Complex I, Iron, and Ferritin in Parkinson's Disease Substantia Nigra

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Elevated iron levels, enhanced oxidative damage, and complex I deficiency have been identified in the substantia nigra of Parkinson's disease patients. To understand the interrelationship of these abnormalities, we analyzed iron levels, ferritin levels, and complex I activity in the substantia nigra of patients with Parkinson's disease. Total iron levels were increased significantly, ferritin levels were unchanged, and complex I activities were decreased significantly in the substantia nigra samples. The failure of ferritin levels to increase with elevated iron concentrations suggests that the amount of reactive iron may increase in the substantia nigra of Parkinson's disease patients. There was no correlation between the iron levels and complex I activity or the iron-ferritin ratio and complex I activity in the substantia nigra samples.

Mann VM, Cooper JM, Daniel SE, Srai K, Jenner P, Marsden CD, Schapira AHV. Complex I, iron, and ferritin in Parkinson's disease substantia nigra. Ann Neurol 1994;36:876–881

The cause of the substantia nigral dopaminergic cell loss that underlies Parkinson's disease (PD) remains unknown. However, specific biochemical abnormalities recently identified in postmortem PD brains now provide some insight into the possible mechanisms involved in nigral cell death. These potential mechanisms include an increase in nigral iron level, oxidative damage [1, 2], and mitochondrial complex I deficiency [3].

The evidence for increased nigral iron is based on histological stains and direct microanalytical and bulk tissue measurement. The levels of nigral iron in PD reportedly are increased by 35 to 77% relative to control values [4, 5]. However, iron also appears to be increased in the basal ganglia in other neurodegenerative disorders, suggesting that the elevated levels seen in PD may simply be a nonspecific response to cell death [6]. The finding of a selective decrease in the levels of the iron-binding protein ferritin in PD might, however, indicate an imbalance of iron homeostasis, perhaps resulting in an increase in free iron, thereby enhancing free radical generation. Some controversy surrounds the question of ferritin levels in PD nigra. Dexter and coauthors [7] reported ferritin levels to be significantly lower in all regions examined, including the substantia nigra, in the PD brain compared to control brains. However, Reiderer and colleagues [5] reported an increase in nigral iron with a concomitant increase in nigral ferritin level.

Evidence for increased oxidative damage in PD substantia nigra includes reports of decreased polyunsaturated fatty acids, increased malondialdehyde levels, and oxidative stress as indicated by increased superoxide dismutase activity and a decrease in reduced glutathione [5, 8, 9]. These changes appear to be specific for the substantia nigra in PD. The normal levels of vitamin E in PD substantia nigra [10] are somewhat at variance with the changes in other parameters of oxidative damage, and this inconsistency awaits explanation. There is also increasing evidence for a deficiency of complex I activity in PD substantia nigra, with levels of this enzyme reduced 30 to 37% in PD [3]. Data also suggest that the complex I defect is selective for the substantia nigra within the central nervous system (CNS) and is specific for PD [11].

Attention is now focused on the complex interrelationship between the three biochemical abnormalities discussed above in an attempt to determine the relevance of each to nigral cell death and to each other. To investigate further the possible role of iron in generating free radicals and mitochondrial damage, we have sought to clarify the issue of changes in ferritin concentration in the substantia nigra of patients with

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Received Feb 15, 1994, and in revised form Apr 21. Accepted for publication May 24, 1994.

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idiopathic PD using two double antibody sandwich enzyme-linked immunosorbent assays (ELISAs). We have also extended our studies on complex I activity in PD nigra to correlate any changes in this enzyme with changes in iron and ferritin levels.

Materials and Methods

Tissue

Postmortem brain samples were provided by the Parkinson's Disease Society Brain Bank. Control samples were removed from matched patients who had died without evidence of neurological or psychiatric disease and without pathology in the substantia nigra. All PD patients showed moderate to severe akinesia with asymmetrical onset, a resting tremor, and a positive response to apomorphine or L-dopa. PD was confirmed pathologically by the presence of Lewy bodies in nigral neurons. A total of 22 control and 18 PD substantia nigra samples were used for the iron and/or ferritin analyses, although because of the limited availability of tissue, not all analyses were performed on all samples. The 22 control and 18 PD samples were matched for age (control, 76.7 ± 10.9 years; PD, 73.7 ± 6.2 years) and time from death to freezing (control, 17.8 ± 7.0 hours; PD, 16.2 ± 7.6 hours). All subgroups used for individual analyses also were matched appropriately with the exception of ELISA method 1 where the age of control subjects was slightly different from that of the PD patients (control, 79.4 ± 10.8 years; PD, 73.3 ± 6.3 years; $p = 0.011$). In practice, this small difference is not considered to affect the interpretation of results.

Enzyme Analysis

Samples were homogenized and assayed for mitochondrial enzyme activity and protein content as previously described [3].

Ferritin Analysis

Brain samples were homogenized as for enzyme analysis, diluted to 5 mg/ml of protein with homogenizing medium, and incubated at 72°C for 10 minutes to inhibit proteases and precipitate nonferritin protein. Samples were then centrifuged at 3,000 *g* for 15 minutes and the supernatants stored at −70°C prior to analysis. Relative levels of ferritin in PD and control substantia nigra were analyzed by two double antibody sandwich enzyme-linked immunosorbent methods. Unless stated otherwise, ELISA plate wells were coated with 100-μl volumes at each step.

Method 1 was a three-layered ELISA. Plates (Nunc Immunoplates 4-39454A) were coated with rabbit anti–human (liver) ferritin capture antibody (Dako, code A133) diluted 1:350 with 50 mM sodium carbonate (Na_2CO_3), pH 9.6, and incubated overnight at 4°C. The wells were then washed six times with phosphate-buffered saline (PBS) buffer (95 mM sodium phosphate [Na_2HPO_4], 1.5 mM KH_2PO_4, 155 mM sodium chloride [NaCl], 2.7 mM potassium chloride [KCl], pH 7.6) and incubated with 125 μl of 50 mM Na_2CO_3, pH 9.6, containing 0.5% bovine serum albumin (BSA) for 1 hour at room temperature. Following six further washes with PBS containing 0.1% Tween-20 (PBST), the wells were coated with substantia nigra samples (in triplicate at 0.25, 0.5, 0.75, and 1.0 μg of protein/well) at room temperature for 75 minutes. Wells were then washed with PBST and incubated with horseradish peroxidase (HRP)–conjugated rabbit anti–human (liver) ferritin detection antibody (Dako, code P145), diluted 1:5,000 with PBST, at room temperature for 75 minutes. After six final wash steps with PBST, the wells were incubated with 0.03% sodium perborate and 22 mM *o*-phenylenediamine dihydrochloride in 50 mM citrate buffer, pH 5.0. After 20 minutes the reaction was stopped by the addition of 50 μl of 4 M sulfuric acid (H_2SO_4) and the absorbance at 492 nm was measured using a BioRad 2550 Microplate reader. For a substrate incubation of 20 minutes, the reaction was linear, with respect to protein, up to 1.8 μg of protein/well.

Method 2 was a four-layered ELISA. Plates were coated with 5 μg/ml of rabbit anti–human (brain) ferritin, diluted in 50 mM Na_2CO_3, pH 9.6, and incubated at 40°C for 2 hours. The wells were then washed six times with TBS (50 mM Tris–hydrochloric acid [HCl], 100 mM NaCl, pH 8.0) and blocked with 0.5% BSA in 50 mM Na_2CO_3, pH 9.6, for 2 hours at 40°C. Samples were then applied to the wells, in triplicate at 0.4, 0.8, 1.2, and 1.6 μg of protein/well and left overnight at 4°C. The wells were washed with TBST and layered with biotinylated rabbit anti–human (brain) ferritin antibody, diluted 1:460 with TBST, for 2 hours at 40°C. After washing with TBST, wells were incubated at 40°C for 2 hours with avidin-biotin-HRP complex (Dako) and washed with TBST and incubated with substrate, as in *method 1*, for 100 minutes, after which time the reaction was terminated and color development was measured. For a substrate incubation time of 100 minutes, the reaction was linear, with respect to protein, up to 2.4 μg of protein/well.

For both methods color development was linear with time up to 2 hours and up to 1.8 absorbance units.

To assess antibody immunoreactivity to the two isoforms of ferritin, plates were coated at room temperature for 2 hours, with recombinant L-ferritin and recombinant H-ferritin (0–40 ng/ml) diluted in 50 mM Na_2CO_3, pH 9.6. The wells were then washed with PBS and blocked with 0.5% BSA in Na_2CO_3, pH 9.6, overnight at 4°C. The wells were washed and incubated with either HRP-conjugated rabbit anti–liver ferritin antibody (Dako, code P145) or the biotinylated rabbit anti–brain ferritin antibody and detected as described for ELISA *methods 1* and *2*.

Elemental Analysis

One hundred microliters of brain homogenate, equivalent to approximately 10 mg of wet weight tissue, was digested and solubilized in 2 ml of 70% nitric acid (Spectrosol grade, BDH-Sharpe) by heating at 170°C down to dryness. Solubilized tissue was then redissolved in 2 ml of 70% nitric acid and diluted 1:6 with distilled, deionized water just prior to elemental analysis. Standard solutions of iron (0–5 μg/ml) and zinc (0–2 μg/ml), with solubilized samples, were analyzed on a Philips PV8490 ICP (inductively coupled plasma) spectrophotometer. Iron was detected at 259.94 nm and zinc at 213.86 nm.

Results

Mitochondrial Function

Only complex I activities are presented in this study. We previously showed that there is no significant dif-

ference in the activities of complexes II/III and IV between control (n = 22) and PD (n = 17) substantia nigra [3]. The complex I activities in the substantia nigra from 7 new PD patients were significantly decreased by 35% ($p \leq 0.026$) compared to control values (citrate synthase corrected). When combined with our previously reported data there was a highly significant decrease of 38% (expressed per milligram of total protein) and 32% (citrate synthase corrected) in complex I activity in PD nigra (Table 1). The spread of the individual data points reveals some overlap between the control and PD activity levels (Fig 1) when results are expressed as per milligram of total protein or citrate synthase corrected.

Iron and Zinc Levels

There was a significant increase (56%) in the mean iron levels in PD nigra compared to control levels ($p < 0.05$); however, no significant difference in the levels of zinc in nigra was seen between PD and control brains (Table 2). There was no correlation between iron levels and complex I activity for individual nigral samples (Fig 2). The lowest levels of zinc (range, 29–220 ng/ml) were approaching the limits of detection. However, duplicate determinations were within the linearity of the assay and showed little variance (≤ 10% of their mean).

Ferritin Analysis

The relative cross-reactivities of the anti–brain ferritin and the anti–liver ferritin (Dako, A145) antibodies against the H and L subunits of ferritin were assessed using ELISA and the respective recombinant forms of ferritin. Both antisera cross-reacted with the L and H forms of ferritin (Fig 3), but the number of epitopes on the H-ferritin were higher than on the L form, independent of the relative subunit composition of the original antigen used to raise the antisera. The specificities of these antisera were confirmed on Western blots against control substantia nigra homogenate. Both the anti–brain ferritin and the anti–liver ferritin (Dako, A145) antisera detected two bands, one corresponding to the L (19 kd) and one the H (21 kd) subunit (data not shown).

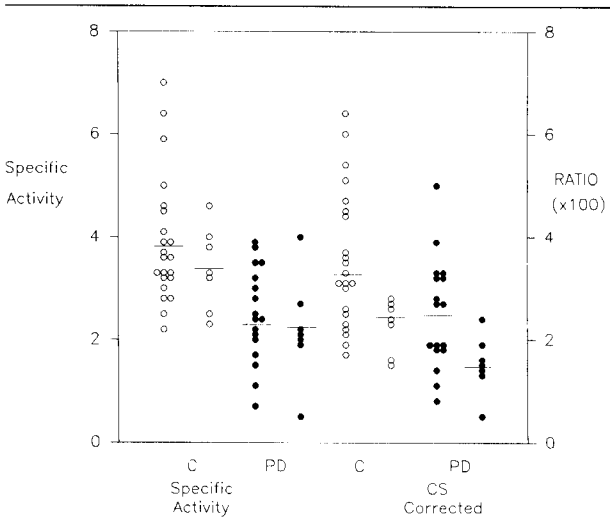

Fig 1. Individual data points for complex I specific activity (nmol/min/mg protein) and complex I/citrate synthase (CS) *ratios for control* (C, *n = 29) and Parkinson's disease* (PD, *n = 24) substantia nigra samples.* Horizontal bars *represent the mean values.*

There was no significant difference in the levels of ferritin in the substantia nigra between PD and control brains when either anti–brain ferritin or anti–spleen ferritin antibodies were used in the ELISA (Table 3). There was no significant correlation between the iron-ferritin ratio and the citrate synthase–corrected values (Fig 4).

Discussion

Increased iron levels, enhanced oxidative damage, and decreased complex I activity have now been identified as a triad of biochemical abnormalities that individuallly or in combination may participate in a cascade of reactions terminating in dopaminergic cell death. Identification of the sequence by which these changes are established will provide important clues to the cause(s) of PD.

Table 1. Complex I Activity in Control and Parkinson's Disease Substantia Nigra[a]

	Complex I (nmol/min/mg protein)		Complex I CS corrected (× 100)	
Control (n)	3.37 ± 0.82 (7)	3.77 ± 1.16 (29)	2.23 ± 0.53 (7)	3.20 ± 1.30 (29)
PD (n)	2.19 ± 1.04 (7)[b]	2.33 ± 0.91 (24)[c]	1.45 ± 0.59 (7)[b]	2.20 ± 1.10 (24)[d]

[a]The data are means ± standard deviations. The 29 and 24 samples were matched for age (control, 74.7 ± 12.7 years; PD, 74.6 ± 7.0 years) and time from death to freezing (control, 18.1 ± 7.0 hours; PD, 15.7 ± 8.6 hours). Significance against corresponding control values was determined by the Mann-Whitney *U* test.
[b]$p \leq 0.026$.
[c]$p < 0.004$.
[d]$p < 0.001$.

PD = Parkinson's disease; CS = citrate synthase.

Table 2. Total Iron and Zinc Content in Substantia Nigra[a]

	Iron	Zinc
PD (n = 14)	1,813 ± 846[b]	133 ± 57
Control (n = 16)	1,159 ± 379	159 ± 80

[a]Results are expressed as ng/mg protein, mean ± standard deviation.
[b]Significantly higher than controls, $p < 0.036$, Mann-Whitney U test.
PD = Parkinson's disease.

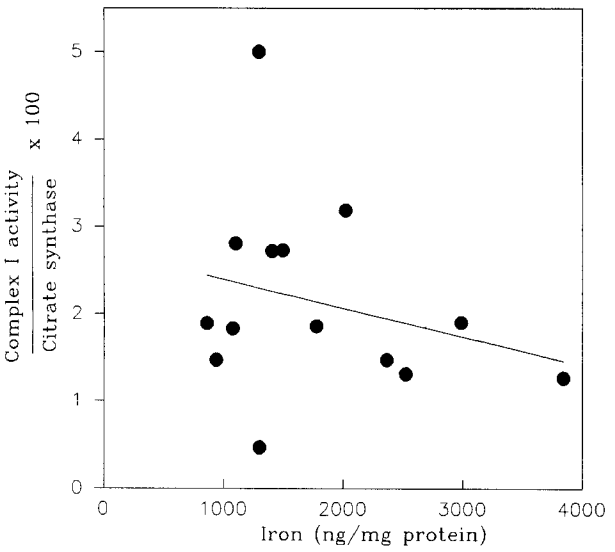

Fig 2. Scatterplot of iron levels against the ratio of complex I and citrate synthase activities in Parkinson's disease substantia nitra samples (n = 14). The linear regression line is shown (r = 0.291, p = 0.313).

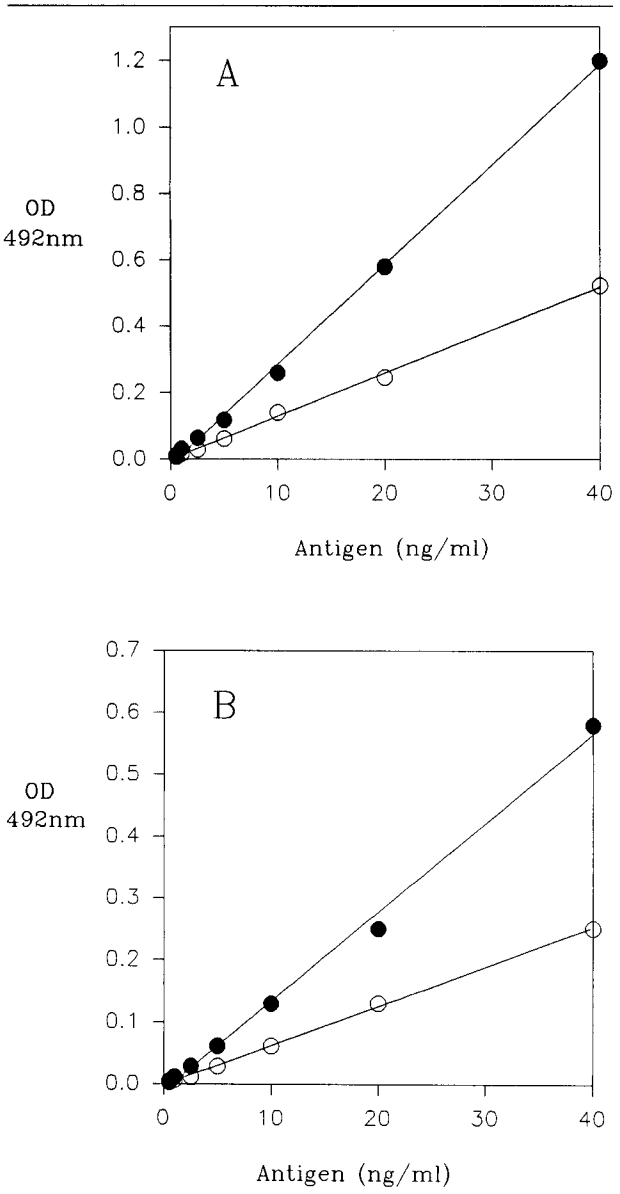

Fig 3. Specificity of antisera for L- and H-ferritin. Recombinant L- (open circle) *or H-* (closed circle) *ferritin was coated onto enzyme-linked immunosorbent assay plates and incubated with antisera raised to either (A) brain ferritin or (B) spleen ferritin (DAKO). See Materials and Methods for details.*

Several studies, including our own work presented here, have established that there are increased levels of iron in PD substantia nigra, with concentrations approximately 50% above control levels. Also, our finding that there is no change in the concentration of zinc between PD and control brains is in agreement with that of Reiderer and colleagues [5] but differs from that of Dexter and coauthors [6] who observed increased levels in PD.

Two groups have investigated ferritin levels in PD and control brain. Using the fluorescent DELFIA kit, which incorporates a polyclonal antibody raised against human liver ferritin in a similar way to a sandwich ELISA, Reiderer and colleagues [5] showed a significant increase (29%) in ferritin levels in substantia nigra and a larger, but nonsignificant increase (37%) in the putamen of 5 PD patients. Details on the sensitivity and linearities for human brain homogenate with time and protein were not given with this method of quantification. However, Dexter and coworkers [7], using a polyclonal antibody raised against human spleen ferritin in a sensitive radioimmunoassay, observed decreased levels of ferritin in all regions assayed in the PD brain (8 PD and 9 control brains). This discrepancy could relate to different specificities of the antibodies for the individual ferritin subunits (light L and heavy H) or indeed to the selection of patients studied. This may be important given that both spleen- and liver-derived ferritin are L-rich ferritins, while normal human brain ferritin is rich (65%) in the H subunit [12]. However, the composition of the original antigen (ratio of L to H antigen) does not appear to alter markedly

Table 3. Relative Ferritin Levels in Substantia Nigra[a]

	Method 1 (× 100)	Method 2 (× 1,000)
PD	7.39 ± 1.25 (n = 16)	2.26 ± 1.07 (n = 15)
Control	7.46 ± 1.99 (n = 19)	2.19 ± 0.59 (n = 18)

[a]Results are expressed as a change in absorbance/min/μg protein, mean ± standard deviation.

PD = Parkinson's disease.

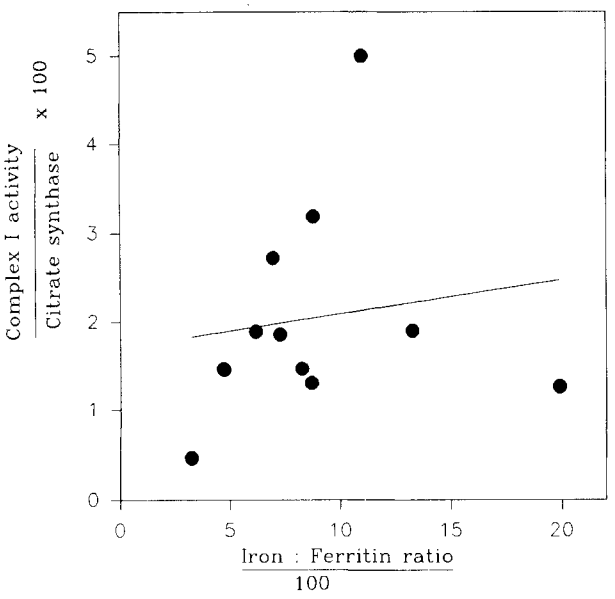

Fig 4. Scatterplot of the iron-ferritin ratio against the ratio of complex I and citrate synthase activities in Parkinson's disease substantia nigra (n = 11). The linear regression line is shown (r = 0.143, p = 0.674).

the subunit specificity of the polyclonal antisera raised. We have extended this analysis to polyclonal antiferritin antisera obtained from Serotec (raised to plasma ferritin) and ICN (raised to spleen ferritin), and confirmed this conclusion (data not shown). Consequently the antisera used by Dexter and coworkers [7] and Riederer and colleagues [5] are also likely to be detecting both L- and H-ferritin.

Our findings, representing the largest of the "ferritin in PD" studies, and based on results using several antibodies and cross-reactivities checked against recombinant ferritin isoforms, show normal ferritin but increased iron levels, and beg the following questions: Are normal levels of ferritin able to sequester such increased levels of iron, as observed in PD, and if not why are ferritin levels not increased?

Iron entering the cell via the transferrin receptor–mediated endocytosis of transferrin may be utilized in a variety of metabolic processes or sequestered in ferritin. Cellular iron homeostasis is achieved by the coordinate regulation of the translation of the transferrin receptor and ferritin messenger RNA (mRNA) [13]. When cells are replete with iron, the levels of transferrin receptor fall and the levels of ferritin rise; the opposite occurs when cells are starved of iron. Under normal circumstances ferritin is believed to be only partially saturated [14] and therefore may be able to sequester further iron prior to ferritin synthesis. However, Dedman and associates [12] showed that a 45% increase in nonhaem iron in the parietal cortex of Alzheimer's disease patients was paralleled by a 38% increase in ferritin levels, and furthermore no significant differences were found in the iron content of ferritin when compared to control values. Also Dexter and coworkers [6] observed increased iron levels and concomitant rises in ferritin concentration in the substantia nigra of patients who had died from either multiple-system atrophy or progressive supranuclear palsy. Consequently, the failure of ferritin levels to increase with increased iron levels supports the conclusion that there is indeed a defect of iron handling in the substantia nigra of PD brains, the excess free and reactive iron possibly enhancing oxidative damage which may contribute to the death of nigral neurons.

The finding of a highly statistically significant 32 to 38% decrease in complex I activity in PD presented here extends our previous observations in this area [3]. The overlap between control and PD activities could represent multiple factors in the cause of nigral cell death in PD or may, of course, simply be a reflection of biological variation. We previously reviewed the complex and reciprocal relationship between respiratory chain dysfunction and oxidative stress [15]. One element of this interaction is whether elevated iron levels can induce complex I deficiency through increased free radical generation. The effect of elevated iron levels on dopaminergic cells was assessed experimentally by Hartley and coworkers [16] using a PC12 cell culture model. Increased intracellular iron resulted in elevated malondialdehyde levels and decreased glutathione levels, consistent with oxidative damage, but a decrease in both complex I and complex IV activities. The pattern and severity of respiratory chain dysfunction observed contrasted with those found in PD and suggest that increased iron levels alone do not play a significant role in the mitochondrial complex I deficiency in PD, although they may contribute to oxidative damage. This is further supported by the lack of correlation between iron levels or iron-ferritin ratios and complex I activity in PD substantia nigra.

Cellular levels of iron are regulated posttranscrip-

tionally by specific mRNA-protein interactions between an iron regulatory factor (IRF) and iron-responsive elements (IREs) contained within the transferrin receptor and ferritin mRNAs. When iron is scarce in the cell, IRFs have a high affinity for IREs and bind to the transferrin receptor and ferritin mRNAs, stabilizing the former but preventing translation of the latter. Conversely, when cells are replete with iron, IRFs possess a low affinity toward IREs, translation of ferritin mRNA proceeds, but transferrin receptor mRNA is more unstable. The precise mechanism of iron sensing and IRF binding to IRE is believed to involve the iron status of an iron-sulfur cluster located near the center of the IRF protein.

Recent data may provide a direct link between the abnormalities of complex I activity and ferritin synthesis in the substantia nigra of the PD brain. Nitric oxide (NO) may be generated from either the constitutive (neuronal and endothelial) or nonconstitutive (macrophages, glial) form of NO synthase. Weiss and associates [17] investigated the potential role of NO in iron metabolism via its interaction with the iron sulfur center of IRFs. They observed that NO activates IRF binding to ferritin mRNA, thereby repressing biosynthesis of ferritin. While there is no direct evidence for the involvement of NO in nigral cell death in PD, it is interesting to speculate that NO may be responsible for the defect in ferritin synthesis observed in the substantia nigra of PD brains.

The debate regarding the sequence of events in PD substantia nigra continues. However, current evidence suggests that the iron changes are unlikely to cause the specific complex I deficiency in PD and are therefore unlikely to be the sole pathogenetically relevant primary defect. Further work is required to establish if the complex I defect is totally independent of excess iron and of the oxidative damage observed in PD.

This work was supported by the Medical Research Council and the Parkinson's Disease Society (UK).

We would like to thank Dr P. Arosio for his gift of recombinant ferritin and Dr A. Treffry for her generous donation of antibodies against human brain ferritin. We also thank Dr P. Walsh, Royal Bedford and Holloway College, for use of his ICP spectrophotometer and Mr B. Jackson for helpful discussions.

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