

---

1. Data from a recent study.
from the two other groups at 40 weeks after 6-OHDA lesioning. Histological analysis of the brains of these rats confirmed that at this longest time point the density of TH-positive fibers in the striatum was reduced in the parkin group to the same level as in the eGFP group (data not shown). Interestingly, the dopaminergic cell numbers in the substantia nigra were still significantly higher in the parkin group (1545 ± 421, n = 5) than in the eGFP group (1004 ± 107, n = 5) (P = 0.02).

DISCUSSION

In this study we demonstrate that targeted overexpression of parkin in rat substantia nigra reduces 6-OHDA-induced degeneration of dopaminergic terminals and cell bodies. We have used a one-site intrastriatal 6-OHDA injection protocol, leading to a partial dopaminergic lesion, which was evidenced by behavioral impairment in three tests. Nigral overexpression of parkin clearly ameliorated the motor behavior of the 6-OHDA-lesioned rats. The improvements were statistically significant in the amphetamine-induced rotational test and in the cylinder test, but not in the skilled reaching test, although the same trend was evident. The amphetamine-induced rotation test and the cylinder test are well-established tests to assess motor performance and are widely used for this hemiparkinsonian model. However, discrepancies between the performance of

![FIG. 2. Nigral overexpression of parkin reduces 6-OHDA-induced dopaminergic cell loss. (A) Stereological quantification of the nigral lesion volume. The data are presented relative to the intact contralateral side ± SEM. The sizes of the dopaminergic lesions in eGFP- versus parkin-treated rats were similar after 1 week (n = 4), but significantly different 3 weeks postlesioning (***P < 0.001, n = 9). There was no difference between 6-OHDA-lesioned rats and eGFP-treated rats. (B) Stereological quantification of the number of dopaminergic neurons in the SN 1 and 3 weeks after 6-OHDA lesioning. The numbers of TH-positive cells in eGFP- versus parkin-expressing rats, relative to the contralateral nonlesioned side (± SEM), were significantly different 3 weeks postlesioning (***P < 0.001, n = 9). There was no difference between 6-OHDA-lesioned rats and eGFP-treated rats. (C) Stereological quantification of the number of dopaminergic neurons in the SN 3 weeks after 6-OHDA lesioning in different control groups. The number of TH-positive cells did not change by nigral transduction with LV-eGFP or LV-parkin in the absence of a 6-OHDA lesion. The number of TH-positive cells after 6-OHDA lesioning was not influenced by LV-eGFP transduction.](image-url)
unilateral dopamine-depleted rats in different tests have been described before [17]. Compared to the two other tests, the skilled reaching test requires more complex motor functions. There are several possible reasons no complete rescue was obtained in this test. First, the protection of the dopaminergic nerve terminals and cell bodies obtained with parkin is only partial. This could be due to incomplete transduction of the substantia nigra or to the fact that parkin does not interfere with all pathways affected by 6-OHDA toxicity. Also, we have scored the reaching performance of the rats only by counting misses and successful reaches, but no qualitative reaching analysis was performed. In addition, a number of studies have reported that unilaterally dopamine-depleted rats display impairment in skilled reaching not only with the paw contralateral to the lesion but also with the ipsilateral paw [18]. The bilateral impairment and the postural imbalance together with the limited recovery obtained with parkin LV probably explain why the motor performance in the skilled

FIG. 3. Overexpression of parkin in the SN protects dopaminergic nerve terminals in the striatum against 6-OHDA toxicity. (A) Immunostaining for TH in the striatum 3 weeks postlesioning. Photomicrographs show serial coronal sections spaced 50 μm apart through the striatum of a rat treated with LV encoding eGFP (top) or parkin (bottom). (B) Stereological quantification of the TH-immunoreactive fibers in the striatum. The volume of TH-positive fibers in the striatum in eGFP- versus parkin-treated rats, expressed relative to the contralateral, nonlesioned side, was significantly higher in the parkin group at 1 week (*P = 0.049, n = 4) and 3 weeks (*P = 0.017, n = 9) postlesioning. There was no difference between 6-OHDA-lesioned rats and eGFP-treated rats.

FIG. 4. Lentiviral vector-mediated overexpression of human parkin attenuates motor impairment after 6-OHDA lesioning. (A) Amphetamine-induced rotation test. The graph shows the ipsilateral rotational net asymmetry scores induced by amphetamine 21 days postlesioning. The ipsilateral turning behavior in rats overexpressing eGFP was similar to that of the 6-OHDA-lesioned control animals, excluding a vector injection artifact. In contrast, rats injected with LV encoding parkin in the SN made significantly fewer full-body turns per minute than the eGFP group (*P < 0.05, n = 5). (B) Cylinder test. The data represent the percentage of impaired forelimb contacts to the cylinder wall 20 days postlesioning. The rats overexpressing parkin used the impaired paw significantly more than the control eGFP-expressing group (*P < 0.05, n = 5). (C) Skilled paw reaching test. The data represent the percentage of successful reaches 3 weeks after 6-OHDA lesioning. The rats overexpressing parkin showed a slight improvement in reaching success compared to the eGFP-injected group. This difference was not significant (P = 0.15, n = 5).
reaching test was not significantly improved. Alternatively, other lesioning protocols (e.g., affecting the entire rostrocaudal extent of the lateral caudate putamen) or larger groups of animals might be required to reach statistical significance.

In terms of the duration of the neuroprotection conferred by lentiviral vector-mediated overexpression of parkin, we could show behavioral improvement in the amphetamine-induced rotations until 20 weeks after lesioning and histological preservation of the dopaminergic cell bodies, but not terminals, until 40 weeks postlesion. At this moment it is unclear why the neuroprotective effect slowly decreases with time, since it has been shown that lentiviral vectors mediate stable transgene expression in the brain. Nevertheless, the time window of the observed therapeutic effect might still be long enough for future clinical applications.

What is known about the cell biology of parkin? Mutations in the parkin gene have been linked to AR-JP [4]. Surprisingly, genetic inactivation of the parkin gene in mice [19–22] and Drosophila [23,24] failed to reproduce the typical dopaminergic cell loss, although more recently shrinkage and degeneration of a subset of dopaminergic neurons were detected in Drosophila parkin mutants [25,26]. Proteomic analysis of parkin knockout mice failed to confirm accumulation of most proposed parkin substrates, except for CDCRel-1 [27] and p38/JTV1 [28].

In cell culture, overexpression of parkin was shown to be neuroprotective against a variety of toxic insults [29–33]. The common denominator of all these toxic insults seems to be proteasomal impairment, which can be rescued by wild-type parkin, but not by the clinical mutants that lack E3 ligase activity. The role of parkin in dopamine-related cell death is still unclear. In PC12 cells, overexpression of parkin was ineffective against a variety of cell death mediators, among which was 6-OHDA [29]. Similarly, in SH-SY5Y cells parkin reduced dopamine-induced aggresome formation, but had no beneficial effect on neuronal survival [34]. In contrast, a more recent report demonstrated a protective effect of parkin overexpression in SH-SY5Y cells against dopamine- and 6-OHDA-induced apoptosis [32].

We reasoned that in vivo experiments were required to determine whether parkin protects against toxin-induced dopaminergic neurodegeneration. In vivo, parkin has been shown to suppress dopaminergic neurodegeneration induced by pael-R and α-synuclein in Drosophila [9,10]. More recently, the neuroprotective effects of parkin against α-synuclein-induced dopaminergic cell loss in rat striatum (L. Vercammen et al., in preparation). Here, we show that parkin overexpression is also protective in this classical toxin-induced PD model. Our results question the proposed mechanism of action of parkin and the role of Levys body (LB) formation. Indeed, the rat 6-OHDA lesion model is the most extensively studied animal model of PD and replicates many of the disease symptoms observed in PD patients [35], but it does not induce LB formation. 6-OHDA toxicity is mostly attributed to mitochondrial complex I inhibition and oxidative stress through the generation of reactive oxygen species [36]. On the other hand, 6-OHDA has also been linked to the ubiquitin–proteasome pathway, endoplasmic reticulum stress, and unfolded protein response [37–40]. Impaired clearance of oxidized proteins may facilitate protein aggregation, which in turn may impair proteasomal activity and cause oxidative stress. The overexpression of parkin could facilitate removal of damaged proteins by the ubiquitin–proteasome pathway. However, reliable and sensitive methods to assess proteasomal degradation in vivo are lacking. We have examined the accumulation of β-catenin as a marker of proteasome function in 6-OHDA and parkin-treated rats. However, no conclusive evidence of proteasomal impairment in 6-OHDA-treated rats has been obtained so far (data not shown). Most reports link parkin to the proteasomal degradation pathway, but the evidence that parkin facilitates α-synuclein degradation directly is controversial. Therefore, a more general neuroprotective effect of parkin should also be taken into consideration. In this regard, it will be interesting to analyze the involvement of (anti-)apoptotic pathways in our model. To narrow down further the mechanism of action of parkin in vivo, it would be worthwhile to investigate its potential benefit in other PD models such as the MPTP mouse model or the rotenone rat model. Future experiments are planned to understand better the kinetics of the neuroprotection, the effect of clinically relevant mutations, and the impact in other animal models of PD. Further mechanism-of-action studies should reveal the molecular base of the observed neuroprotection.
In conclusion, our data strongly support the potential of parkin-based gene or drug therapy for PD. Moreover, our results point to a benefit from parkin treatment not only for those patients carrying pathogenic parkin mutations but also for the majority of sporadic PD patients.

**Materials and Methods**

**Lentiviral vector construction and production.** The cDNA encoding human parkin (kind gift from Professor Alexis Brice, human GenBank sequence AB009973) was cloned into a lentiviral pHIV-derived transfer plasmid containing a central polyuridine tract sequence, the SIN-18 deletion, and the woodchuck hepatitis posttranscriptional regulatory element [13]. HIV-1-derived vector particles encoding hParkin or eGFP and pseudotyped with the envelope of vesicular stomatitis virus were produced in two-layer cell factories (Sanbio, Uden, The Netherlands) by transfecting 293T cells in serum-free medium, essentially as described before [41]. After concentration, the LV were resuspended in PBS.

**Cell culture and transduction.** One day prior to transduction, 25,000 SH-SY5Y cells were seeded in a 24-well plate. The next day the medium was replaced by Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum, 4 μg/ml Polybrene, and LV corresponding to 4 × 10^5 pfu per well. Two hours after transduction, the medium was refreshed. Two days posttransduction the cells were harvested in PBS containing 5 mM EDTA. The cells were lysed with 1% SDS containing 10 mM phenylmethylsulfonyl fluoride (Sigma, Belgium) and boiled for 5 min.

**Western blotting.** Ten micrograms of total protein was diluted in SDS-PAGE loading buffer and run on a 4–20% SDS-polyacylamide gel. The proteins were transferred to a PVDF membrane (Bio-Rad, Watford, UK). Detection was performed with a polyclonal rabbit anti-parkin antibody 1:10000 (Cell Signaling Technology, Beverly, MA, USA) using the ECL+ detection system (Amersham-Pharmacia, The Netherlands). Blots were stripped using Re-blot (Chemicon, Temecula, CA, USA) according to the manufacturer’s protocol and reprobed with rabbit polyclonal anti-a-tubulin antibody (1:10000, T5168; Sigma, Belgium) for 1 h and detected as above.

**Stereotactic injections.** All animal experiments were approved by the bioethical committee of the K.U. Leuven. Young adult female Wistar rats (Janvier, France) weighing 200–250 g were housed with free access to food and water under a 12:12 h dark:light cycle. All surgical procedures were performed under ketamine (60 mg/kg ip) and medetomidine (0.4 mg/kg) anesthesia using aseptic procedures. Surgery was performed in two sessions with use of a stereotactic head frame (Stoelting, IL, USA). All injections were made using a 30-gauge needle and a 10-μl Hamilton syringe. The various experimental groups are described in Table 1. First, some animals received unilateral injections of lentiviral vectors encoding hParkin or eGFP into the SN. Coordinates used for the SN were anteroposterior (AP) 5.3, lateral (LAT) +/−2.0, dorsoventral (DV) 7.2, calculated from the dura (tooth bar −3.3) using bregma as a reference. Two microliters of highly concentrated vector (10^5 pfu of p24/ml) supplemented with Polybrene (4 μg/ml) was injected at a rate of 0.25 μl/min. Two weeks later, a unilateral injection of 20 μg 6-OHDA into the striatum was administered (Sigma, calculated as free base, dissolved in 4 μl of 0.05% ascorbate saline). Coordinates used for the striatum were AP 0, LAT +/−2.0, DV −5.5 (tooth bar −3.3). The injection rate was 0.5 μl/min. The 6-OHDA solution was kept on ice, used fresh, and protected from light to minimize oxidation.

**Behavioral testing.** Amphetamine-induced rotational behavior was monitored in automated rotometer bowls (San Diego Instruments, San Diego, CA, USA) for 50 min after ip injection with 2.5 mg/kg d-amphetamine (Certa, Braine-l’Alleud, Belgium). Data are expressed as number of complete body turns per minute (net rotations = ipsilateral − contralateral turns). Nonlesioned control rats make fewer than 1 net rotation per minute. The cylinder test [42] was used to quantify forelimb use. Contacts made by each forepaw with the wall of 20-cm-wide clear glass cylinder were scored from the videotapes by an observer blinded to the animal’s identity. A total of 20 contacts were recorded for each animal. The number of impaired forelimb contacts was expressed as a percentage of total forelimb contacts. Nonlesioned control rats should score around 50% in this test. We used the skilled reaching test [43] mainly to determine the paw of preference of each individual animal. The rats were trained to perform the test correctly prior to testing, after food deprivation to about 90% of their initial body weight. On testing days, the rats were presented with 20 food pellets and the reaching session was video recorded. Reaching performance was scored as the percentage of successful reaches to total reaching.

**Histology.** Rats were sacrificed 1 and 3 weeks after 6-OHDA lesioning with an overdose of pentobarbital, followed by intracardial perfusion with 4% paraformaldehyde in PBS. After postfixation overnight, 50-μm-thick coronal brain sections were made with a Vibratome (Leica Microsystems, Wetzelern, Germany). Immunohistochemistry was performed on free-floating sections using antibodies raised against eGFP (rabbit polyclonal, 1:10,000, made in-house), Parkin (rabbit polyclonal, 1:50; Cell Signaling; or rabbit polyclonal, 1:2000 made in-house), and tyrosine hydroxylase (rabbit, 1:3000; Chemicon). Sections were pretreated with 3% hydrogen peroxide and incubated overnight with primary antibody in 10% normal swine serum (Dako, Glostrup, Denmark). As secondary antibodies we used a 1:300 dilution of biotinylated anti-rabbit IgG (Dako), followed by incubation with streptavidin–biotin–horseradish peroxidase complex (ABC Kit; Dako). EGF and TH immunoreactivity was visualized using 3,3-diaminobenzidine (DAB) as a chromogen; parkin was visualized using a nickel-enhanced DAB staining method. For fluorescent double staining for parkin/TH and parkin/NeuN, sections were rinsed three times in PBS and preincubated in 5% normal goat serum (NGS) for 1 h at room temperature. They were then incubated overnight in 5% NGS, 0.1% Triton X-100, rabbit anti-parkin (1:200; made in-house), and mouse anti-TH (1:200; Chemicon) or mouse anti-NeuN (1:200; Chemicon). After three rinses in PBS-0.1% Triton X-100 the sections were incubated in the dark for 1 h in fluorochrome-conjugated secondary antibodies: goat anti-mouse Alexa 633 (1:200, A21050; Molecular Probes, Invitrogen, The Netherlands) and goat anti-rabbit Alexa-488 (1:200, A11070; Molecular Probes). After being rinsed in PBS and mounted, the sections were coverslipped with Mowiol.

**Stereological quantification.** The number of TH-positive cells in the substantia nigra was estimated using an optical fractionator sampling design [44] in a computerized system (Stereoinvestigator; MicroBrightField, Magdeburg, Germany). Sections used for counting covered the entire SN. Every fifth section throughout the entire SN was analyzed, with a total of six sections for each animal. The coefficients of error, calculated according to the procedure of Schmitz and Hof as estimates of precision [44], varied between 0.05 and 0.10. The volume of TH-immunoreactive neurons in the SN- and TH-positive fibers in the striatum was determined by stereological volume measurements using the Cavalieri method as described before [13]. Every fifth section covering the entire extent of the SN or striatum was included in the counting procedure. For each section the volume of TH-positive fibers or cells was calculated based on area measurements in the lesioned and the intact hemisphere. The values for the lesioned side were expressed relative to the control hemisphere. The coefficients of error for SN and striatum varied between 0.07 and 0.13.

**Statistical analysis.** Statistical analysis was performed using the Statistics software package (StatSoft, Inc.). Results are expressed as means ± standard error of the mean. Analysis of variance with post hoc Sheffe’s test was used for intergroup comparisons. Statistical significance level was set as follows: * if P < 0.05, ** if P < 0.01.

**Acknowledgments.** We are very grateful to A. Brice (Groupe Hospitalier Pitié-Salpêtrière, Paris, France) for the parkin cDNA. We acknowledge excellent technical assistance from F. Courn, A. Cuppers, K. Eggemont, M. Michiels, and S. Willems. V.B. and C.V.d.H. are Postdoctoral Fellows of the Flemish Fund for Scientific Research.
REFERENCES


