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Brain iron and ferritin in Parkinson's and Alzheimer's diseases

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Summary. Semiquantitative histological evaluation of brain iron and ferritin in Parkinson's (PD) and Alzheimer's disease (DAT) have been performed in paraffin sections of brain regions which included frontal cortex, hippocampus, basal ganglia and brain stem. The results indicate a significant selective increase of Fe^{3+} and ferritin in substantia nigra zona compacta but not in zona reticulata of Parkinsonian brains, confirming the biochemical estimation of iron. No such changes were observed in the same regions of DAT brains. The increase of iron is evident in astrocytes, macrophages, reactive microglia and non-pigmented neurons, and in damaged areas devoid of pigmented neurons. In substantia nigra of PD and PD/DAT, strong ferritin reactivity was also associated with proliferated microglia. A faint iron staining was seen occasionally in peripheral halo of Lewy bodies. By contrast, in DAT and PD/DAT, strong ferritin immunoreactivity was observed in and around senile plaques and neurofibrillary tangles. The interrelationship between selective increase of iron and ferritin in PD requires further investigation, because both changes could participate in the induction of oxidative stress and neuronal death, due to their ability to promote formation of oxygen radicals.

Keywords: Iron, ferritin, Parkinson's disease, Alzheimer's disease, melanin, Lewy body.

Introduction

There have been numerous hypotheses regarding the etiology of Parkinson's disease (PD). However, the cause of this neurodegenerative disorder remains

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obscure, in spite of the discovery of synthetic neurotoxins such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and of 6-hydroxydopamine which can induce degeneration of nigrostriatal dopamine neurons (Langston et al., 1984; Heikkila et al., 1986; Davis et al., 1979; Burns et al., 1984). The possibility exists that rather than a neurotoxin, an altered endogenous metabolic activity in substantia nigra (SN) can lead to oxidative stress and, eventually, to cell death. The evidence for oxidative stress in SN includes: increase in monoamine oxidase B in the striatum with ageing (Adolfsson et al., 1979) and in the SN of PD (Riederer et al., 1984), decreased glutathione peroxidative activity (Kish et al., 1985), peroxidase and catalase (Ambani et al., 1975), altered cellular calcium homeostasis as a result of decreased calcium binding protein (Iacopino and Christakos, 1990), decreased activity of mitochondrial electron transport systems Complex I and Complex III (Mizuno et al., 1989; Schapira et al., 1989; Reichmann and Riederer, 1989), and increase of iron (Earle, 1968; Dexter et al., 1987, 1989 a; Sofic et al., 1988; Riederer et al., 1989), and basal lipid peroxidation (Dexter et al., 1989b; Zaleska et al., 1989).

Abnormalities of iron metabolism, resulting from iron tissue overload, have more often been cited as being involved in systemic organs oxidative stress than other metals (Halliwell and Gutteridge, 1986; Youdim et al., 1989). Within the brain, most iron is stored as ferritin (Hallgren and Sourander, 1958; Octave et al., 1983) with relatively little in a free and reactive form. Under normal circumstances, iron is stored as complex with ferritin and modulates the biosynthesis of ferritin; so increased iron levels should trigger ferritin synthesis (Casey et al., 1988). However, in free state iron, as a transitional metal, can interact with available H_20_2 to produce cytotoxic oxygen free radicals such as the hydroxyl radical ($^{\cdot}OH$) and Fe^{3+}. Such radicals are though to induce lipid peroxidation of the cell membrane and alter the internal homeostasis of calcium with resultant cell death, as observed in iron initiated ischaemia of the heart (Gutteridge et al., 1983). A similar hypothesis has recently been proposed to be of possible significance in the pathogenesis of PD (Youdim et al., 1989). This may be valid since a selective increase of iron has been demonstrated in SN (Earle, 1968; Dexter et al., 1987; Sofic et al., 1988; Riederer et al., 1989), and this increase is only significant in the pars compacta, whereas no changes have been detected in the SN zona reticulata (Sofic et al., 1991).

Materials and methods

This study was performed on human postmortem brain tissue obtained from the following groups of patients:

(1) Eight patients with PD with an age range of 66 to 81 (mean 74.7) years, all clinical stage V (Hoehn and Yahr, 1967), with duration of illness ranging from one to 10 (mean 7.7) years. All patients had undergone long-term treatment with levodopa plus Benserazide with or without anticholinergic drugs and/or dopamine agonists. None of them developed severe cognitive deficits or fulfilled the NINDCS-ADRDA criteria for AD (McKhann et al., 1984).

(2) Five cases of PD with senile dementia of Alzheimer's type (PD/DAT) with an age

range of 72 to 82 (mean 76) years showing clinical signs of PD (akinesia-rigidity type), with duration of illness ranging from 4 to 10 (mean 7.0 years), all dying in PD stage V having developed severe dementia and fulfilling the clinical criteria for probable AD (McKhann et al., 1984).

(3) 15 patients with DAT, age range 72 to 82 (mean 76) years with duration of dementing illness from 2 to 13 (mean 5.7) years, all fulfilling the clinical criteria of probable AD.

(4) Eight controls dying of non-neuropsychiatric disorders, none with clinical dementia (age range 57 to 88, mean 80 years).

All brains were examined histologically, using tissue blocks from multiple brain regions, including neocortex, hippocampus, brain stem and cerebellum. Paraffin sections were stained with routine methods, including hematoxilin-eosin, cresyl violet, PAS, Bodian, methenamine, and Bielschowsky silver impregnation methods, and immunostains with monoclonal antibodies against tau and ubiquitin (see Jellinger et al., 1990 a, b). Neuropathological diagnosis of PD, PD/DAT and DAT followed current criteria, including those for AD by Khachaturian (1985) and Tierney et al. (1988). Details of the patients' characteristics and post mortem findings are given in Table 1.

For histologic evaluation of iron, paraffin sections of frontal cortex, hippocampus, basal ganglia and brain stem were processed using Perls' or Berlin blue stain for Fe^{3+} after pretreatment with ferrocyanid-HCl (Gomori, 1936), Turnbull blue for Fe^{2+}, and Quincke's reaction for both Fe^{2+} and Fe^{3+}, reducing Fe^{3+} to Fe^{2+} and ensuing transformation to Turnbull's blue. The sections were semiquantitatively assessed for the distribution and degree of iron staining using four randomly selected degrees: 0 = no iron detectable; 1+ = very few deposits visible; 2+ = moderate number of deposits; 3+ = large number of deposits visible. Histological assessment of iron staining was performed independently by two observers (K. J. and W. P.) on laboratory coded slides without knowledge of the clinical and pathological diagnosis. Ratings of both observers were used as individual data points and the results were averaged for each case and considered as a single data point. Interobserver variability was minimal. For statistical evaluation Kruskal-Wallis test for p values was used. Immunohistochemistry for ferritin was performed by incubation of deparaffinized sections with anti-ferritin antisera isolated from human liver raised in New Zealand White rabbits (Fleming and Joshi, 1987) at dilutions of 1:1000 to 1:2500. The bound antibodies were detected using biotinylated species-specific anti-rabbit immunoglobulines (Amersham, IL; 1:2000) and avidin/horseradish peroxidase complex (Sigma, Mo; 14 mg/ml) followed by development with 0.05% (W/V) 3,3'-diamino-

Table 1. Characteristics of patient groups and neuropathology

	PD	PD/DAT	DAT	Controls
No. of cases	8	5	15	8
Age	66–81 (74.7)	72–82 (76)	72–82 (76)	57–88 (80)
Sex (m/f)	3/5	1/4	10/6	3/5
Duration illness (yr)	1–10 (7.7)	4–10 (7)	2–13 (5.7)	0
Brain pathology:				
Nigral cell loss	3+	3+	0/1+	0
Lewy bodies brain stem	3+	3+	1+	0
NFT isocortex	0	3+	3+	0
Plaques hippocampus	0/1+	3+	3+	0/1+

Numbers = means

benzidine and 0.01% H_2O_2. In human brain autopsy material, anti-liver ferritin serum almost exclusively reacts with microglia cells (identified by their positive reaction with anti-CD 45 and MRP 14) and macrophage, while some diffuse, but weaker reactivity is found in astrocytes and oligodendroglial cells (Grundke-Iqbal et al., 1990).

Since the intensity of ferritin immunostaining varied considerably from case to case, suggesting that fixation, autolysis and/or other factors might influence the immunological preservation of the ferritin molecules, no semiquantitative assessment was performed. Glial fibrillary protein (GFAP) was demonstrated with antisera of the kit 507 DAKO (Dako, Santa Barbara, Calif., U.S.A.) at dilutions of 1 : 5 using the PAP technique (Sternberger et al., 1970).

Results

Iron

Positive staining was seen exclusively with Perls' stain for Fe^{3+} and with Quincke's reaction reducing Fe^{3+} with ammonium sulfide to Fe^{2+} that is visualized by Turnbull's blue, while Turnbull's blue stain for Fe^{2+} alone was constantly negative in all cases and brain areas examined.

Within any of the examined brain regions, iron stain for Fe^{3+} was most constantly observed in astrocytes, macrophages, microglial cells, and in the walls of arterioles and veins. In putamen and globus pallidus, Fe^{3+} was also seen in occasional oligodendrocytes and, rarely, in neurons. By contrast, calcifications frequently seen around blood vessels and pericapillary calcospherites in the lentiform nucleus showed strongly positive Fe^{3+} reaction. In cerebral cortex and hippocampus, only a few iron deposits in and around the walls of small blood vessels, in pericytes and occasional macrophages were present.

In substantia nigra zona reticulata (SNZR), Fe^{3+} was most frequently observed in macrophages, microglia that could be identified by the shape and characteristic elongated nuclei, and around small vessels, in axonal spheroids and, rather rarely, in non-pigmented large neurons. In substantia nigra zona compacta (SNZC), Fe^{3+} was localized particularly in macrophages around small vessels, in microglia and astrocytes, often located in the vicinity of, but not immediately adjacent to, pigmented and non-pigmented neurons (Fig. 1). However, in most cases of PD, the majority of melanin-bearing neurons were not surrounded by Fe^{3+} containing cells or free iron within the neuropil, while iron-positive cells were rather often seen in areas depleted of melanin-loaden nigral neurons (Fig. 2). In general, both melanin-containing cells and free melanin were not associated with Fe^{3+} deposits nor with iron-containing microglia, and no overlap between both intracytoplasmic and free melanin and Fe^{3+} deposits was detected (Fig. 3). Neither intracytoplasmic nor free melanin showed any reaction with Perls' stain. Finely granular intracytoplasmic deposits of Fe^{3+} were seen in occasional large non-pigmented neurons of both SNZR (Fig. 4) and SNZC (Fig. 5), and locus ceruleus (not shown). Only exceptionally few Lewy bodies in nigral neurons displayed an extremely faint, weakly positive iron-staining of the peripheral halo (Fig. 6). Large SNZC neurons containing granular Fe^{3+} deposits were seen in six among 13 cases of PD and PD/DAT

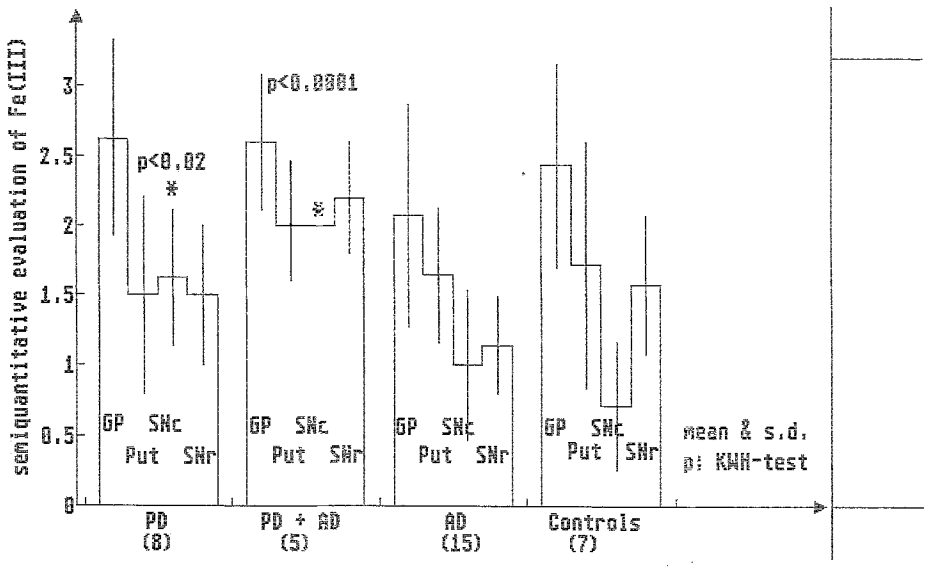

Fig. 7. Semiquantitative evaluation of Fe^{3+} in PD and AD and controls using Perls' stain. The results are expressed as mean ± SD, using Kruskal-Wallis test for p values

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Fig. 1. Substantia nigra zona compacta in case of DAT without significant loss of melanin-containing neurons showing negative Fe^{2+} staining, while iron deposits are located in adjacent glia. Perls' stain × 1000. (#421–485)

Fig. 2. Substantia nigra zona compacta (ventral part) in PD showing focal neuronal depletion with increased iron pigment clearly demarcated from disseminated, iron-negative melanin pigment. Perls' stain × 400 (#84–89)

Fig. 3. Substantia nigra zona compacta in PD with some preserved melanin-containing neurons and a Lewy body, all showing negative iron stain. Perls' stain × 400 (#306–389)

Fig. 4. Unpigmented neuron in substantia nigra zona reticulata in case of PD/DAT showing finely granular iron deposits in cytoplasm. Melanin-containing neuron iron negative. Perls' stain × 1125 (#71–89)

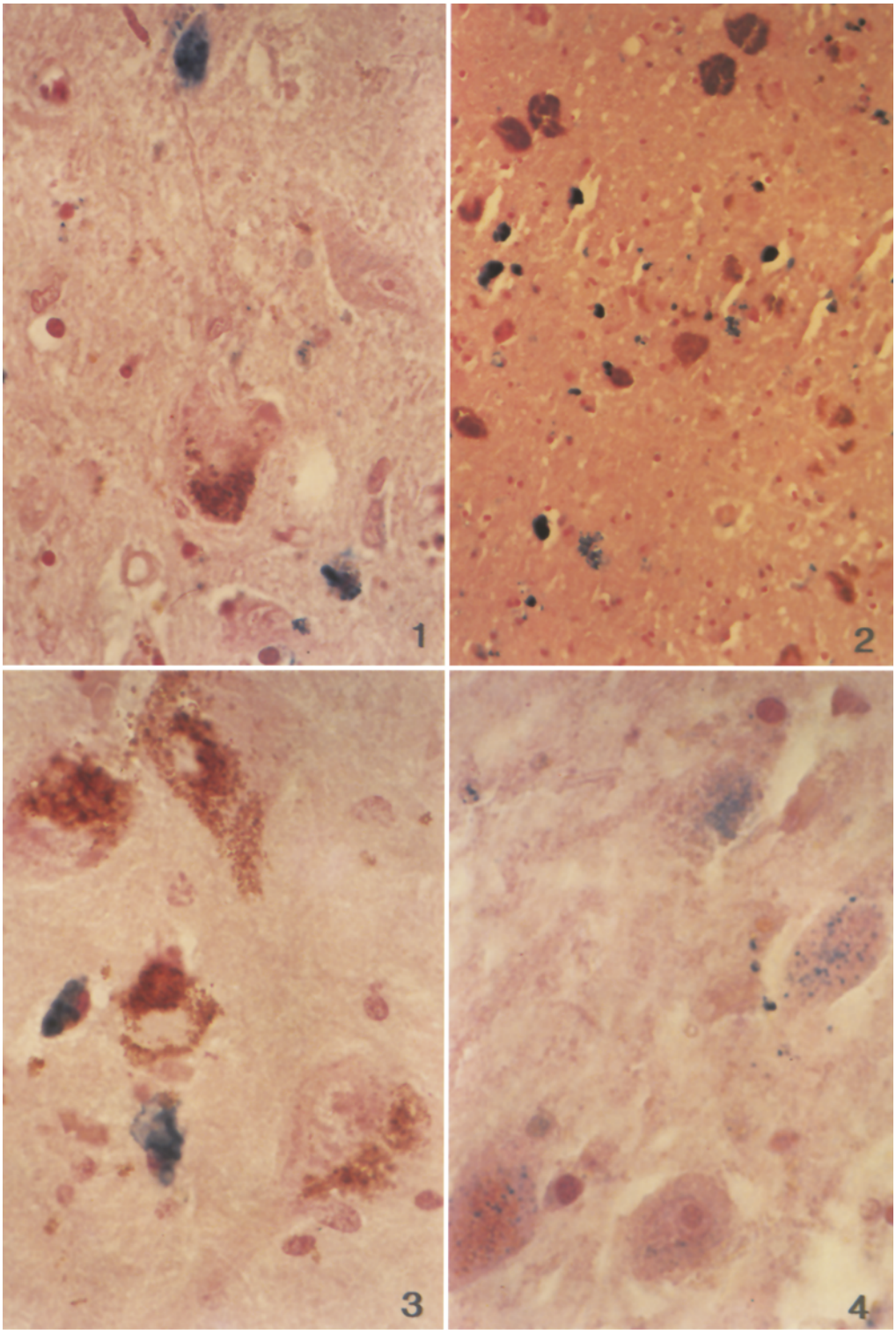
Fig. 5. Finely granular iron deposits in non-pigmented neurons of substantia nigra zona compacta of same case of PD/DAT. Perls' stain × 800

Fig. 6. Lewy body in pigmented nigral neuron showing faint iron positive peripheral halo, while other parts are negative. Perls' stain × 1125 (#71–89)

Fig. 8. Substantia nigra zona compacta of control brain, almost totally lacking ferritin + microglia (#299–385)

Fig. 9. Numerous ferritin-positive microglia in substantia nigra zona compacta of PD brain. Ferritin × 400 (#480–486)

Fig. 10. Substantia nigra zona compacta in PD. Loss of pigmented neurons with increase in ferritin-positive microglial cells often attached to neurons. Ferritin × 400 (#481–488)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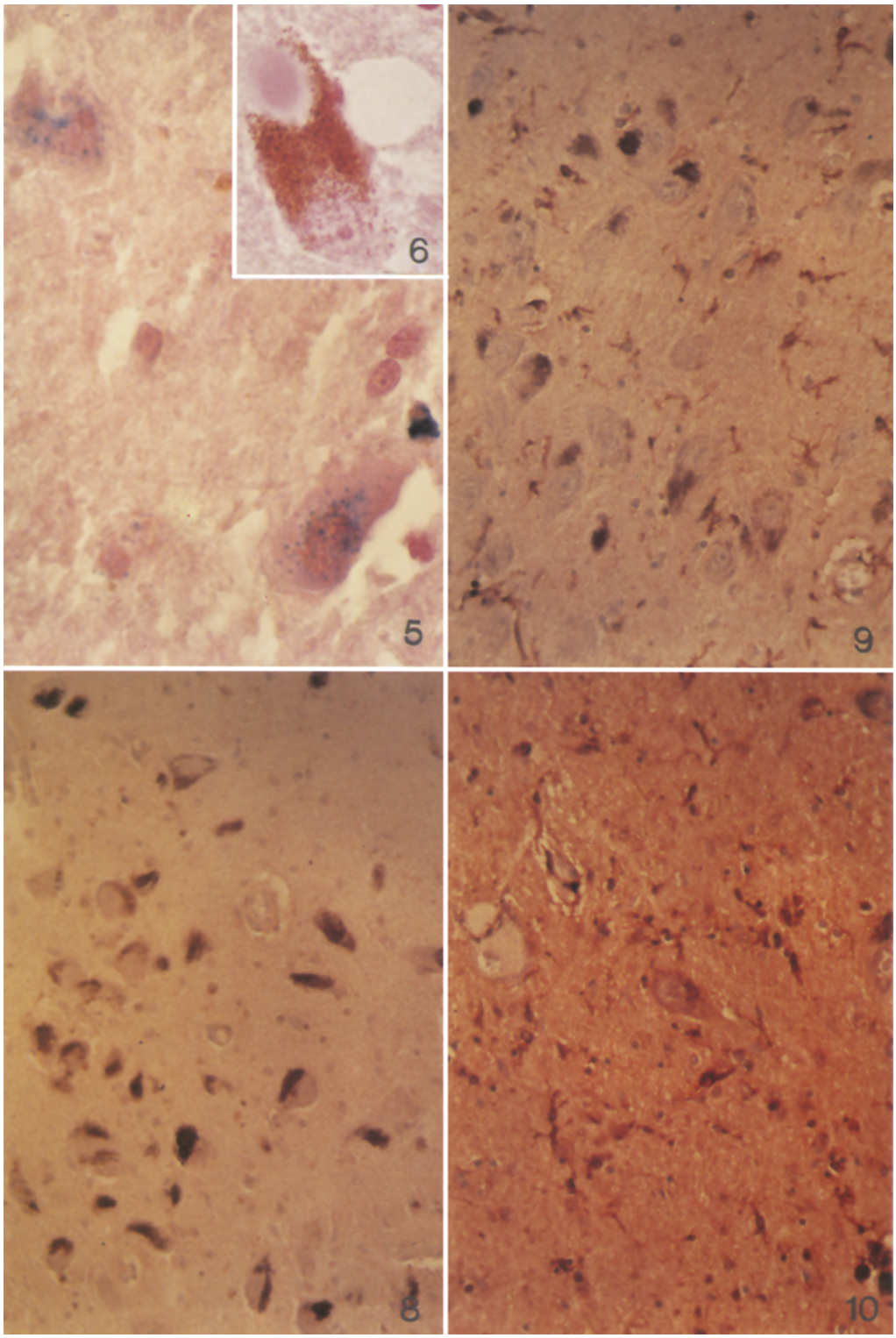
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(3/8 PD and 3/5 PD/DAT brains), but only in one each of 15 DAT and 8 control cases. Finely granular iron deposits in non-pigmented SNZR neurons were observed only in 3 PD brains and in one single case of DAT, but not in controls. In both globus pallidus and SNZR, the neuropil often showed weak diffuse staining for Fe^{3+}, while in the SNZC finely granular or coarser iron deposits were rather randomly distributed in the neuropil, particularly in damaged areas devoid of pigmented neurons. The results of semiquantitative assessment of Fe^{3+} in the basal ganglia and in SN in the different disease groups demonstrate a significant increase of Fe^{3+} in SNZC. This increase is evident in both PD and PD/DAT, but not in DAT and aged controls (see Fig. 7).

Ferritin

Ferritin reactivity in cortex, hippocampus, striatum and SN was almost exclusively associated with microglial cells that were identified by their shape and fine processes and characteristic elongated nuclei (Figs. 8 and 9). Positive reaction was also seen in perivascular macrophages. In addition, occasional, but usually weaker staining of reactive astrocytes also showing positive GFAP reaction, e.g. in small vascular scars, and of a few oligodroglial cells was observed.

Ferritin immunoreactivity was present in normal brains in oligodendroglia and microglia and, with weaker intensity, in occasional astrocytes. In striatum and hippocampus of subjects with PD, their number and intensity did not considerably differ from that in aged controls, while in SNZC and SNZR, the number of ferritin-immunoreactive cells was dramatically increased in both PD and PD/DAT (Fig. 9), and somewhat less in DAT. In SN of both PD and PD/DAT, a strong reactivity for ferritin was observed that was almost exclusively associated with greatly proliferated microglia. Numerous reactive microglia cells are in close proximity to melanin-containing or degenerating nigral neurons and to free melanin in the neuropil (Fig. 9), while such ferritin-positive cells are lacking in unaffected tissue of controls (Fig. 8). The contrast between affected and unaffected SN tissues is not seen when conventional dyes are used, since these do not stain the surface membranes of glial cells. Often, ferritin-immunoreactive microglia are in close association or apparently attaching to a pigmented nigral neuron (Fig. 10). Due to methodological reasons, semiquantitative assessment of ferritin immunoreactivity was not performed. In DAT and PD/DAT, a strong ferritin immunoreaction associated with proliferated microglia was also observed in and around amyloid and neuritic plaques and neurofibrillary tangles (not shown, but see Grundke-Iqbal et al., 1990).

Discussion

The data described in this study illustrate two important findings. The first is the selective increase of Fe^{3+} in SNZC in PD with or without associated DAT, while the reticulata nigrae and striopallidum in both PD and DAT remain

largely unchanged. Immunohistochemically, Fe^{3+} is particularly localized in macrophages, astrocytes and reactive microglia. In addition, iron is seen in the cytoplasm of occasional non-pigmented neurons in both zones of SN and in striopallidum, while it can not be detected in melanin-containing dopaminergic neurons of the SNZC nor in intracytoplasmic melanin nor in extracellular melanin granules in the neuropil. Lewy bodies, cytoplasmic inclusions in neurons of SN, locus ceruleus and other brainstem nuclei, being the anatomical hallmark of PD (Forno, 1983), are consistently negative for Fe^{3+} except for an occasional and extremely weak reaction in the peripheral halo.

The second finding illustrated by the presented data is that ferritin immunoreactivity, which is present in the normal brain in oligodendroglia (Gerber and Connor, 1989) and microglia (Kaneko et al., 1989), is selectively and dramatically increased in the PD and PD/DAT brain in what appears to be mostly activated microglia. Cells of similar shape as the anti-ferritin positive cells have been described recently to show an intense HLA-DR reactivity (McGeer et al., 1988 b) and to express various monocyte/macrophage antigens (Rozemuller et al., 1989; Grundke-Iqbal et al., 1990). While the increase in ferritin reactivity in AD/DAT is primarily restricted to the areas of the greatest neurofibrillary pathology and is closely interconnected with both the tangles and neuritic (senile) plaques (Grundke-Iqbal et al., 1990), large numbers of HLA-DR positive macrophages and microglia have been observed in the SN of PD and AD brains (McGeer et al., 1988 a, b). The immunoreactions for both ferritin and HLA-DR can outline reactive microglia and areas of phagocytosis indicating an active pathological process which could easily be overlooked by standard neuropathological staining methods. McGeer et al. (1988 b) observed almost six times as many HLA-DR positive microglia phagocytosing dopamine neurons in PD brains and a 3,6 fold increase in DAT brains as in controls. Our study, although not presenting quantitative data on ferritin-immunoreactive cells, confirms the dramatic increase of such cells, particularly in the SN of PD and PD/DAT brains, and much less in DAT without severe SN damage.

Several authors have described iron (ferritin and hemosiderin) storage in the basal ganglia in PD and other movement disorders by MR imaging (Drayer et al., 1986 a, b; Rutledge, 1989), but previous histological examinations of intact brains demonstrated very poor correlations between both biochemical assays of ferritin and iron concentrations and with T 2 values measured by MR spectrometry (Chen et al., 1989). On the other hand, recent biochemical studies in PD clearly documented an increase in total iron (Earle, 1968; Sofic et al., 1988; Dexter et al., 1989 a; Riederer et al., 1989) and particularly of Fe^{3+} in SN of PD (Riederer et al., 1989; Sofic et al., 1988, 1991), with a shift of Fe^{2+}: Fe^{3+} ratio in SNZC from almost 2:1 in normal brain to 1:2 in PD brain (Sofic et al., 1991). This increase in both total iron and Fe^{3+} is highly significant in severe cases of PD (clinical stage IV and V), while iron content is not considerably changed in brains from subjects with mild forms of PD (Riederer et al., 1989; Youdim et al., 1990).

The increase of ferritin-immunoreactive cells in SN of both PD and DAT brain is at variance with the results of measurements of ferritin levels in post-mortem brain by Dexter et al. (1990) using a RIA (rapid radioimmunoassay) procedure. These authors found decreased ferritin levels in SN, striatum, pallidum, cerebral cortex and cerebellum of PD patients. These discrepancies might be related to different techniques and antibodies. Ferritin is a ubiquitous protein consisting of a shell of 24 subunits surrounding an iron core. The protein subunits are of two types: light, or L (19 kDA), and heavy, or H (21 kDA), the proportions of which vary between tissues (Theil, 1987). In the present immunohistochemical study, the antibody used was raised in rabbits using human liver ferritin as antigen. On immunoblots, anti-liver ferritin sera label both the 19 kDA and 21 kDA subunit bands of isolated brain and liver ferritin (Grundke-Iqbal et al., 1990). By contrast, Dexter et al. (1990) used a monoclonal antibody directed against splenic ferritin consisting mainly of L-ferritin subunits. So, the values reported by them likely reflect the distribution of the L-ferritin subunit in brain that is preferentially synthesized in response to iron stimulation of mRNA for ferritin formation (see White and Munto, 1988). Thus, the results of the two studies may not necessarily be comparable due to methodological reasons.

The demonstration of increased immunoreactivity to ferritin in both PD and DAT brain may be significant from different points of view. Ferritin, an iron binding protein, also effectively binds other metallic ions such as zinc and aluminium, and is therefore thought to play a role in the defense of the brain against toxic metals (Joshi and Clauberg, 1989). Furthermore, a role of ferritin via the release of iron in the peroxidative inactivation of enzymes, membranolysis, and nonenzymatic dephosphorylation has been postulated (Joshi and Clauberg, 1989). Hence, the prominence of ferritin in the stimulated microglia may not only be an indicator of the active degenerative process leading to neuronal cell death (McGeer et al., 1988 a, b), but may also be of some functional consequence. Fe^{3+} stored in ferritin is released after reduction to Fe^{2+}. The oxygenated free radicals generated during this reduction may self-propagate by interacting with transition metal ions such as copper and iron. These free radicals are known to react with and thereby modify several cellular components. Proteins modified by such reactions would become inactive and more prone to proteolysis during phagocytosis by microglia. The often close connections of the ferritin-positive microglial processes with pigmented nigral neurons most probably indicate an involvement of the microglia in either the damage and/or removal of degenerating nigral neurons. Thus, the proliferation of the ferritin-positive microglia may reflect their active role in the degenerative process of dopaminergic neurons that, on the other hand, could be induced by the presence of free iron that may participate in autoxidation of dopamine to melanin, resulting in the formation of the cytotoxic hydroxyl and increased membrane lipid peroxidation (Dexter et al., 1988 b). Striatal dopamine synthesis is extremely susceptible to lipoperoxidative processes, and it has been shown that

iron-induced lipid peroxidation inhibits dopamine synthesis and may induce damage to the dopamine synthesizing striatal system (Zaleska et al., 1989). However, the relationship between selective increase of Fe^{3+} and ferritin in PD brain remains to be further elucidated. This problem appears of particular importance, since, under normal conditions, iron does not cross the blood brain-barrier (Ben-Shachar et al., 1986; Dwork et al., 1990). The presented data on increased Fe^{3+} and increased ferritin-immunoreactivity of cells in SN of the PD brain are based on two different methods, i.e. semiquantitative histological demonstration of Fe^{3+} using Perls' stain, and immunostaining for ferritin using a monoclonal antibody directed against liver ferritin (consisting of both L and H subunits) that has been used to show accumulation of ferritin in AD brain (Joshi et al., 1990), whereas Dexter et al. (1990), using a monoclonal antibody directed against splenic (L-) ferritin, reported decreased levels in PD brain. There is general agreement that cellular concentration of iron controls ferritin synthesis (Hentze et al., 1987; White and Munro, 1988; Casey et al., 1988). Increased iron levels activate the ferritin formation leading to rapid loading and detoxification of the metal. Consequently, there may be some interrelationship between the excess iron in SN of PD and increased ferritin-immunoreactivity on the one hand and decreased L-ferritin levels on the other, that may suggest some dysregulation in the induction or transfer of the mRNA for the production of ferritin (Hentze et al., 1987). Therefore, an explanation for the selective increase of iron and of ferritin-immunoreactivity in the SN of PD patients reported here and reduction of L-ferritin levels in SN and other brain areas in PD suggesting a defect in iron handling in this disorder (Dexter et al., 1990) will be necessary, since these changes may be related to the pathogenesis of degeneration of nigrostriatal dopamine neurons in PD and related disorders.

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