Kinetics of Microglial Activation and Degeneration of Dopamine-Containing Neurons in a Rat Model of Parkinson Disease Induced by 6-Hydroxydopamine

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Abstract

In both Parkinson disease and in animal models of Parkinson disease, there is a microglial reaction in addition to the loss of dopaminergic neurons in the ventral midbrain. To determine the pathological role of this microglial reaction, we analyzed the kinetics of microglial activation and dopaminergic cell death induced in rats with the neurotoxin 6-hydroxydopamine. As early as Day 1 after the injection, there was a decline in the motor performance of the 6-hydroxydopamine–lesioned rats that correlated with a reduction of dopaminergic innervation of the contralateral striatum. Loss of dopaminergic neurons in the ventral midbrain developed a few days later and seemed to follow a specific temporospatial pattern. Degenerating neurons and activated microglia were seen only in areas in which dopaminergic cells were no longer observed, suggesting that the loss of the dopaminergic phenotype preceded the degenerative process. In sham-lesioned rats, there was a transient activation of microglia in the vicinity of the needle tract without any cell degeneration. This chronological pattern supports the hypothesis that microglial activation is a secondary rather than primary phenomenon in dopaminergic cell degeneration induced by 6-hydroxydopamine.

Key Words: 6-OHDA, Microglia, Neurodegeneration, Parkinson disease, Tyrosine hydroxylase.

INTRODUCTION

Parkinson disease (PD) is a neurodegenerative disorder characterized by progressive cell loss of dopamine-containing neurons of the substantia nigra pars compacta (SNpc) that leads to a dopamine deficiency in the striatum, which is responsible for most of the motor manifestations in patients, including akinesia, rigidity, and tremor (1). Symptomatic treatments aimed at replacing dopamine or correcting the effects of dopamine deficiency on the basal ganglia have proved extremely beneficial in most patients (2). In the absence of effective neuroprotective therapies, however, the degenerative processes progress after several years of disease and cause disabling symptoms that are not amenable to current treatments. Knowledge of the exact mechanisms responsible for dopaminergic cell death in PD is a prerequisite for developing treatment to stop or slow the neuronal cell loss.

Brain microglia change from a quiescent to an activated phenotype under various circumstances, such as traumatic or ischemic injury, infection, and neurodegenerative disease. Activated microglia are identified by morphological changes and the expression of specific markers, such as major histocompatibility complex class II and ED1 in the rat (3, 4). After the report of McGeer et al (5) of HLA-DR II microglial cells in the SNpc of PD patients, a number of studies have provided evidence indicating that dopaminergic cell death is associated with microglial activation in PD patients both postmortem (6, 7) and in vivo using positron emission tomographic scan (8, 9); this has also been reported in N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–intoxicated humans (10) and in animal models of PD (11). There are, however, many questions as to the role of activated microglia in these conditions, including 1) whether activated microglia are primarily responsible for the neuronal cell death or solely secondary to the degenerative process and 2) whether they play a protective or a deleterious role in the SNpc.

Microglia exert a key homeostatic role in the brain by scavenging excess neurotoxins and removing dying cells and cellular debris (12, 13). Activated microglia release trophic factors, such as brain-derived neurotrophic factors (14, 15) and glial-derived neurotrophic factors (16); they also produce proinflammatory molecules, such as interleukin-1β (17), tumor necrosis factor (18), and nitric oxide (19), which are known to have neurotoxic properties. The normal SNpc is one of the brain areas with the highest microglial density (20). This probably reflects its key role in protecting dopaminergic neurons, which are particularly exposed through dopamine metabolism to a high concentration of free radicals (21). Experimental activation of nigral microglia in rodents induces dopaminergic cell loss. For example, the bacterial antigen lipopolysaccharide added to a mixed dopaminergic neuron/glia culture activated the microglia, which released proinflammatory molecules that led to dopaminergic cell death (22); dopaminergic cell loss was observed in vivo after direct bacterial (23) or viral (24) infection and after injection.
of lipopolysaccharide into the SNpc (25–27), and anti-inflammatory treatment prevented the cell loss induced by lipopolysaccharide injection (28).

To clarify the role of activated microglia in dopaminergic cell loss in PD, we analyzed the kinetics of the degenerative process in a rat model of PD. Dopaminergic neurons and their microglial surroundings were studied sequentially from Day 1 (D1) to D35 after injection into the medial forebrain bundle (MFB) of 6-hydroxydopamine (6-OHDA), a neurotoxin that leads to deleterious oxidative stress within dopaminergic neurons.

**MATERIALS AND METHODS**

**Lesion Surgery**

A total of 31 male Sprague-Dawley rats (Charles River, Rouen, France; 200–250 g body weight at the beginning of the study) were used. They were housed at 22°C under 12-hour light/12-hour dark conditions with ad libitum access to food and water. The local ethics committee on animal research approved the treatment of the animals and their conditions. Each animal was handled daily for several minutes during the week before surgery.

The animals were anesthetized with an intraperitoneal injection of Rompun/ketamine (1 mL/kg) and were placed in a stereotaxic frame (Stoelting, Wood Dale, IL). 6-Hydroxydopamine was dissolved in 0.02% ascorbate saline at a concentration of 1.5 μg/μL and was injected (0.5 μL/minute) in 2 deposits (2.25 and 2.85 μg, respectively) at the following coordinates (in millimeters relative to bregma and the surface of the dura mater): anterior (A), +1.2; L, 2.4, respectively (29). Injections of 6-OHDA in the right MFB were done in 21 rats; 7 rats underwent similar surgery but with injection of vehicle alone (sham-lesioned rats); 3 other naïve rats served as controls.

**Motor Behavioral Assessments**

On the day before surgery and on the day of death (both between 10:30 and 11:30 AM), the motor behavior of the animals was assessed using a modified version of the stepping test (30, 31). Briefly, the experimenter firmly suspended the hindquarters of the rat while it supported its weight on its forelimbs. The experimenter then moved the rat backward along the table (0.9 m in 5 seconds) 3 times consecutively per session. All the sessions were video recorded to allow the number of adjusting steps to be counted by an investigator blinded to the state of the rat (i.e. 6-OHDA-lesioned or sham-lesioned, before or after surgery). For each session, the total score calculated was the sum of the number of adjusting steps observed in the 3 tests (a score for the right paw and a score for the left paw).

**Tissue Preparation**

Groups of 3 lesioned rats and 1 sham-lesioned rat were randomly assigned for death at 1, 5, 7, 14, 21, 28, and 35 (D1–D35) days after surgery. The animals were deeply anesthetized with Rompun/ketamine (1 mL/kg, i.p.) and perfused transcardially with ice-cold 4% paraformaldehyde in PBS, pH 7.4. Brains were rapidly removed, immersed in the same fixative for 1 hour at 4°C, and stored in 25% PBS sucrose for 24 to 48 hours before cutting. Coronal sections, 40-μm thick, through the midbrain and through the striatum were cut as serial sections on a freezing cryostat (Leica, CM 3050) and collected in sequence in 24-well culture plates (Falcon, Becton Dickinson Labware S.A., Le Pont de Claix, France) containing 1 mL of PBS in each well.

**Immunohistochemistry and Fluoro-Jade B Staining**

For each rat, every sixth section was processed free-floating for tyrosine hydroxylase (TH) and OX42 (CD11b/c antibody supernatant from American Type Culture Collection) immunohistochemistry, respectively, as previously described (32, 33). Briefly, sections were incubated overnight in a 1:500 dilution of primary anti-rat antibodies (rabbit anti-TH; Pel-Freeze, Brown Deer, WI) or in a mouse anti-OX42 supernatant. The sections were first immersed in a 1:500 dilution of secondary biotinylated antibody (anti-rabbit IgG raised in donkey; Jackson ImmunoResearch Laboratories, West Grove, PA; and anti-mouse IgG raised in goat; Jackson ImmunoResearch Laboratories) and then in the peroxidase-conjugated avidin-biotin complex (4.5 μL Solution A and 4.5 μL Solution B of the Vectastain ABC kit; Vector Laboratories, Burlingame, CA; for 1 mL PBS, prepared 30 minutes before use). Finally, the peroxidase was revealed by immersion in a solution of 3,3′-diaminobenzidine (Vector Laboratories) processed according to the manufacturer’s instructions.

Every sixth section of the ventral midbrain of D1, D7, D14, and D28 6-OHDA-lesioned rats was processed for TH-OX42 double labeling immunohistochemistry. Free-floating sections were incubated overnight with a mixture of a 1:500 dilution of the rabbit anti-TH and a 1:500 dilution of the mouse anti-OX42 and then immersed in a mixture of secondary antibodies, 1:200 dilution of Cy3-conjugated donkey anti-mouse and a 1:200 dilution of Cy2-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 2 hours. After staining, sections were mounted on gelatin-coated slides, air-dried, and coverslipped with GEL/MOUNT (Biomeda, Foster City, CA). For each rat, every sixth section was stained for Fluoro-Jade B (34). The sections were mounted with distilled water onto gelatin-coated slides and dried for 20 minutes on a slide warmer at 45°C. Slides were then immersed successively in 100% ethyl alcohol (3 minutes), 70% alcohol (1 minute), and distilled water (1 minute) in a solution of 0.06% potassium permanganate (15 minutes) in distilled water (1 minute) and in a 0.001% Fluoro-Jade B (Histo-Chem, Inc., Jefferson, AR) 0.1% acetic acid solution (30 minutes). After staining, the sections were rinsed with 3 immersions 1 minute each in distilled water, dried for 10 minutes on a slide warmer at 45°C, immersed in xylene (2 minutes), and coverslipped with EUKIT (Sigma, St. Louis, MO).

**Image Acquisition, Processing, Quantification**

For qualitative analysis, sections were examined under a Nikon Eclipse E600 microscope (Tokyo, Japan) equipped for fluorescence with a filter suitable for visualizing fluorescein isothiocyanate or Alexa 487. Images were acquired...
FIGURE 1. Two types of microglial cells immunostained for OX42 on a ventral midbrain section of a 6-hydroxydopamine-lesioned rat. An amoeboid intensely stained (i.e. activated) cell (black arrowhead) and a ramified slightly stained (i.e. quiescent) microglial cell (black arrow). The densities of these 2 OX42-positive cell types were measured using an unbiased stereological counting method. A counting frame (50 × 50-μm squares regularly spaced by 200 μm horizontally and vertically) was systematically superimposed after a random start point was determined by the Mercator software. Only stained cell somata included within the square and not crossing the red lines (left and bottom) and cells crossing the green line (right and top) were counted. Scale bar = 10 μm.

FIGURE 2. The kinetics of nigral dopaminergic lesion and of motor behavioral changes before (D0) and 1, 5, 7, 14, 21, 28, and 35 days after (D1–D35) 6-hydroxydopamine injection in the medial forebrain bundle. (A) Stepping test performance for the paw contralateral to the injection site; 1-way analysis of variance (ANOVA) significant effect, *p < 0.001) followed by post hoc tests: ***p < 0.001 compared with before surgery (D0), #p < 0.05 compared with the first day after surgery (D1). (B) Optical density measurement on lesioned striatum section stained for tyrosine hydroxylase (TH) immunohistochemistry; 1-way ANOVA (significant effect, p < 0.001) followed by post hoc tests: ***p < 0.001 compared with controls. (C) Correlations between the mean score on the stepping test performed just before the animals were killed and the TH optical density of the contralateral striatum (black square) and the percentage of remaining TH-positive cells in the contralateral substantia nigra (black triangle).
in right and left ventral midbrains for TH staining and only in the right ventral midbrain (lesioned side) for OX42 staining. For each section, a line was drawn around the perimeter of the SNpc and the ventral tegmental area (VTA), the boundaries being defined with reference to the Paxinos and Watson atlas (37). A counting frame (50 × 50-μm squares regularly spaced by 200 μm horizontally and vertically) was superimposed on the section at random. Only stained cells included within the squares were counted (Fig. 1). For TH staining, a cell was considered to be stained when staining was observed in more than half of the section thickness and when a stained nucleus was clearly visible within it. For OX42, a cell was considered to be stained when the largest diameter of stained somata was more than 5 μm. Ramified slightly stained and amoeboid and intensely stained OX42-positive cells were differentiated (Fig. 1). To characterize the OX42-positive cells further, the density of OX42-positive cells from a series consisting of every sixth ventral midbrain section and the density of ED1-positive cells (staining with 1:500 mouse anti-ED1 supernatant [American Type Culture Collection]) from a series of immediately adjacent sections were measured in 6 rats lesioned with a similar 6-OHDA injection in the MFB. The density of amoeboid and intensely stained OX42-positive cells was 30% lower than the density of ED1-positive cells (significant correlation between the 2 densities, r = 0.85, p < 0.001).

Extensive counting of stained cells was performed for TH immunohistochemistry and Fluoro-Jade B staining at 5 different rostrocaudal levels of the SNpc at the level of the exiting third nerve, 200 μm and 400 μm rostral and 200 μm and 400 μm caudal to this level. For the left side and the right side, digitized images comprising the whole SNpc from the accessory terminal tract medially to the lateral border of the mesencephalon were obtained using the 40× magnification of an optical microscope (Nikon Eclipse E600). The perimeter of the SNpc and of the VTA was drawn as previously described, and all stained cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD). As previously described (33), split-cell counting errors were corrected using the formula of Abercrombie (38), where \( N = n(\pi t d) \) (\( N \), total number of cells; \( n \), number of cells counted; \( t \), section thickness; and \( d \), cell diameter). The total number of cells in the SNpc was calculated using the formula of Konigsmark (39), where \( N_t = N_x X S_y/S_x \) (\( N_x \), total number of cells; \( N_y \), number of cells counted; \( S_x \), total number of sections through the SNpc; \( S_y \), number of sections in which cells were counted). There was no significant difference in the number of TH-positive cells per ventral midbrain calculated by the formula of Konigsmark (39) applied to the extensive counting of positive cells on 5 rostrocaudal levels of the midbrain and the number of TH-positive cells calculated by the unbiased stereological counts with the optical fractionator method; an intraclass correlation (p < 0.001) verified the consistency between the 2 methods of calculation.

**Data Analysis**

Data are expressed as mean ± SEM. The stepping test scores before and after lesion in 6-OHDA– and sham-lesioned rats and the number of remaining TH-positive cells, the density of OX42-positive cells, and the number of Fluoro-Jade B–positive cells in the sham-lesioned rats and the control rats were analyzed using a t-test. After having verified the normal distribution of the values, a 1-way analysis of variance was used to analyze the number of remaining TH-positive cells, the density of OX42-positive cells, and the number of Fluoro-Jade B–positive cells in the 6-OHDA–lesioned rats, with the different time points after the toxic injection as an independent variable. If the factor time-point showed a significant main effect, post hoc Newman-Keuls tests were performed to make all possible comparisons. Correlations were analyzed using Pearson test. All statistical tests were performed using Prism 4.0 software (GraphPad, San Diego, CA).

**RESULTS**

**Evaluation of Motor Behavior After Unilateral 6-OHDA Injection**

In the stepping test, the performance of the left limb (contralateral to the injection site) in the sham-lesioned animals was not significantly different before (20.29 ± 0.75 steps) and after surgery (19.48 ± 0.69 steps) and was similar to the performance observed in control rats (21.67 ± 0.88 steps). In both sham-lesioned rats (before and after surgery) and controls, there was no significant difference in performance between the left and the right forelimb. The performance of the left forelimb in the 6-OHDA–lesioned rats at every time point after surgery was significantly altered compared with that of control rats (p < 0.001) and to their own scores before surgery (20.89 ± 0.65, p < 0.001). There was no statistical difference between the scores obtained at D1, D5, and D7 or between the scores obtained at D14, D21, D28, and D35. The latter scores were significantly lower than the score at D1 (p < 0.05) (Fig. 2A).

**Characterization of the Unilateral 6-OHDA Lesion**

The OD of TH immunostaining in the right striatum was not significantly different between sham-lesioned (percentage of OD on the lesioned side compared with the non-lesioned side, 99.14% ± 0.63%) and control rats (99.67% ± 1.45%). Compared with control rats, the OD of TH immunostaining in the right striatum of the 6-OHDA–lesioned rats was significantly lower at each time point after surgery (p < 0.001). There were no significant differences between the OD measurements at the different time points, but there was a tendency for the OD to be lower in rats analyzed 7 days or more after surgery (Fig. 2B). There was a significant correlation between the mean score on the stepping test performed just before the animals were killed and the OD (r = 0.91, p < 0.001) (Fig. 2C).

The numbers of TH-positive cells were not significantly different between sham-lesioned and control rats in either the SNpc (percentage of cells on the lesioned side compared with the nonlesioned side, 100.8% ± 2.06% and 95.52% ± 1.12%, respectively) or the VTA (100.6% ± 1.98%...
and 101.3% ± 4.84%, respectively). Compared with control rats, in which a similar number of TH-positive cells was observed in the right and left SNpc and in the right and left VTA, there was a loss of TH-positive cells on the lesioned side compared with the nonlesioned side; this reached significance in the SNpc for animals analyzed 14 days or more after surgery and in the VTA for animals analyzed 7 days or more after surgery (Figs. 3A, E). Compared with

FIGURE 4. Tyrosine hydroxylase (A) and OX42 (B) immunostaining and Fluoro-Jade B (D) staining on a section of the ventral midbrain 7 days after 6-hydroxydopamine injection. Section double-stained for tyrosine hydroxylase and OX42 merged with the immediately adjacent section stained for Fluoro-Jade B (C), showing the concomitant localization of intensely stained OX42-positive cells (i.e., activated microglia) and Fluoro-Jade B-positive cells in dopaminergic regions (substantia nigra pars compacta and ventral tegmental area) where no more tyrosine hydroxylase-positive cells were visualized. Scale bar = 100 μm.

FIGURE 3. The kinetics of the degenerative process in the substantia nigra pars compacta (SNpc) analyzed before (D0), and 1, 5, 7, 14, 21, 28, and 35 days after (D1–D35) 6-hydroxydopamine injection in the medial forebrain bundle (A–D) and in the ventral tegmental area (VTA) (E–H). (A, E) Percentage of remaining tyrosine hydroxylase (TH)-positive cells on the lesioned side compared with the nonlesioned side; 1-way analysis of variance (ANOVA) significant effect, p < 0.001 for both SNpc and VTA) followed by post hoc tests: *p < 0.05, **p < 0.01, ***p < 0.001 compared with D0. (B, F) Number of Fluoro-Jade B-positive (FJ-B+) cells within the lesioned side; 1-way ANOVA (significant effect, p < 0.001 for both SNpc and VTA) followed by post hoc tests: *p < 0.05, **p < 0.01, ***p < 0.001 compared with D0. (C, G) Density of OX42 amoeboid and intensely stained cells (activated microglia) on the lesioned side; 1-way ANOVA (significant effect, p < 0.001 for the SNpc, p < 0.05 for the VTA) followed by post hoc tests: *p < 0.05, **p < 0.01, ***p < 0.001 compared with D0 and, ##p < 0.01 D7 compared with other time points. (C', G') Density of all OX42-positive cells on the lesioned side. (D, H) Correlations between the numbers of FJ-B+ cells and the densities of activated microglia on the lesioned side (for the SNpc, r > 0.95, p < 0.001; for the VTA, r > 0.90, p < 0.01). Error bars represent SEM.

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FIGURE 5. Distribution of amoeboid and intensely stained OX42-positive cells on 5 rostrocaudal sections (5.93 to 5.09 mm from bregma) of the ventral midbrain of rats 1, 7, 14, and 28 days after the injection of 6-hydroxydopamine (A) or the vehicle alone (B) in the medial forebrain bundle. Data were generated using MapTools V1.10 software (Explora Nova), which provides the cell count within each square of the stereological analysis performed with the Mercator software. The gray scale indicates the number of cells counted within each 50 × 50-μm² square of the counting frame superimposed on the substantia nigra pars compacta and the ventral tegmental area.
control rats, TH-positive cell loss was mainly observed in the medial and the posterior part of the SNpc in rats analyzed 5 and 7 days after 6-OHDA injection. From D14, the loss was also observed in the more anterior part of the nucleus. From D28, the spatial pattern of cell loss did not change; there was preservation of the most lateral TH-positive neurons. In the VTA, there was no specific temporospatial pattern of TH-positive cell loss, but the lesion was slightly more severe in its lateral part. There was a significant negative correlation between the mean score on the last stepping test performed before animals were killed and the percentage of remaining TH-positive cells ($r = 0.62, p < 0.001$) (Fig. 2C).

**Degeneration of Dopamine-Containing Cells**

A few sparsely scattered Fluoro-Jade B–positive cells were observed in the control rats (2.28 ± 0.75 in the SNpc; 3.00 ± 1.08 in the VTA) and in the sham-lesioned rats (4.00 ± 1.47 in the SNpc; 3.75 ± 1.38 in the VTA). There was no significant difference in the number of Fluoro-Jade B–positive cells between these 2 groups of animals.

Numerous Fluoro-Jade B–positive cells were observed in the 6-OHDA–lesioned rats, but only in the ventral midbrain (i.e. SNpc and VTA) ipsilateral to the injection site. Fluoro-Jade B–positive cells had a similar morphology to TH-positive cells observed in sections at the same level in control rats, but they were slightly smaller in diameter (Fig. 4D). Compared with control rats, the number of Fluoro-Jade B–positive cells was significantly higher in the SNpc of animals analyzed 5, 7, 14, and 21 days after the lesion and in the VTA of animals analyzed 5, 7, 14, and 14 days after the lesion (Figs. 3C, G). Amoeboid and intensely stained OX42-positive cells were observed almost exclusively in the ventral midbrain areas where Fluoro-Jade B–positive cells were localized (Fig. 4). There was a significant correlation between the density of amoeboid and intensely stained microglial cells and the number of Fluoro-Jade B–positive cells in both the SNpc ($r = 0.95, p < 0.001$) (Fig. 3D) and the VTA ($r = 0.90, p < 0.01$) (Fig. 3H).

**DISCUSSION**

We describe the detailed kinetics of the degenerative processes in a rat model of PD, in which dopaminergic cell death was induced by 6-OHDA injection in the MFB. The main results are as follows: 1) the loss of TH in striatal terminals leading to the dopamine deficiency responsible for abnormal motor behavior preceded the disappearance of TH in cell somata within the ventral midbrain; 2) the loss of TH phenotype preceded the cell degenerative process in both the VTA and the SNpc; and 3) the microglial activation was strictly concomitant with the neuronal degeneration and seemed to be a secondary rather than a primary phenomenon associated with dopaminergic cell death. The injection site (i.e. the MFB) and the dose of 6-OHDA were chosen to obtain, with the highest reproducibility, a partial midbrain dopaminergic lesion sufficient to induce a quantifiable level of abnormal motor behavior (32). The topographic pattern of dopaminergic cell lesion in this model is similar to that observed in PD, with the most severe lesion in the posterior and lateral part of the SNpc and a moderate lesion within the VTA (33). 6-Hydroxydopamine enters dopaminergic neurons through the dopamine transporter and induces strong oxidative stress by both monoamine B enzymatic oxidation and auto-oxidation and a defect in the mitochondrial respiratory chain within the cell (40). In contrast, the mechanisms of dopaminergic cell death in PD patients are not yet well known (41). They are likely caused by an interaction between multiple genetic and environmental factors and might, therefore, vary among patients (42).

Nevertheless, oxidative stress and a defect in the mitochondrial respiratory chain have also been identified in the ventral midbrain of PD patients (43, 44). Thus, despite the likelihood of mechanistic differences between the rat model and the
human disease, they share some similarities, and oxidative stress might represent a common final pathway in dopaminergic cell degeneration in both.

Abnormalities in motor behavior assessed by the stepping test were evident as early as D1 after the injection of 6-OHDA. These abnormalities were contemporaneous with decreased TH staining in the striatum, and their severity correlated with the staining level decrease in the level of staining. The decrease of TH staining in the striatum preceded that within the ventral midbrain. This chronology could be explained either by a dysfunction of the vesicle axonal transport that prevents TH transport to the striatal terminals or by a dying-back neuropathopshy with a loss of striatal terminals preceding the loss of neuronal cell bodies. There is evidence for the latter mechanism in MPTP-intoxicated mice (45) and a monkey model of PD (46). Moreover, a recent study reconciles the 2 mechanisms by demonstrating that the dysfunction of the vesicle axonal transport induced by MPP+, the MPTP metabolite, leads to a dying-back neuropathopshy (47).

The first change that we observed in the ventral midbrain was the disappearance of TH-positive neurons. Initially, the loss of TH-positive cells, observed from the fifth day after the 6-OHDA injection, was restricted to the posterior ventral midbrain at the junction between the VTA and the SNpc (because of the small number of cells lost relative to the total number of TH-positive cells in the whole region, the loss was significant only from Day 7 in the VTA and from Day 14 in the SNpc). It then seemed to spread from posterior to anterior and from medial to lateral, as has also been observed in the PD midbrain (48). In 6-OHDA-lesioned rats at D35, some TH-positive cells were still observed within the VTA without any specific localization. The ventromedial region of the SNpc was almost devoid of any TH-positive cells, whereas the density of TH-positive cells in the lateral region of the SNpc seemed to be similar to that observed in control rats. This specific pattern of lesion could be caused by a concentration gradient of 6-OHDA within the ventral midbrain, that is, a high concentration in the vicinity of the needle and a progressive decrease with distance. Another explanation could be differences in the vulnerability of dopaminergic cells. Cells in the VTA might be less vulnerable than those of the medial region of the SNpc, which might explain the less severe lesion in the former region despite their being at a similar distance from the injection site. The Fluoro-Jade B histochemical staining was used to identify neurons undergoing degeneration. Fluoro-Jade B is an anionic fluorescein derivative reported to specifically stain degenerating neurons regardless of the mechanism (apoptosis or necrosis) (34, 49). Fluoro-Jade B–positive cells observed in our study are likely previously TH-positive cells. They were localized exclusively in regions, namely, the VTA and the SNpc, where most of the neurons are TH-positive neurons; their shapes and orientations were similar to the TH-positive cells observed at the same level in control rats. The Fluoro-Jade B–positive cells, however, seemed to be slightly smaller than the TH-positive cells usually seen in these regions. This is likely caused by the cell shrinkage typical of cells in apoptosis, that is, the mechanism of cell death that occurs in dopaminergic cells exposed to 6-OHDA (50). Chemical processes used for Fluoro-Jade B staining prevent double labeling with TH immunohistochemistry being performed, but careful analysis of Fluoro-Jade B–stained and adjacent TH-immunostained sections did not suggest that many (if indeed any) cells were concomitantly stained. Moreover, Fluoro-Jade B–positive cells were observed almost exclusively in regions devoid of TH-positive cells. These results strongly suggest that the loss of TH phenotype precedes the cell degeneration per se. The postmortem observation of TH-negative cells containing neuromelanin, a pigment typical of dopaminergic cells in human SNpc of advanced PD patients and not in controls, suggests a similar chronology in the human disease (51). The loss of TH phenotype first may also explain the recovery observed in monkeys treated with 1 or only a few MPTP injections. In these animals, some dopaminergic cells may have transiently stopped producing dopamine, thereby inducing parkinsonian symptoms but without an irreversible degenerative process being triggered. Because the toxic exposure was not maintained, the animals recovered.

The OX42 antigen (CD11b/c), associated with the complement type-3 receptor, is a marker of both activated and quiescent microglia (3). In the present study, we used the morphological criteria of intense OX42-positive staining and amoeboid shape to identify activated microglia. Most of the cells identified as activated had the characteristics of macrophages, as confirmed by ED1-positive staining on adjacent sections (data not shown). Very few sparsely scattered activated microglia were observed within the control rat brains. In sham-lesioned rats, a high density of activated microglia was observed as early as 1 day after surgery in the vicinity of the needle trajectory as a reaction to the traumatic injury. In sham-lesioned rats at D28 and D35 after surgery, microglia were similar to those of control brains. None of the sham-lesioned rats had Fluoro-Jade B–positive neurons or a significant decrease in the number of TH-positive cells in this region. Much stronger microglial activation was observed in the 6-OHDA–lesioned rats, with a higher density of activated microglia in the vicinity of the needle trajectory and diffusion to the VTA and the medial part of the SNpc.

The exact role of microglial activation in dopaminergic neuronal loss in PD has been intensively debated (52–54). Our results suggest that microglial activation is mainly a phenomenon secondary to the dopaminergic cell degeneration induced by 6-OHDA. If microglial activation were a primary phenomenon, we would have observed activated microglia infiltrating apparently still normal regions, that is, with TH-positive neurons or at least with nondegenerating cells. In fact, we did not observe activated microglia in areas without Fluoro-Jade B–positive degenerating neurons. Moreover, the activation of microglia induced by the needle injury did not induce any degeneration of dopaminergic cells in its vicinity. These conclusions are fully in line with the previously observed atypical microglial activation in a similar 6-OHDA rat model (55). Microglia might in fact exacerbate dopaminergic cell death induced by 6-OHDA only in the event of its primed peripheral activation through a systemic inflammation; a recent observation suggests a putative...
accelerating role of systemic inflammation in PD degenerative process (56). As a phenomenon secondary to neuronal cell death, the main role of activated microglia would be to clear away degenerating cell debris. A few activated microglia were still observed in the SNpc 35 days after the 6-OHDA injection. Whether they could play a role in maintaining ongoing deleterious inflammation after the initiating agent has disappeared, as has been suggested to occur with MPTP intoxication (57), merits further investigation.

Degeneration of dopaminergic cells induced by 6-OHDA lesion in rats is probably different from what occurs in the ventral midbrain of PD patients. The results of our study indicate that microglia likely play only a secondary role in activation of cell death. If this observation in an animal model resembles the reality in PD, this might account for disappointing results of clinical trials of various anti-inflammatory treatments in this disease (58) and why the regular use of anti-inflammatory treatment does not seem to reduce the risk of PD (59, 60).

REFERENCES