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Time Course of Dopaminergic Cell Death and Changes in Iron, Ferritin and Transferrin Levels in the Rat Substantia Nigra after 6-Hydroxydopamine (6-OHDA) Lesioning

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Parkinson's disease is characterized by dopaminergic cell death in the substantia nigra. The underlying mechanism is, however, unknown. Though there are increasing lines of evidence showing iron accumulation in the Parkinsonian substantia nigra, it still remains obscure whether increased iron is the primary cause of dopaminergic cell death, or just a consequence of the pathological process. It is also unclear how iron gains access to the Parkinsonian SN. To gain more understanding in these areas, the present study investigated the time course of dopaminergic cell death and of changes in the level of iron, ferritin and transferrin. The results showed that iron was increased after the significant nigral dopaminergic cell death induced by 6-hydroxydopamine injection into the rat substantia nigra. On the other hand, the expression of transferrin was decreased. However, there was a temporal increase in the number of ferritin positive microglia. The results indicated that iron increase was not the primary cause of dopaminergic cell death in the Parkinsonian rat. It was most likely the result of an accumulation of iron-laden microglia.

Keywords: Dopaminergic cell death, iron, ferritin, transferrin, substantia nigra, 6-hydroxydopamine, Parkinson's disease

INTRODUCTION

Dopaminergic cell death in the substantia nigra (SN) is the main pathology of Parkinson's disease. However, the mechanism underlying the cell death is still unknown. Recently, iron accumulation and iron-induced oxidative stress has been implicated in the mechanism of nigral cell death.

Oxidative stress, due to the excessive formation of hydrogen peroxide and other reactive oxygen species such as hydroxyl, superoxide and other oxygen-derived free radicals, can cause cell damage, even cell death.[1] There is a considerable body of evidence supporting an oxidative stress in the Parkinsonian SN.[2–7] In the SN, hydrogen peroxide is mainly generated by the deamination and auto-oxidation of dopamine.[8,9] Hydrogen peroxide is relatively inert and not toxic to cells.[1]

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However, damage occurs when hydrogen peroxide interacts with the reduced forms of transition metal ions (for example, Fe^{2+}), and decomposes to the highly reactive hydroxyl radical (the Fenton reaction). Hydroxyl radicals react at great speed and affinity with almost every molecule found in the living cell. These result in single or double DNA strand breakage and chemical alterations of the deoxyribose, purine and pyrimidine bases, membrane lipids, and carbohydrates,[1] leading to a cascade of events with subsequent damage to mitochondrial electron transport system, decompartmentation of intracellular calcium homeostasis, induction of proteases, increased membrane lipid peroxidation, and finally cell death.[1]

The increasing evidence indicates that iron is elevated in the Parkinsonian SN except for one recent study.[10] Sofic *et al.*[7] demonstrated that within the SN, total iron levels were increased about 77% in tissue obtained from Parkinsonian patients, compared with control subjects. Subsequent study showed that the increase of total iron content was confined to the substantia nigra pars compacta (SNc).[11] These results have been confirmed in the SN in PD by inductively coupled plasma spectroscopy,[2] spectrophotometric method[12] and laser microprobe mass analysis.[13] Iron accumulation has been demonstrated in the SN of MPTP induced Parkinsonian monkeys.[14,15] Previous study of Oestreicher *et al.*[16] and the results from our laboratory[17] also demonstrated an increase in the levels of iron in the SN of 6-hydroxydopamine (6-OHDA) induced Parkinsonian rats. However, all these data are not sufficient to conclude whether increased iron is the primary cause of cell death, or just a consequence of the pathological process, even though intranigral injection of iron could induce the degeneration of nigrostriatal neurones in rats.[18,19] Another unresolved problem is how iron gains access to the Parkinsonian SN, even though lactoferrin mediated iron mobilization has been implicated as one of the mechanisms.[20,21]

As the main iron mobilization proteins, transferrin and ferritin are involved in the iron homeostasis in tissue. Transferrin is an 80-kD glycoprotein which transports iron between cell surfaces by a receptor mediated process.[22] There is roughly 10 times more ferritin than transferrin in the cerebral cortex.[23] Most soluble brain iron is bound to ferritin, where presumably it is sequestered in a relatively accessible but nontoxic form.[23,24] Each ferritin molecule can bind up to 4500 iron atoms[25]. Ferritin exists in two isoforms: heavy (H-chain) and light (L-chain). The ratio between H- and L-subunits of ferritin differs among different organs. In general, H-chain ferritin is found predominantly in organs which have high iron utilization and normally little iron storage as in heart, whereas the L-chain ferritin is associated with long-term iron storage, for example, in liver and spleen.[26]

Using iron histochemistry (Perls' stain) and immunohistochemistry for tyrosine hydroxylase (TH), transferrin and ferritin, the present study investigated the time course of dopaminergic cell death and time dependent changes in the levels of iron, transferrin and ferritin in the rat SN after 6-OHDA lesioning. It aimed to understand the relationship between iron accumulation and cell death and the possible mechanism of iron accumulation.

MATERIALS AND METHODS

6-OHDA Induced Parkinsonian Rats

Male Sprague–Dawley rats, weighing from 180 to 200 g, were obtained from the Laboratory Animal Centre (Sembawang, Singapore), and housed under environmentally controlled conditions. These rats were anaesthetized by intraperitoneal injection of 7% chloral hydrate at a dosage of 0.4 mg/g. Four μl of 0.2% 6-OHDA (Sigma, USA) dissolved in 0.01% ascorbic acid solution was then stereotaxically injected into the right SN at AP – 4 mm, ML – 1 mm, DV – 7.5 mm from bregma, according to the rat brain atlas of

Paxinos and Watson.[27] Control rats received 4 μl of vehicle injection into the right SN. For all animals, the infusate solution was delivered over a 5-min period via a Hamilton 10 μl syringe mounted on a probe drive of the stereotaxic frame (David Kopf Instruments). The needle was left *in situ* for 5 additional minutes prior to syringe withdrawal and wound closure. After survival periods of 3 days, 1 week, 2 weeks, 1 month, 2 months and 3 months, the 6-OHDA lesioned rats were killed by decapitation. Control rats were decapitated at 3 months after vehicle injection. The brains were quickly dissected and freshly frozen on dry ice, then kept at −70°C for use.

Immunohistochemistry

Sections of rat brains were cut at 20 μm in thickness in a cryostat (Jung Frigocut 2800E) operating at −16°C. Those sections from 3.2 to 4.2 planes of SN according to the rat brain atlas of Paxinos and Watson were picked up for TH immunohistochemical staining. The sections were fixed with 4% paraformaldehyde for 15 min immediately after cutting, washed three times for 5 min in phosphate-buffered saline (PBS). They were quenched in 2% hydrogen peroxide for 10 min at room temperature; washed three times for 5 min in PBS; then incubated with a monoclonal mouse anti-TH antibody (Boehringer Mannheim Biochemica) at a dilution of 1:100 in PBS-TX (PBS with 0.1% Triton X-100) overnight at room temperature. Thereafter the sections were treated with a biotinylated anti-mouse IgG (Vector) at a dilution of 1:200 in PBS-TX for 1 h at room temperature and subsequently incubated with avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, USA) for 1.5 h. The peroxidase was revealed by incubation with a solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma Co., USA) containing 0.015% hydrogen peroxide in Tris buffer (pH 7.6). The sections were washed with 4 changes in Tris buffer for 5 min each, then dehydrated and coverslipped.

For transferrin immunohistochemical staining, the sections were incubated in 4% normal rabbit serum for 1 h at room temperature (25°C) before incubation in primary antibody (goat anti-human transferrin, 1:50; Sigma Co., USA) overnight. The biotinylated rabbit anti-goat Ig was used as the secondary antibody at a dilution of 1:200. Sections of rat liver were processed for transferrin staining in parallel with those of SN as the positive control. The primary antibody was omitted in the staining for negative control.

Sections were incubated in 4% normal goat serum for 1 h at 25°C, then transferred in rabbit anti-horse spleen ferritin (Sigma Co., USA; 1:100) overnight at room temperature. The biotinylated goat anti-rabbit Ig was used as the secondary antibody at a dilution of 1:200. Sections of rat liver were processed for ferritin staining as positive control. For negative control, the primary antibody was omitted in the staining procedure.

Iron Histochemistry

The sections adjacent to those used for immunohistochemistry were processed for iron histochemical staining (Perls' stain). The fresh frozen sections were fixed in cold 4% paraformaldehyde for 5 min immediately after cutting, then immersed in Perls' solution (1:1, 2% HCl and 2% potassium ferrocyanide) at room temperature for 30 min, rinsed in deionized water for 30 min, and subsequently immersed for 15 min in 0.5% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in cold phosphate buffer (pH 7.4). Thereafter 0.5 ml of 1% H_2O_2 was added for every 100 ml of DAB solution and the sections remained in this solution for 15 min. The sections were rinsed in deionized water for 30 min, and finally mounted after being air dried.

Cell Counting and Image Analysis

After immunohistochemical staining, 5 sections of the SN of each rat sacrificed at different time intervals after 6-OHDA or vehicle injection were

examined. These sections were taken from different levels of the SN, and matched as far as possible from animal to animal. The immunolabelled positive cells in both right and left SN were counted with a Leitz Laborlux II microscope at 40× magnification. Slides were coded and the investigator counting the cells was kept from knowing the lesioned from the control side.

Optical densities, represented by grey means (average of optical density per area), of iron histochemical staining (Perls' stain) in the SN were measured by computer-aided image analysis. For the above purpose, 3 sections, containing both right and left SN of each brain were examined. The SN was outlined first, and the average of the density per area (grey mean) of the outlined region was then obtained, using a Carl Zeiss AXIOPHOT upright microscope and PROGRESS 3012 camera based IBAS 2.5 image analysis system (Kontron Elektronik, Germany).

RESULTS

Iron Histochemistry

Iron reaction products were found in some cells bearing the morphology of neurons, oligodendrocytes, and microglia (Figure 1) in the 6-OHDA lesioned SN of rats. Neurons appeared as large cells having triangle or fusiform outline. Oligodendrocytes were much smaller, having round or ovate shape and few processes. Microglia were also small cells, no larger than 10 μm, but unlike oligodendrocytes, they had many branching processes. However, a considerable degree of the increased iron staining could not be clearly associated with cell bodies and was therefore considered to be within the neuropil. According to computer-aided image analysis, the density of iron reaction products was significantly increased in the 6-OHDA lesioned SN ($p < 0.05$, paired t-test), compared to the contralateral side, from 1 to 3 months after 6-OHDA injection (Table I and Figures 2 and 3). However, there was no such difference between the 6-OHDA lesioned SN and the contralateral side from 3 days to 2 weeks after 6-OHDA injection. There was no significant change in the densities of iron reactive products in vehicle injected SN, compared to those in the contralateral side.

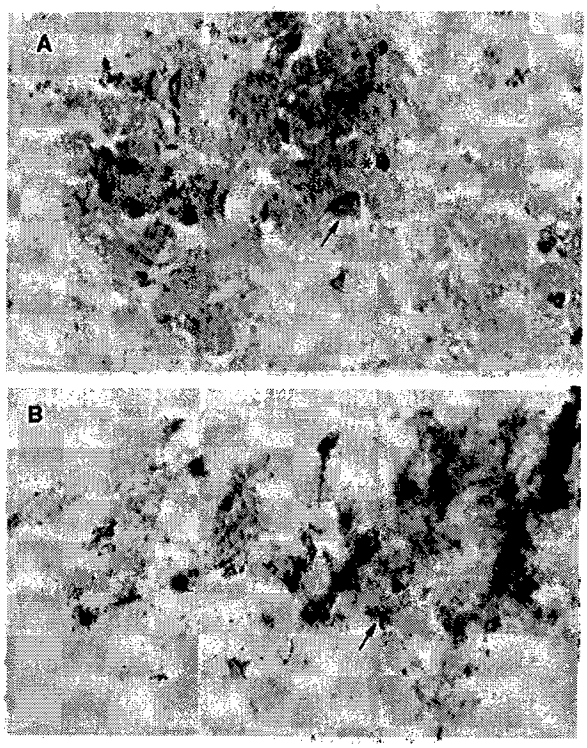


FIGURE 1 Photomicrographs showing two sections of the SN stained by Perl's iron histochemical technique. A neuron in A is arrowed and an oligodendrocyte, indicated by a star. A microglial cell in B is also arrowed. Patches of iron staining are also seen in the neuropil. The rat was perfused at 1 month after 6-OHDA lesioning. (A and B: ×270).

TABLE I Time dependent changes in the density of iron reaction products in 6-OHDA lesioned SN examined

Survival period	Density of iron reaction products in SN	
	Lesioned	Contralateral
3 days	103 ± 6	102 ± 8
1 week	107 ± 14	105 ± 15
2 weeks	102 ± 7	97 ± 8
1 month	115 ± 9*	103 ± 10
2 months	129 ± 12*	109 ± 12
3 months	131 ± 10*	110 ± 9

*Indicates significant increase in the density of iron reaction products in the 6-OHDA lesioned SN ($p < 0.05$, paired t-test), compared to that in the contralateral SN. Mean ± SD. Five rats were included in each group.

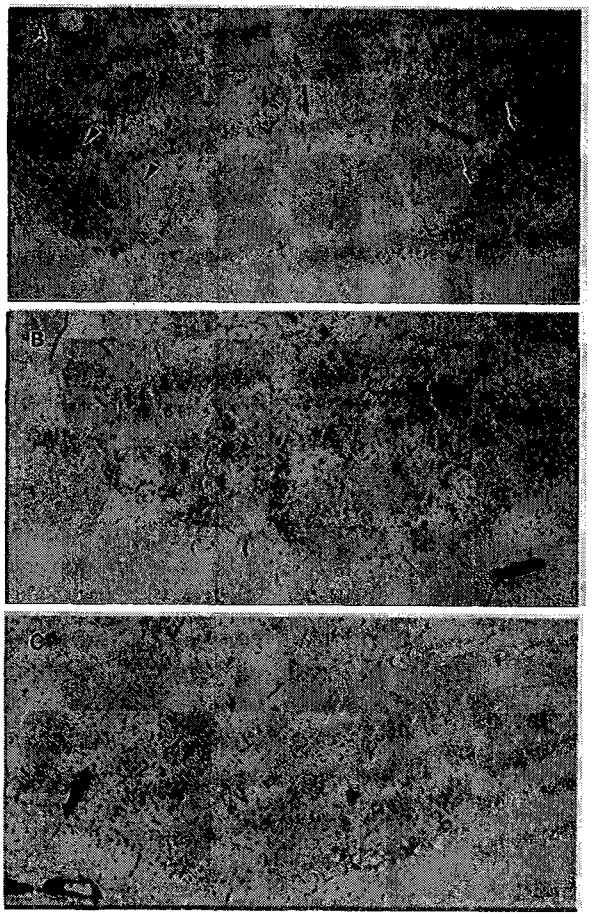


FIGURE 2 Photomicrographs showing iron histochemical staining in the SN. The density of iron reaction products is increased in the lesioned SN (arrowed in A) at one month after 6-OHDA injection, compared to that in the contralateral SN (arrowheads in A). B and C are magnified pictures of the lesioned and contralateral side respectively of the SN shown in A (A: ×16; B and C: ×50). (See Color plate I at the end of this issue.)

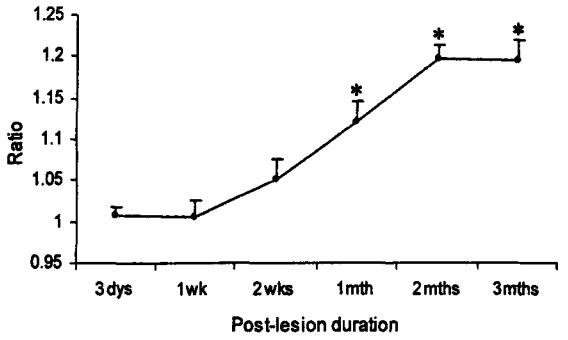


FIGURE 3 Chart showing ratios of iron staining densities (grey mean) in the lesioned and contralateral SN, plotted against survival periods. The ratios are significantly increased from 1 to 3 months after 6-OHDA injection ($p < 0.05$; one-way ANOVA and Dunnett method).

TABLE II Time dependent changes in the number of TH positive cells (per section) in the rat SN examined after 6-OHDA lesioning

Survival period	Number of TH stained cells in SN	
	Lesioned	Contralateral
3 days	113 ± 14	118 ± 16
1 week	61 ± 9*	109 ± 9
2 weeks	30 ± 6*	124 ± 14
1 month	22 ± 5*	103 ± 13
2 months	13 ± 6*	100 ± 15
3 months	17 ± 6*	106 ± 16

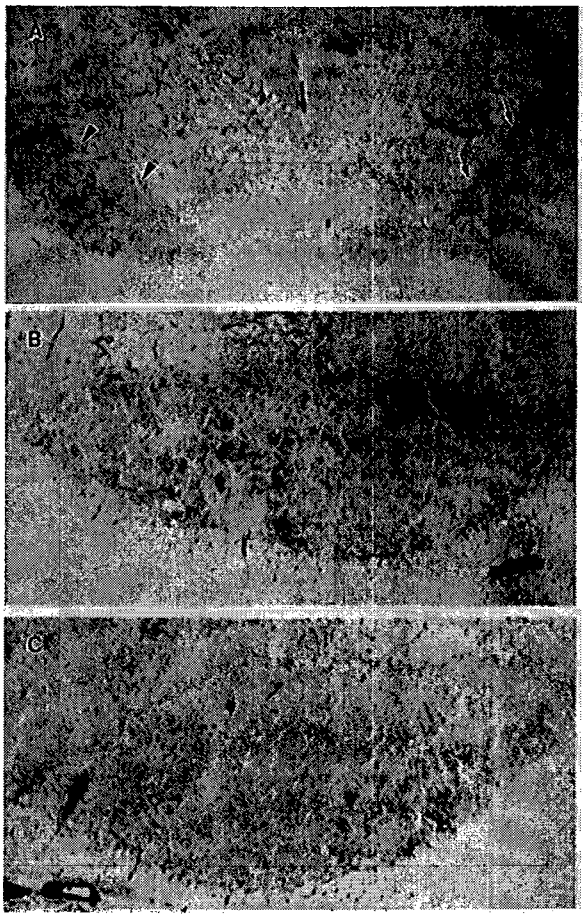
*Denotes significant decrease in the number of TH positive cells in 6-OHDA lesioned SN ($p < 0.05$, paired t-test), compared to that in the contralateral SN. Mean ± SD. Five animals were included in each group.

TH Immunohistochemistry

TH positive cells were located mainly in the SNc, scattered in the pars lateralis (SNl) and pars reticularis (SNr). They were fusiform or trigonal in shape and arranged in layers. There was a gradual loss in the number of TH positive cells in the 6-OHDA lesioned SN, compared to the contralateral side, from 1 week to 3 months after 6-OHDA injection. The loss became significant ($p < 0.05$), paired t-test) from 1 week to 3 months after the injection (Table II). There was no TH positive cell loss in the vehicle injected SN, compared to the contralateral side.

Ferritin Immunohistochemistry

Ferritin immunostaining was found in oligodendrocytes and microglial cells in the SN of normal rats (Figure 4). These cells were identified morphologically as described above. The staining was relatively light in microglial cells, but strong in oligodendrocytes. The ferritin positive oligodendrocytes were distributed throughout the SN. A decrease in the number of these cells was noticeable in the SN from 1 week to 3 months



Color Plate I (see page 107, figure 2) Photomicrographs showing iron histochemical staining in the SN. The density of iron reaction products is increased in the lesioned SN (arrowed in A) at one month after 6-OHDA injection, compared to that in the contralateral SN (arrowheads in A). B and C are magnified pictures of the lesioned and contralateral side respectively of the SN shown in A (A: ×16; B and C: ×50).

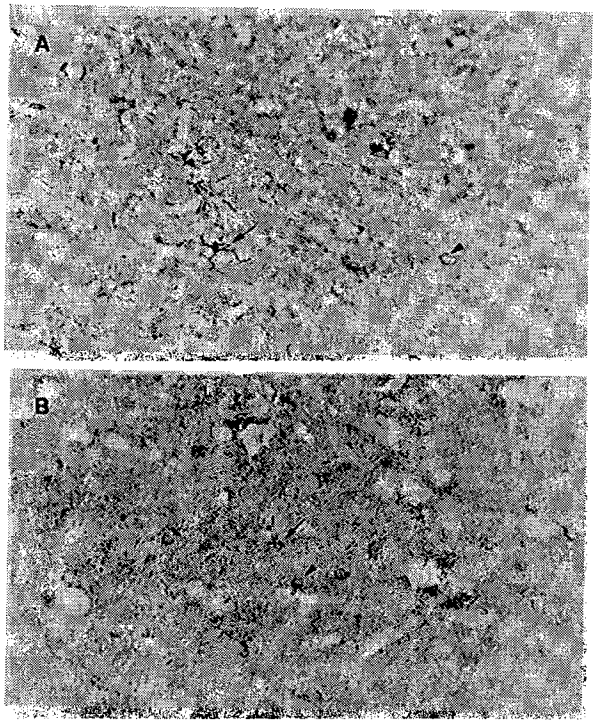


FIGURE 4 Photomicrographs showing ferritin immunopositive cells in the rat SN. These are most likely microglia (arrowed in A) and oligodendrocytes (arrowed in B). The number of ferritin positive microglia is increased in the lesioned SN (A) at 1 week after 6-OHDA injection, while ferritin positive oligodendrocytes are reduced in number, compared to the contralateral SN (B). The staining is strong in oligodendrocytes and relatively weak in microglial cells in the non-lesioned SN (B), but it is strong in microglial cells and faint in oligodendrocytes after 6-OHDA lesioning (A) (A and B: ×270).

TABLE III Time dependent changes in the number of ferritin positive oligodendrocytes per section in the rat SN examined after 6-OHDA lesioning

Survival period	Number of cells in SN	
	Lesioned	Contralateral
3 days	76 ± 7	76 ± 9
1 week	51 ± 4*	78 ± 5
2 weeks	43 ± 3*	80 ± 5
1 month	36 ± 3*	80 ± 5
2 months	39 ± 5*	74 ± 9
3 months	41 ± 5*	85 ± 7

*Figures showing that compared to the contralateral side, the ferritin positive oligodendrocytes are significantly less in the lesioned SN ($p < 0.05$, paired t-test). Mean ± SD. Five animals were included in each group.

after 6-OHDA lesioning, compared to the contralateral side ($p < 0.05$), paired t-test) (Table III).

Ferritin positive microglial cells were scattered in the SN. The number of these cells was increased in the SN from 1 week to 1 month after 6-OHDA lesioning, but appeared normal from 2 to 3 months (Table IV), compared to the contralateral side ($p < 0.05$, paired t-test). In 6-OHDA lesioned SN, especially in SNc, some of the ferritin positive microglial cells appeared hypertrophic (Figure 4).

Transferrin Immunohistochemistry

In the rat SN, transferrin immunopositive cells were morphologically identified as neurons and oligodendrocytes (Figure 5). Transferrin positive neurons were mainly located in the SNc, and scattered in the SNl and SNr. Transferrin positive oligodendrocytes were almost evenly distributed in the SNc, SNr and SNl. These neurons were fusiform or trigonal in shape, and medium to large in size. The number of transferrin positive neurons was decreased in the ipsilateral SN from 1 week to 3 months after 6-OHDA lesioning, compared to the contralateral side ($p < 0.05$, paired t-test) (Table V). Transferrin positive oligodendrocytes were also reduced in number in the ipsilateral SN from 1 week to 3 months after 6-OHDA lesioning, compared to the contralateral side ($p < 0.05$, paired t-test) (Table VI).

TABLE IV Time dependent changes in the number of ferritin positive microglia per section in the rat SN examined after 6-OHDA lesioning

Survival period	Number of cells in SN	
	Lesioned	Contralateral
3 days	37 ± 6.55	35 ± 9
1 week	57 ± 9*	44 ± 4
2 weeks	62 ± 14*	38 ± 8
1 month	59 ± 11*	41 ± 9
2 months	41 ± 9	39 ± 8
3 months	36 ± 9	36 ± 6

*Denotes significant increase in the number of ferritin positive microglia in the lesioned SN, compared to that on the contralateral side ($p < 0.05$, paired t-test). Mean ± SD. Five animals were included in each group.

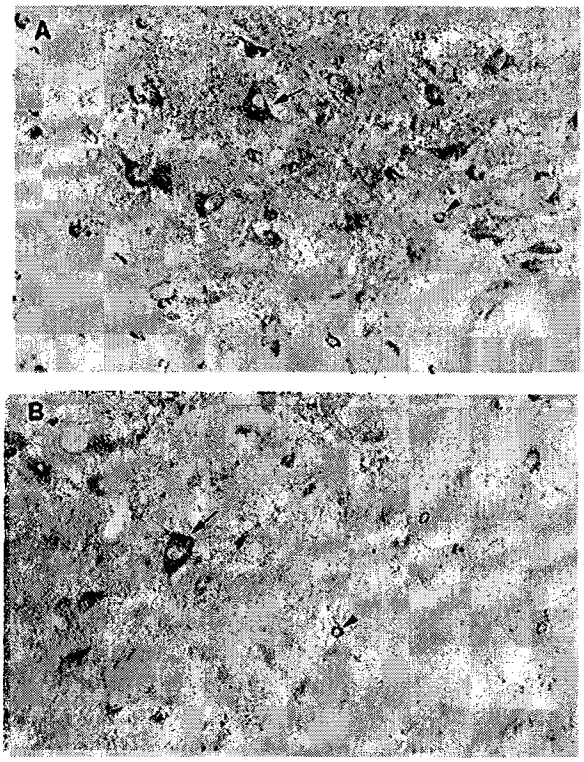


FIGURE 5 Photomicrographs showing transferrin immunoreactive neurons (arrowed) and oligodendrocytes (indicated by arrowheads) at one week after 6-OHDA lesioning. Their numbers are reduced in the ipsilateral (B), compared to the contralateral (A) SN (A and B: ×270).

TABLE V Time dependent changes in the number of transferrin positive neurons per section in the rat SN examined after 6-OHDA lesioning

Survival period	Number of cells in SN	
	Lesioned	Contralateral
3 days	185 ± 19	187 ± 21
1 week	163 ± 14*	189 ± 16
2 weeks	122 ± 10*	176 ± 16
1 month	116 ± 18*	181 ± 19
2 months	107 ± 12*	192 + 21
3 months	112 ± 11*	175 ± 14

*Denotes significant decrease in the number of transferrin positive neurons in the lesioned SN, compared to that on the contralateral side ($p < 0.05$, paired t-test). Mean ± SD. Five animals were included in each group.

TABLE VI Time dependent changes in the number of transferrin positive oligodendrocytes per section in the rat SN examined after 6-OHDA lesioning

Survival period	Number of cells in SN	
	Lesioned	Contralateral
3 days	116 ± 10	118 ± 10
1 week	80 ± 12*	110 ± 13
2 weeks	67 ± 8*	112 ± 9
1 month	60 ± 9*	99 ± 8
2 months	56 ± 10*	100 ± 9
3 months	62 ± 6*	103 ± 15

*Compared to the contralateral side, the number of transferrin positive oligodendrocytes is significantly less in the lesioned SN ($p < 0.05$, paired t-test). Mean ± SD. Five animals were included in each group.

DISCUSSION

In the present study, Perls' staining revealed the presence of iron in some neurons, oligodendrocytes and microglial cells in the rat SN. This is in agreement with previous findings.[28–30] A very faint staining was said to be present in astrocytes of the rabbit brain.[31] The present study could not, however, demonstrate iron reaction product in the astrocytes of the rat brain. This difference may be attributed to the different animal species used in the investigations or differences in the staining process.

Some previous studies of human patients or animal models demonstrated increased iron in the Parkinsonian SN.[2,12,14–17] However, other studies[10,32] failed to show any increase in the iron content of Parkinsonian SN. The discrepancies may be related to the different specimens investigated. For example, Dexter[2] and Riederer[12] showed that a significant increase of total iron content was only seen in the SN of severely affected patients, whereas total iron levels were unaltered in the SN of Parkinsonian patients with a mild grade of nigrostriatal degeneration.[12] Of course, methodological interferences may also be decisive. These include, the sensitivity of iron detecting methods and the processing of specimens, as iron may be washed out or added in by

perfusion or staining. In agreement with the results of our previous study carried out by nuclear microscopy, the present investigation demonstrated an increased iron level in the Parkinsonian SN of rats after 6-OHDA lesioning. Furthermore, the present time course study showed that iron started to increase significantly in the Parkinsonian SN about 1 month after 6-OHDA lesioning, while the loss of TH positive dopaminergic cells became significant as early as 1 week after 6-OHDA lesioning. Moreover, the ratio of iron increase in the SN of lesioned and non-lesioned sides was significantly correlated to the percentage of TH positive cell loss. These results indicate clearly that iron accumulation in the Parkinsonian rat SN is secondary and therefore consequential to the nigrostriatal dopamine cell death.

Even though increased iron is not the primary cause of the acute dopaminergic cell death in the Parkinsonian rat model, it could still be an important factor in the cascade of events leading to oxidative stress. An excess of iron in the SN may mean that, when injured, the dopaminergic cells would release iron ions that can stimulate free radical reactions, leading to a progression of dopaminergic cell death in Parkinson's disease.

The specificity of ferritin and transferrin immunohistochemical staining was confirmed by both positive and negative controls. Both ferritin and transferrin stainings were observed in hepatic cells, and were absent when the respective primary antibodies were omitted. In the present study, ferritin immunohistochemistry was carried out with antibodies against the horse spleen ferritin, which is classified as L-rich ferritin. Ferritin immunostaining was demonstrated in microglial cells and oligodendrocytes. This is in agreement with previous work of Connor *et al.*[33] who, using monoclonal antibody to L-chain ferritin, showed the same cellular distribution of staining in the brain of monkeys. Using transferrin antibodies against human transferrin which does not cross-react with lactoferrin[34] the present study demonstrated transferrin immunoactivity in oligodendrocytes and neurons. This is consistent with some previous findings. Using *in situ* hybridization and immunohistochemistry, Bloch *et al.*[35] located transferrin mRNA and transferrin immunolike product in oligodendrocytes in the rat brain. Later on, transferrin immunoreactivity was also shown in neurons in the brain of human and rat.[3,28,31,36]

There are only a few investigations to determine the level of transferrin in Parkinson's disease, and the results are conflicting. Thus, while van-Kamp[37] demonstrated an increase in serum transferrin in Parkinsonian patients, Cabrera-Valdivia[38] showed that serum transferrin levels in Parkinson's disease did not differ from those of control. Furthermore, a decreased transferrin/iron ratio, a measure of iron mobilization capacity, was shown in the globus pallidus and caudate nucleus in Parkinson's disease.[39] The present time course study showed that the numbers of both transferrin positive neurons and oligodendrocytes were decreased in the rat SN from 1 week to 3 months after 6-OHDA lesioning. This implies that transferrin expression was decreased in the Parkinsonian rat SN after lesioning. Therefore, transferrin may not be involved in the mechanisms underling iron accumulation in the Parkinsonian SN.

Previous studies on ferritin levels in the Parkinsonian SN also gave conflicting results. Using radioimmunoassay, Dexter found that ferritin levels were reduced in the Parkinsonian SN.[40] In contrast, Riderer[12] and Jellinger[42] reported an increase in ferritin levels in the SN. The discrepancies may be related to the different methods used in the studies. A recent study indicated that L-ferritin (most of the spleen and liver ferritin) which seems to promote iron storage at the core and lack peroxidase activity,[24,26] was not changed in human Parkinsonian SN.[43] Goto *et al.*[44] also showed that ferritin levels in the monkey SN were not changed at 6 months after MPTP lesioning. The present study demonstrated that the number of ferritin positive oligodendrocytes was decreased in the rat SN

from 1 week to 3 months after 6-OHDA lesioning. The reason for the loss of transferrin positive neurons and oligodendrocytes is unclear. As the loss of neurons and oligodendrocytes occurred at about the same time, it is possible that both the cell types were affected by 6-OHDA

The number of ferritin positive microglia remained unchanged from 2 to 3 months after the lesioning but was increased from 1 week to 1 month after 6-OHDA lesioning. This increase is most likely due to the increased microglial cells as revealed by OX42 and OX6 immunohistochemistry (data not shown). However, since ferritin positive microglial cells may be derived from the blood stream after nigral cell damage, one cannot rule out the possibility that the iron increase could be partially attributed to an increase in the number of these cells during the very early stage of this condition. Moreover, previous study has shown that activated microglia cause superoxide-mediated release of iron from ferritin.[45] Reactive microglia can also produce nitric oxide (NO), which would in turn affect the cellular iron metabolism.[41]

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