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A nuclear microscopic and histochemical study of iron concentrations and distribution in the midbrain of two age groups of monkeys unilaterally injected with MPTP

Min-Qin Ren,[a] Wei-Yi Ong,[b,*] Xin-Sheng Wang,[b] and Frank Watt[a]

[a] *Research Centre for Nuclear Microscopy, Department of Physics, National University of Singapore, Singapore 119260*
[b] *Department of Anatomy, National University of Singapore, Singapore 119260*

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Abstract

The present study was carried out to elucidate the concentration and distribution of iron in the substantia nigra of two age groups of monkeys after experimental hemi-Parkinsonism induced by unilateral internal carotid injections of MPTP. Iron levels and distribution were detected using the nuclear microscope, which is able to provide structural and quantitative elemental analysis of biological tissue down to the parts per million (ppm) level of analytical sensitivity. Five weeks after unilateral lesioning with MPTP, we observed a 30–65% loss of neurons in the injected substantia nigra of each monkey, compared with the contralateral control 'non-lesioned' side. In monkeys less than 7 years of age, the iron was distributed fairly uniformly and showed little evidence of focal deposits. In monkeys greater than 7 years of age, we observed many dense focal deposits of iron in the substantia nigra. A comparison between iron distributions in nuclear microscopic scans and cell distributions in the same sections stained by the Nissl technique showed that areas containing high iron concentrations were present not where large-diameter neurons with abundant Nissl substance (presumed dopaminergic neurons) were located but in a region ventral to these cell bodies, i.e., in the substantia nigra pars reticulata. These distributions were present on the control side as well as the MPTP-injected side. Since a previous study has shown that unilateral MPTP injection results in lesions of the substantia nigra of the same side but negligible injury to the opposite side, this implies that the iron deposits existed in the older monkeys *before* MPTP injections (i.e. they occurred normally). The accumulation of iron in the substantia nigra with age suggests the possibility of localised damage to neurons through the catalysis of free radicals.

Keywords: Iron; Nuclear microscopy; Substantia nigra pars reticulata; MPTP; Parkinson's disease

Introduction

Parkinson's disease (PD) is a movement disorder of uncertain etiology characterised by the accelerated loss of dopaminergic neurons, astrogliosis, and mitochondrial insufficiency in the pars compacta of the substantia nigra. Aging is a significant risk factor for the development of PD, although the cellular and molecular changes that render the aging nervous system prone to PD remain to be elucidated. There is currently evidence implicating oxidative stress in the pathogenesis of PD (Cohen and Werner, 1994). Abnormal deposits of non-haem iron in the substantia nigra of PD subjects are thought to be a major generator of neurotoxic free radicals in this condition (Jellinger et al., 1990; Jenner, 1992; Morris and Edwardson, 1994; Reiderer et al., 1989; Youdim, 1994). The excess iron may facilitate the reduction of hydrogen peroxide derived from the oxidation of dopamine by monoamine oxidases, and from the mitochondrial electron transport chain, to highly cytotoxic hydroxyl radicals. Further, ferrous iron may catalyse the oxidation of catecholamines, such as dopamine, to neurotoxic quinones and semiquinone radicals (Goldfischer et al., 1966; Metodiewa et al., 1989; Schipper et al., 1991).

Much remains to be learned, however, concerning the cellular–subcellular substrates for and the mechanisms responsible for deposition of redox-active iron in the aging and degenerating nervous system. In rats, human, and other

* Corresponding author. Department of Anatomy, National University of Singapore, Lower Kent Ridge Road, Singapore 119260. Fax: +65-67787643.
E-mail address: antongwy@nus.edu.sg (W.-Y. Ong).

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vertebrates, aging populations of subcortical glial cells progressively exhibit an affinity for Gomori stains, orange red autofluorescence, and intense, non-enzymatic peroxidase activity mediated by ferrous iron and possibly other transition metals (Schipper and Ciessé, 1995). The presence of these iron-laden glial cells has been observed in the aging mammalian hippocampus and striatum and in the aging substantia nigra, and it has been proposed that this may lead to the development of senescence-dependent disorders of cognition and locomotor activity and PD (Justino et al., 1997; Schipper et al., 1998).

Nuclear microscopy utilises a variety of high-energy (MeV) ion beam techniques at submicron spatial resolutions to provide structural and quantitative elemental analysis of biological tissue down to the parts per million level of analytical sensitivity. The techniques used in this work are particle induced X-ray emission (PIXE), Rutherford backscattering spectrometry (RBS), and scanning transmission ion microscopy (STIM). These three techniques, which can be carried out simultaneously, are extremely useful for mapping and measuring the concentrations of major, minor, and trace elements in biological tissues (Watt, 1995; Watt et al., 1994).

In view of the potential importance of iron in generation of toxic free radicals in the substantia nigra, the present study was carried out using nuclear microscopy to elucidate the distribution of iron in the substantia nigra in two age groups of monkeys after MPTP injections. The possible presence of ferrous iron in the substantia nigra was also studied, using a histochemical stain (Turnbull's blue stain for ferrous iron).

Materials and methods

Monkeys

A total of six adult macaque monkeys (*Macaca fascicularis*) weighing 1.1–3.7 kg were used for the present study. The ages of the monkeys were estimated from their weight, the amount of wear on the dentition, and from facial photographs by two vets, and classified into two age groups, three young monkeys weighing 1.1–1.3 kg and aged less than 7 years; and three monkeys weighing 3.5–3.7 kg and aged greater than 7 years. All monkeys were housed individually under natural day–night cycle conditions, and provided with food and water ad libitum.

MPTP injections

The six monkeys were unilaterally lesioned on the right side with MPTP. The left substantia nigra in each case was used as controls. A previous study has shown that unilateral MPTP injections result in lesions of the substantia nigra on the same side but negligible injury to the opposite side (Bankiewicz et al., 1986).

MPTP injections were carried out as described previously (Emborg and Colombo, 1994; Emborg et al., 2001). In brief, the monkeys were tranquilised with ketamine (10 mg/kg, im) and anaesthesia was induced and maintained with isofluorane (1–2%). The animal was positioned in the supine position with neck hyperextended and turned slightly. Under sterile conditions, the skin was opened along the medial edge of the sternocleidomastoid muscle. The muscles were dissected, and the carotid sheath exposed and opened using fine iris scissors. The common carotid artery, internal jugular vein, and vagus nerves were identified. The common carotid artery was fully exposed until the carotid bifurcation. The medial artery from the bifurcation was identified as the external carotid artery with the superior thyroid artery seen branching distal to the bifurcation. The external carotid artery was temporarily clamped. A 27-gauge butterfly needle was inserted into the common carotid artery in a direction anterograde to the blood flow, and MPTP (0.8 mg/kg) in saline solution (0.005%) was infused at a rate of 1.33 ml/min. After the infusion was completed, a 3-ml post-flush of saline was delivered. The clamp was removed from the external carotid artery. The needle was withdrawn from the carotid artery, and a small piece of Surgicel was used to apply focal pressure to the penetrated vessel. The musculature, subcutaneous tissue, and skin were then closed. All monkeys were sacrificed 5 weeks after MPTP injection, a time interval known from our previous experiments to correspond to an approximate average neuronal cell death of 40–50% in the lesioned substantia nigra. All procedures involving monkeys were approved by the local Animal Care and Use Committee.

Tissue blocks and sections

Five weeks after MPTP injection, the monkeys were anaesthetised by intraperitoneal injection of Nembutal (30 mg/ml) followed by injection of sodium pentobarbital (Sagattal, 0.5 ml/kg of a 60 mg/ml solution). The brains were quickly removed, and blocks containing the midbrain removed and frozen in liquid nitrogen within minutes of removal. The location of the substantia nigra was estimated from the characteristic dark regions in the frozen tissue blocks and marked by inserting pinholes in the blocks around the boundaries of the substantia nigra. The blocks were sectioned at 20 μm thickness using a freezing microtome (Jung Frigocut 2800E) operating at −22°C box temperature. The sections were grouped into four sets as follows:

(a) The first set of sections was mounted on glass slides and standard Nissl staining was carried out for cell identification and cell counting in both the right, lesioned, and left, non-lesioned, substantia nigra. The characteristic large-diameter neurons with abundant Nissl substance, characteristic of dopaminergic neurons in the substantia nigra, were counted.
(b) The second set of sections was mounted on nuclear microscope holders supported on submicron pioloform

film (a self-supporting plastic film used in nuclear microscopy studies) and air-dried. These (unstained) sections were used for accurate measurements of iron concentrations using nuclear microscopy.

(c) The third set was mounted on nuclear microscope holders and air-dried. These sections were Nissl stained, and the iron distribution mapped using nuclear microscopy in the same section to determine any correlation between the location of iron and that of neurons. Previous scans on unstained and stained tissue have indicated that the iron distribution, particularly that of focal deposits of iron, is not significantly affected by the Nissl staining process. Standard Nissl staining procedures were carried out, except that the dehydration step using xylene was omitted because of its chemical reaction with the pioloform support film. Immunocytochemical staining for dopamine or tyrosine hydroxylase was not carried out as the multiple-step procedures involved could interfere with the very sensitive, nuclear microscopic measurements of iron.

(d) A fourth set of sections was cut to ascertain the possible presence of ferrous iron using Turnbull's blue staining. This staining was carried out by following a protocol described previously (McManus and Mowry, 1960). The sections were mounted on glass slides and fixed using a solution of 4% paraformaldehyde in phosphate buffer pH 7.4 for 20 min. They were then washed in distilled water and immersed in a potassium ferricyanide mixture (containing one part of 2% potassium ferricyanide and one part of 2% hydrochloric acid) for 30 min, followed by washing with distilled water for six times at 5 min each.

Nuclear microscopy

The left and right substantia nigra, red nucleus, and the cerebral peduncles (a white matter tract) were scanned using the NUS Nuclear Microscope operating with a 2-MeV proton beam (Watt, 1995). The complementary techniques of particle induced X-ray emission (PIXE), Rutherford backscattering (RBS), and scanning transmission ion microscopy (STIM) were simultaneously employed in the analysis. PIXE was used for simultaneous multi-elemental analysis for sodium and upward elements of high atomic numbers in the Periodic Table at the parts per million (ppm) level. RBS was used to provide information on the matrix composition (carbon, nitrogen, and oxygen), thickness, and density to facilitate normalisation of elemental concentrations, and off-axis STIM was used for structural imaging and fast positioning of the sample. The strength of nuclear microscopy lies in its elemental mapping capability at the parts per million levels coupled with the ability to measure trace element concentrations at high quantitative accuracy independent of the chemical state of the element.

Results

Cell counts

The number of neurons remaining on the side of the MPTP injections was reduced compared with the contralateral (control) non-lesioned side. A trend towards increased cell loss after MPTP injection was observed in the younger monkeys, compared to older monkeys, although the difference was not statistically significant. In monkeys aged greater than 7 years, an average of 36% cell loss was observed, whereas in monkeys aged less than 7 years, an average of 56% cell loss was observed.

Iron concentrations

Representative sections (unstained) through the midbrain from the three young and three old monkeys were scanned using nuclear microscopy and several regions analysed for their iron content. Iron levels in the substantia nigra, red nucleus, and the cerebral peduncles were obtained (Table 1, Fig. 1). The average iron concentration in the substantia

Table 1
Iron concentrations in the midbrain of monkeys unilaterally injected with MPTP

Age	Non-lesioned side			Lesioned side		
	SN	RN	CP	SN	RN	CP
< 7 years						
Monkey-1	155	61	55	131	59	35
Monkey-2	265	57	64	289	50	71
Monkey-3	305	64	31	257	122	84
Mean ± SE	242 ± 77	61 ± 3.5	50 ± 17	226 ± 84	77 ± 39	63 ± 25
>7 years						
Monkey-1	617	50	64	574	50	40
Monkey-2	766	53	31	846	65	77
Monkey-3	2035	126	55	2205	144	234
Mean ± SE	1139 ± 779	76 ± 43	50 ± 17	1208 ± 874	86 ± 51	117 ± 103

Iron concentrations (parts per million dry weight) in the substantia nigra (SN), the red nucleus (RN), and the cerebral peduncles (CP) of monkeys less than 7 years of age (<7 years), and greater than 7 years of age (>7 years).

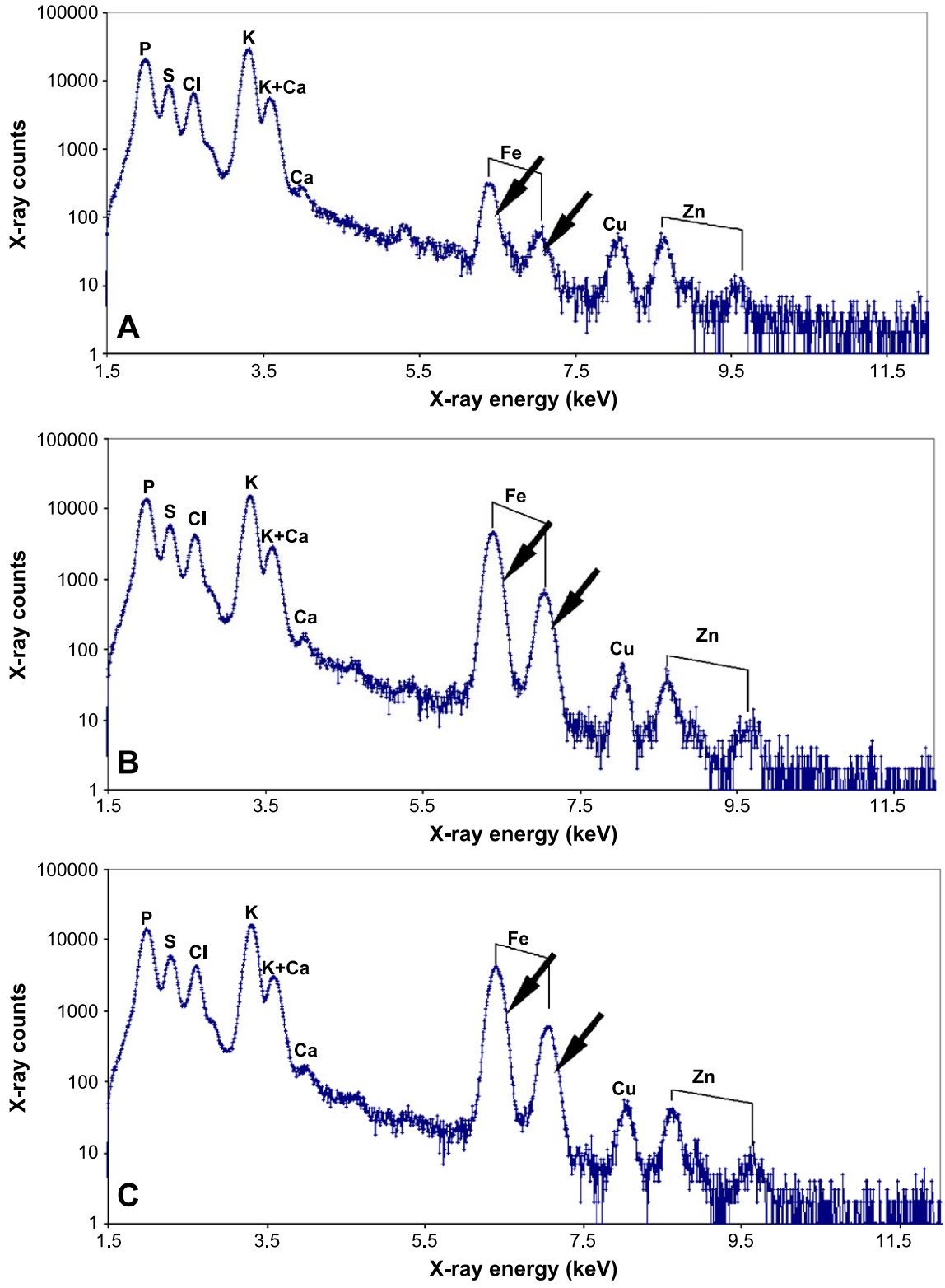

Fig. 1. Iron levels in the substantia nigra. The *Y* axis indicates X-ray counts. The various elements are indicated on the *X* axis. (A) The un-lesioned side of a monkey aged less than 7 years. The arrows indicate the iron peaks. (B) The un-lesioned side of a monkey aged greater than 7 years. The iron peaks (arrows) are greater than those in monkeys aged less than 7 years (c.f. A, above). (C) MPTP-injected side of a monkey aged greater than 7 years. Approximately the same level of iron is present as on the non-lesioned side.

nigra, in monkeys aged greater than 7 years, was 1174 ppm. This was a five times elevation in iron levels when compared to an average iron level of 224 ppm in the substantia nigra of monkeys aged less than 7 years ($P < 0.05$). For the red nucleus and the white matter, the elevation in iron levels with age was 1.2 and 1.5 times, respectively. These differences were, however, not statistically significant. No significant difference in iron levels was also found between the lesioned and the non-lesioned sides, for the three areas analysed.

Iron distribution in the substantia nigra

Areas containing high iron concentrations are indicated as closely spaced dots, whereas those containing lower iron

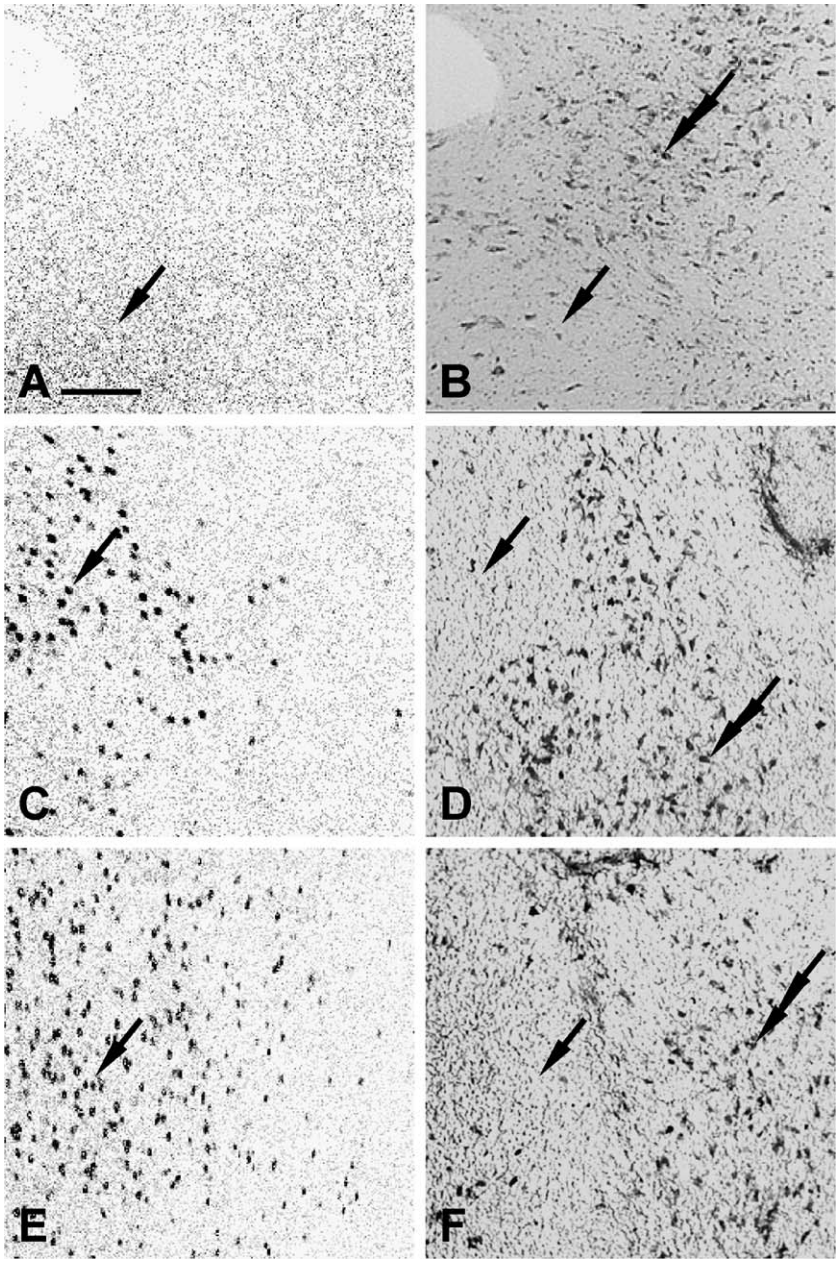

Fig. 2. Iron distribution in the substantia nigra. (A and B) Iron (A) and Nissl stained (B) map of the same section from the lesioned side of a monkey aged less than 7 years. There are no focal deposits of iron in this and the other two animals in this group. An area of slightly higher density of iron is visible (arrow in A), but this region is away (arrow in B) from the location of large neuronal cell bodies (double arrow in B) with abundant Nissl substance, and characteristic of type I dopaminergic neurons. Immunocytochemical staining for tyrosine hydroxylase was not carried out as it could interfere with the nuclear microscopic measurements of iron. (C and D) Iron (C) and Nissl stained (D) map of the same section from the non-lesioned side of a monkey aged greater than 7 years. Focal deposits of iron are observed even in the un-lesioned side (arrow in C). Their location is away (arrow in D) from large neurons with abundant Nissl substance (double arrow in D). (E and F) Iron (E) and Nissl stained (F) map of the same section from the MPTP-lesioned side of a monkey aged greater than 7 years. As in the un-lesioned side, focal deposits of iron (arrow in E) are present away (arrow in F) from large neurons with abundant Nissl substance (double arrow in F). Scale bar = 250 μm.

concentrations are represented as less closely spaced dots in nuclear microscopic scans. In monkeys less than 7 years of age, the iron appeared fairly uniformly distributed and showed little evidence of focal deposits. A close comparison between iron distributions in nuclear microscopic scans and cell distributions in sections stained by the Nissl technique, however, showed some areas containing higher iron concentrations. These were present, not where densely packed large cell bodies (presumed dopaminergic neurons in the pars compacta) were located, but rather, in areas away from the cell bodies (Figs. 2A, B). These distributions were present on the control side as well as the MPTP-injected side.

The substantia nigra of monkeys aged greater than 7 years showed not only an increase in iron levels but also the appearance of a large number of focal deposits of iron. There was also a much more obvious 'inverse correlation' between the distribution of iron and neuronal cell bodies in these monkeys. Areas of high iron concentrations were observed, which contained focal accumulations of extremely high densities of 'dots'. As in the younger animals, the iron-rich areas were present, not where densely packed large cell bodies (presumed dopaminergic neurons in the pars compacta) were located, but rather, in a region ventral to these cell bodies, i.e., in the pars reticulata. These distributions were present on the control side (Figs. 2C, D) as well as the MPTP-injected side (Figs. 2E, F), implying that they were present before MPTP injections.

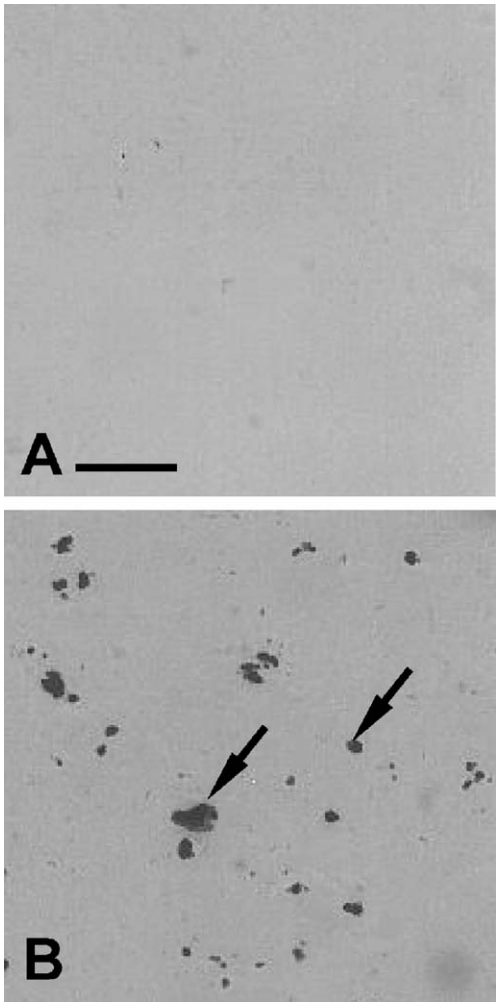

Fig. 3. Sections of the substantia nigra on the MPTP-lesioned side, stained by Turnbull's blue stain for ferrous iron deposits. (A) Monkey aged less than 7 years showing lack of ferrous iron deposits. (B) Monkeys aged greater than 7 years showing presence of ferrous iron deposits (arrows). Scale bar = 150 μm.

Ferrous iron distribution

There was an absence of ferrous iron staining on both the lesioned and non-lesioned sides, in monkey aged less than 7 years (Fig. 3A). In contrast, focal deposits of ferrous iron granules were observed in the substantia nigra of both the lesioned and non-lesioned sides, in all three monkeys aged greater than 7 years (Fig. 3B). Ferrous iron was not observed in any other regions of the midbrain, in any of the monkeys.

Discussion

Our results show that there is a general increase in iron levels in specific regions of the brain with age, with up to a 5-fold increase in the substantia nigra. No significant differences in iron levels with age were observed in the red nucleus, or in the cerebral peduncles, a white matter tract. In addition, no significant increase in iron was observed in the lesioned MPTP side compared with the contralateral non-lesioned control side despite a cell loss of 36% in the monkeys aged greater than 7 years and 56% for the younger monkeys. The differences in cell loss between the two age groups of monkeys were not statistically significant.

High-resolution nuclear microscope scans over the substantia nigra showed that there were substantial focal deposits of iron in the substantia nigra of each monkey greater than 7 years of age but not in the younger monkeys. A comparison between iron distributions in nuclear microscopic scans and cell distributions in the same sections stained by the Nissl technique showed that areas containing high iron concentrations were present not where large-diameter neurons with abundant Nissl substance (presumed dopaminergic neurons) were located but in areas away from these cell bodies. These distributions were present on the control side as well as the MPTP-injected side. Since a previous study has shown that unilateral MPTP injection results in lesions of the substantia nigra of the same side but negligible injury to the opposite side (Bankiewicz et al., 1986), this implies that the iron deposits existed in the older monkeys *before* MPTP injections, i.e., they occurred normally. No iron deposits were observed in the substantia nigra of untreated young monkeys (unpublished observations).

An accumulation of iron could be harmful to the surrounding neurons. Ferrous iron could be converted to the ferric form with release of an electron, and free radicals could be formed as a result. Free radicals could lead to lipid peroxidation and further damage to the surrounding neurons. This possibility of a ferrous iron catalysed free radical formation occurring in the substantia nigra of monkeys aged greater than 7 years is supported by the observation of the presence of ferrous iron granules by Turnbull's blue staining in the substantia nigra of these monkeys. The fine structure of the ferrous iron granules and the type of cells in which the granules are present could not be elucidated in the present study, since the technique of nuclear microscopy precludes the use of fixatives for fixing the tissues for electron microscopy. One possibility is that the focal deposits could be due to an increase in iron storage in glial cells. A study of the kainate-lesioned hippocampus showed an increase in ferrous iron in the degenerating hippocampus with time after kainate injections. The ferrous iron was present in glial cells that had morphological features of oligodendrocytes and were double-labeled for CNPase, a marker for oligodendrocytes (Wang et al., 2002).

It thus appears that a very high accumulation of iron in the substantia nigra in the older monkeys is associated with some of the iron being present in the ferrous state. These observations are consistent with those of human studies, which showed increasing iron deposition in the basal ganglia with age (Hallgren and Sourander, 1958). An accumulation of iron has also been observed with age in the substantia nigra pars reticulata in MRI studies (Bartzokis et al., 1999). The ability of iron to cause free radical damage is, however, not only dependent on iron concentration but also on the ability of iron binding proteins such as ferritin to sequester the iron in a non-reactive, bound form. Iron bound to iron binding proteins is present in the ferric state (Aisen and Listowsky, 1980; Faucheaux et al., 1995; Ford et al., 1984), but an increase in iron without a concomitant increase in iron binding proteins might lead to more iron being available in an unbound form to catalyse free radical reactions. With normal aging, both H and L ferritin increased in the substantia nigra but the age-associated increase in isoferritins generally failed to occur in Alzheimer's disease and PD brain tissue (Connor et al., 1995). A decrease in ferritin-bound iron had been also observed in the substantia nigra pars reticulata of late-onset PD patients compared to age-matched controls in magnetic resonance imaging studies (Bartzokis et al., 1999). These studies, together with our present observations that the increased iron is present in the form of focal deposits of very high iron concentrations, suggest that a 'mismatch' between iron and iron binding may be an important factor in the pathogenesis of late-onset PD. In contrast to late-onset PD, an increase, rather than decrease, in ferritin-bound iron had been observed in the substantia nigra of patients with early-onset PD. This may indicate a difference in etiologies between these two forms of PD (Bartzokis et al., 1999).

The factors that result in the greater accumulation of iron in the subtantia nigra of the monkeys aged greater than 7 years compared to those less than 7 years are unknown. It is possible the iron accumulation could be due to increased transport of iron across the blood–brain barrier. Further studies with larger sample sizes and accurate information on age are needed to elucidate the distribution and function of iron transport proteins, and possible age-related changes in these proteins, in the primate substantia nigra.

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