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Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease

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ABSTRACT **There is growing evidence that oxidative stress and mitochondrial respiratory failure with attendant decrease in energy output are implicated in nigral neuronal death in Parkinson disease (PD). It is not known, however, which cellular elements (neurons or glial cells) are major targets of oxygen-mediated damage. 4-Hydroxy-2-nonenal (HNE) was shown earlier to react with proteins to form stable adducts that can be used as markers of oxidative stress-induced cellular damage. We report here results of immunochemical studies using polyclonal antibodies directed against HNE–protein conjugates to label the site of oxidative damage in control subjects (ages 18–99 years) and seven patients that died of PD (ages 57–78 years). All the nigral melanized neurons in one of the midbrain sections were counted and classified into three groups according to the intensity of immunostaining for HNE-modified proteins—i.e., no staining, weak staining, and intensely positive staining. On average, 58% of nigral neurons were positively stained for HNE-modified proteins in PD; in contrast only 9% of nigral neurons were positive in the control subjects; the difference was statistically significant (Mann–Whitney *U* test; $P < 0.01$). In contrast to the substantia nigra, the oculomotor neurons in the same midbrain sections showed no or only weak staining for HNE-modified proteins in both PD and control subjects; young control subjects did not show any immunostaining; however, aged control subjects showed weak staining in the oculomotor nucleus, suggesting age-related accumulation of HNE-modified proteins in the neuron. Our results indicate the presence of oxidative stress within nigral neurons in PD, and this oxidative stress may contribute to nigral cell death.**

The major pathologic change in idiopathic Parkinson disease (PD) is the neuronal degeneration of melanized neurons in the substantia nigra (SN). The exact cause of melanized neuronal death in PD is still unknown; however, in recent years, oxidative stress has been implicated as one of the most important contributors to nigral cell death in PD (1). A role of oxidative stress in PD is indicated by the observations that PD is associated with an increase in the level of iron in SN (2–6), a decrease in the level of glutathione (7, 8), an increase in the level of Mn superoxide dismutase (9), and mitochondrial respiratory failure (10–12). Moreover, free radicals are likely involved in the pathogenesis of several other neurological disorders (13).

Nigral dopaminergic neurons are particularly exposed to oxidative stress because the metabolism of dopamine, whose concentration is high in SN, can lead to formation of H_2O_2, which in the presence of ferrous iron can give rise to the highly toxic hydroxyl radical (14, 15). Dexter *et al.* (16) demonstrated an increase in basal levels of malondialdehyde, a product of lipid peroxidation, and suggested that lipid peroxidation could be a cause of the nigral cell death. In addition, several recent studies have shown selective and highly significant elevation in iron, especially Fe^{3+} in SN of patients with PD (2, 3, 17). This could lead to enhancement of oxygen radical formation and consequently to nigral degeneration. Unfortunately, it is not possible based on biochemical analysis of tissue homogenates to determine whether neurons or glial cells or both are preferential targets of oxidative stress in PD. We have therefore used a histochemical approach to examine the sites of oxidative damage associated with PD. Specifically, antiserum raised against 4-hydroxy-2-nonenal (HNE)-modified protein, which recognizes HNE–protein epitopes (18), was used to detect HNE–protein adducts in midbrain sections of normal individuals and of patients with PD. HNE is an α,β-unsaturated aldehyde that is produced during oxidation of membrane lipid polyunsaturated fatty acids. It is one of the major products of membrane peroxidation (19) and is considered to be largely responsible for cytotoxic effects observed under the oxidative stress (19–21). HNE exhibits variable adverse effects such as inhibition of DNA, RNA, and protein synthesis, interference with certain enzyme activities, and induction of heat shock proteins (19). In an earlier report (20–22), we described the preparation of polyclonal antibodies against HNE-modified proteins and demonstrated that they can be used for the immunohistochemical detection of HNE adducts in tissue slices.

MATERIALS AND METHODS

Clinical Subjects. Clinical subjects consist of seven patients with PD, three patients with multiple system atrophy (MSA), and seven control subjects. Their age, sex, and clinical and pathologic characteristics are shown in Table 1. The histological diagnosis was confirmed by the Department of Pathology of Juntendo University School of Medicine (Tokyo) in all the patients and control subjects studied. All the patients with PD showed nigral degeneration with Lewy bodies in the remaining neurons. Two of the three patients with MSA had striatonigral degeneration plus olivopontocerebellar atrophy (SND); the remaining one patient showed olivopontocerebellar atrophy (OPCA) alone. None of the control subjects had nigral or striatal lesions. The postmortem delay between death and fixation of brains with formalin was 2.5–10 hr in the control subjects, 3.5–12 hr in the PD patients, and 3–6 hr in the MSA patients. All the patients with PD and MSA had been treated with levodopa with a peripheral dopa decarboxylase inhibitor until their death, except for the terminal agonal period in some patients. Average maintenance dose of levodopa that had been taken by PD patients shortly before death was 540 ± 89 mg/day

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Abbreviations: HNE, 4-hydroxy-2-nonenal; SN, substantia nigra; PD, Parkinson disease; MSA, multiple system atrophy; SND, striate nigral degeneration; OPCA, olivopontocerebellar atrophy.

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Table 1. Clinical backgrounds of the control subjects and PD patients studied

Patient	Age, yr	Sex	Duration of disease, yr	L-Dopa therapy, yr	Postmortern time, hr
PD					
1	51	M	12	11	12.0
2	63	F	7	6	5.5
3	65	F	5	5	4.0
4	66	F	10	9	11.5
5	75	M	10	9	4.0
6	76	F	8	6	3.5
7	78	M	10	0.5	4.0
Control					
1 Duchenne PMD	18	M		None	3.5
2 ovarian cancer	38	F		None	4.0
3 gastric cancer	47	F		None	4.0
4 cervical spondyrosis	64	M		None	3.5
5 lung cancer	70	M		None	2.5
6 lung cancer	71	M		None	10.0
7 heart failure	99	M		None	6.0
MSA					
1 MSA	52	F	6	5	3.0
2 SND	69	F	7	2	5.0
3 OPCA	63	M	8	3	6.0

PMD, progressive muscular dystrophy.

(mean ± SD), and that for SND and OPCA patients was 450 ± 220 mg/day and 300 mg/day. The duration of levodopa therapy in MSA patients was much shorter (2–5 years) than that in PD patients (5–11 years) except for one patient (0.5 year).

Immunohistochemistry. Immunohistochemical studies were performed on paraffin-embedded midbrain sections at the superior colliculus level. The 3-μm-thick transverse sections were deparaffinized with xylene and refixed with Bouin's solution for 20 min and immersed in 70%, 90%, and 100% ethanol to remove picric acid. For inhibition of endogenous peroxidase, the sections were incubated with 0.3% H_2O_2 in methanol for 30 min. After rinsing in 0.01 M phosphate-buffered saline (PBS), the sections were incubated with normal goat serum (Dako; diluted to 1:10) to inhibit nonspecific binding of the antibodies. To preclude the possibility that pretreatment with H_2O_2 yields HNE epitopes, we also stained our specimens with alkaliphosphatase-conjugated avidin instead of peroxidase-conjugated avidin (data not shown). Because the results were qualitatively identical to those obtained with the H_2O_2-treated samples, we conclude that the H_2O_2 treatment did not generate HNE–protein adducts under our experimental conditions. Polyclonal antiserum (hereafter referred to as G_3HG_3) used throughout this study was raised by immunizing New Zealand White rabbits with a HNE-modified histidyl peptide (Gly_3-His-Gly_3) conjugated with keyhole limpet hemocyanin. Characterization of this antibody has been confirmed by biochemical and immunoblotting methods (K.U. and E.R.S., unpublished data). After rinsing with 0.01 M PBS, sections were incubated with biotin-labeled goat anti-rabbit IgG antiserum (Dako; diluted 1:100) for 60 min and then with avidin–biotin complex (Vectastain ABC kit, Vector laboratories; 1:100) for 60 min. After rinsing, sections were finally incubated with 0.02% 3,3-diaminobenzidine and 0.03% H_2O_2 in deionized water for 6–9 min. Nuclear counterstaining was performed with Harris's hematoxylin solution. As a control staining, sections were incubated with normal rabbit serum as well.

Analysis of Immunostaining. For a semiquantitative analysis, microscopic photographs of the whole nigral area were taken, and then the substantia nigra was divided into three approximately equal parts—that is, the medial, central, and lateral parts. The number of neurons containing neuromelanin in each part of one of the midbrain sections was counted and each neuron was arbitrarily classified into one of three groups according to the intensity of the immunostaining of the cytoplasm using a scale consisting of intense staining (++), weak staining (+), and no staining (−). Initially, we attempted to divide the nigra into the ventral and dorsal tiers as suggested by Gibb and Lees (23); however, this division was frequently unclear in patients with PD. In addition, in PD patients, the ventrolateral as well as the dorsolateral tier showed severe degeneration. The intensity of immunostaining was assessed independently by two investigators (A.Y. and N.H.). As a reference, we analyzed the immunostaining of the oculomotor nucleus that was located in the same section with SN. Statistical analysis was performed using the Mann–Whitney *U* test.

RESULTS

In preliminary studies, we confirmed that Bouin's solution refixed sections were suitable for immunohistochemistry compared to neutral formalin-fixed paraffin-embedded sections. Neutral formalin-fixed paraffin-embedded sections exhibited lower sensitivity than Bouin's solution refixed ones.

Control Subjects. Although the number of melanized neurons in SN appeared to decrease somewhat with the age of the subjects, although the correlation was not statistically significant ($y = -3.997x + 729.307$; $r = 0.508$). More than 90% of melanized neurons in SN did not show positive immunostaining with G_3HG_3 (Fig. 1*C*). As shown in Table 2, most of the positively stained neurons showed only weak staining. Oculomotor neurons could be observed in six of seven control subjects. Oculomotor neurons and surrounding neuropils were stained with variable intensity ranging from negative to weak staining (Fig. 2*A* and *B*); glia cells were not stained. In younger control subjects (patients 1, 2, and 3), most of the oculomotor neurons as well as neuropils were not stained. In elderly control subjects (patients 4, 5, and 7), scattered oculomotor neurons with weak immunostaining were observed; neuropils also showed some immunostaining, whereas glia cells were not stained; patient 7 was the eldest control subject (99 years old) and the immunostaining intensity was stronger than that in patient 4 (64 years old). Other neurons in the midbrain section such as those in the red nucleus, superior colliculus, and midbrain raphe were not positively stained.

PD. Six of the seven PD patients studied showed a moderate to severe degree of melanized neuronal loss in SN. The remaining nigral neurons showed various degrees of immu-

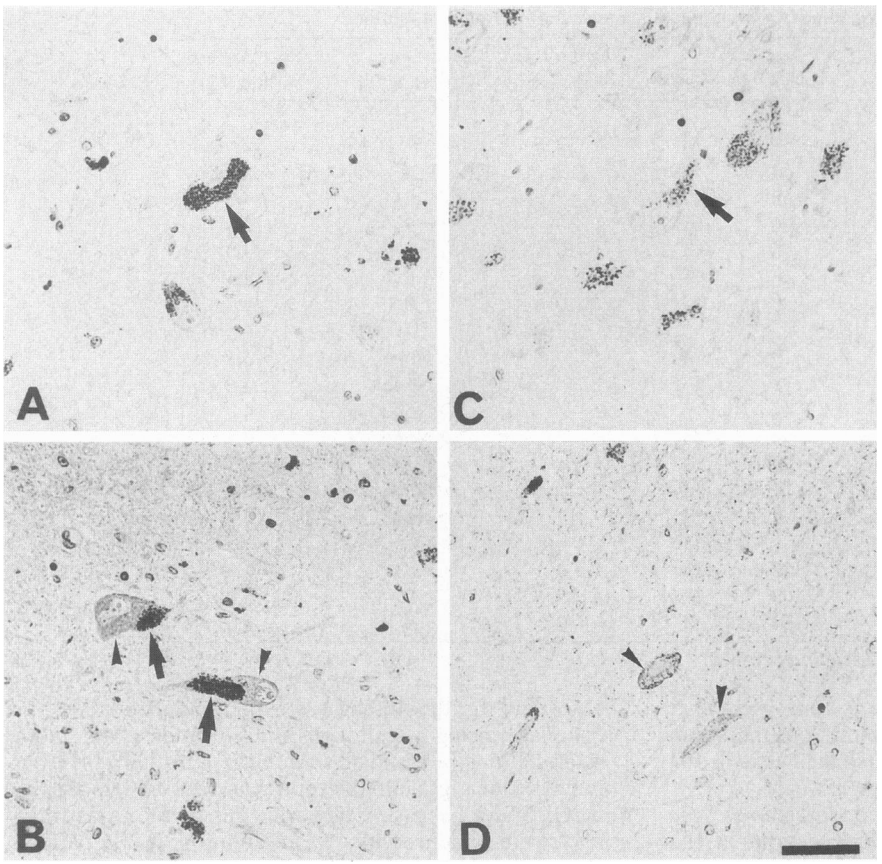

FIG. 1. Immunostaining of nigral neurons with G_3HG_3. (*A*) SN of PD patient 2 stained without primary antibody. Dark pigments represent neuromelanin; cytoplasm and other glial cells are not stained. (*B*) SN of PD patient 2 stained with G_3HG_3; cytoplasms of the two neurons show positive staining. (*C*) SN of control 2 stained with G_3HG_3; cytoplasm is not stained. (*D*) SN of MSA patient 1 stained with G_3HG_3; cytoplasm shows intense staining in granular fashion. Arrows, melanin; arrowheads, reactive products. (×260.) (Bar = 50 μm.)

nostaining for HNE (Table 2). The overall impression on immunostaining intensity was that PD patients showed higher proportions of positively stained neurons and more intense immunostaining compared with control subjects. However, rather marked differences in the proportion and intensity of immunostaining from one patient to another and even within the same individual were observed. Lewy bodies were not immunostained in any of the patients studied. Positively stained melanized neurons showed uniform and granular staining in the cytoplasm; both degenerated and remaining neurons were positively stained (Fig. 1 *A* and *B*). In patient 4, whose nigral neuronal loss was most severe among the patients

Table 2. Proportion of positively staining neurons in HNE immunostaining using G_3HG_3 in three parts of the SN

	Total cells	Medial, %			Central, %			Lateral, %			Total, %		
		−	+	++	−	+	++	−	+	++	−	+	++
PD													
1	169	76.7	13.3	10.0	67.5	29.9	2.6	60.7	26.2	13.1	66.3	19.5	7.7
2	178	37.6	37.6	24.7	67.4	23.9	8.7	73.0	10.8	16.2	52.2	28.1	18.5
3	266	43.8	44.9	11.2	35.0	41.0	23.9	48.1	38.9	13.0	39.8	41.0	16.9
4	75	33.3	25.0	41.7	39.5	39.5	21.1	0.0	40.0	60.0	30.7	33.3	32.0
5	274	9.0	27.0	64.0	58.6	37.5	4.6	94.4	5.6	0.0	42.0	30.7	25.9
6	183	35.3	27.9	36.8	74.2	23.7	2.1	100.0	0.0	0.0	59.6	23.0	14.8
7	151	13.6	20.5	65.9	27.9	35.3	36.8	0.5	38.5	56.4	17.9	31.8	50.0
Average	185	35.7	28.1	36.4	52.8	32.9	14.4	53.8	22.9	22.6	44.1	29.6	28.5
Control													
1	605	82.1	17.1	0.8	88.4	11.1	0.5	92.4	6.5	1.1	87.6	11.6	0.1
2	1221	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
3	559	91.1	5.4	3.6	83.9	13.8	2.3	85.7	12.1	2.1	85.5	11.8	2.5
4	438	97.4	2.6	0.0	98.1	1.5	0.5	100.0	0.0	0.0	98.4	1.6	0.0
5	250	91.9	5.4	2.7	91.0	7.2	1.8	90.2	6.6	3.3	89.6	6.4	2.4
6	486	97.5	2.5	0.0	85.6	12.2	2.3	89.8	9.5	0.7	87.9	9.7	1.4
7	309	94.4	4.2	1.4	92.0	7.0	1.0	90.2	9.1	0.8	90.0	7.1	1.0
Average	553	93.5	5.3	1.2	91.3	7.5	1.2	92.6	6.3	1.1	91.3	6.9	1.1
MSA													
1	86	0.0	17.1	82.9	33.3	38.1	28.6	75.0	12.5	12.5	23.3	26.7	48.8
2	47	42.9	14.3	42.9	40.0	45.0	15.0	27.8	44.4	27.8	34.0	38.3	23.4
3	304	85.3	14.0	0.7	84.4	12.8	2.8	90.5	9.5	0.0	84.9	12.8	0.1

Melanized neurons were classified into three groups according to the intensity of immunostaining of cytoplasm using the following scale: ++, intense staining; +, weak staining; −, no staining. Total cells, total melanized neurons in SN. Numbers represent proportions of positively stained neurons in each part of the SN against total number of melanized neurons in each part according to the intensity of immunostaining.

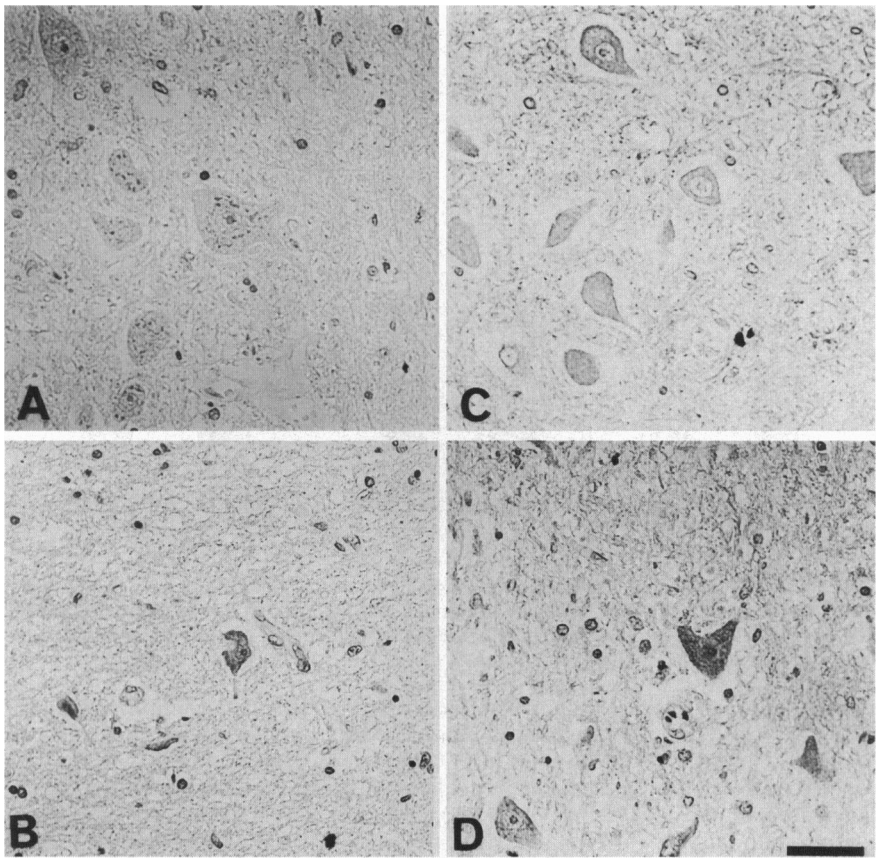

FIG. 2. Immunostaining of oculomotor and Edinger–Westphal nuclei with G_3HG_3. (*A*) Oculomotor neurons of control patient 2; no immunostaining is seen. (*B*) Oculomotor neurons of control patient 7; cytoplasm is weakly stained. (*C*) Edinger–Westphal nucleus of PD patient 3 stained with G_3HG_3; cytoplasm is stained weakly. (*D*) Edinger–Westphal nucleus of MSA patient 1 stained with G_3HG_3; cytoplasm is stained more intensely than that of the aged control and PD patients. (×260.) (Bar = 50 μm.)

studied, the proportions of positively stained neurons in the medial, central, and lateral parts were 67%, 61%, and 100%, respectively. In the medial part, several neurons were seen which showed highest immunostaining intensity among all the patients studied. Patient 7, who received only 6 months of levodopa therapy, showed the highest proportion of positively stained neurons among all the PD patients studied; proportions of positively stained neurons in the medial, central, and lateral parts were 86%, 72%, and 95%, respectively. No correlation appeared to exist between the amount of neuromelanin and the intensity of immunostaining. Neuropils of SN showed some immunostaining in the medial part but not in the lateral part. Glia cells were not positively stained except for one most severely involved patient (patient 7). The oculomotor nucleus could be analyzed in three of seven patients (as only half of the brainstem was histopathologically observed at times, the oculomotor nucleus was not included in the midbrain section even at the superior colliculus level). In these three patients, most of the oculomotor neurons and the neuropils showed weak immunostaining. The Edinger–Westphal nucleus of one of three PD patients, patient 3, showed weak staining (Fig. 2*C*). Other neurons in the midbrain section such as those in the red nucleus, superior colliculus, and midbrain raphe were not positively stained in either the PD patients or the control subjects.

MSA. There was severe melanized neuronal loss in the SN of patients with SND, but in the patient with OPCA the loss was mild. The MSA patient had combined striatonigral and olivopontocerebellar degeneration. Nigral neuronal loss was severe and the remaining neurons were shrunken. Melanized neurons were more intensely immunostained than those of PD patients (Fig. 1*D*). The immunostaining intensity in SN and the degree of degeneration of melanized neurons were much higher in the SND patients than in the patient with OPCA. In the one of three MSA patients that could be analyzed, most of the oculomotor neurons showed weak staining; the intensity of immunostaining was higher than that of PD patients. The Edinger–Westphal nucleus of MSA patient 1 showed intense staining (Fig. 2*D*). Other neurons in the midbrain section such as those in the red nucleus, superior colliculus, and midbrain raphe were not positively stained.

Semiquantitative Analysis. Results of semiquantitative analyses are shown in Table 2 and Fig. 3. The proportion of intensely stained neurons in the medial part of SN was 36.4 ± 7.7% (mean ± SE) in PD and 1.2% ± 0.6% in control subjects ($P < 0.01$), that in the central part was 14.4% ± 3.5% in PD and 1.2% ± 0.6% in the control ($P < 0.01$), and that in the lateral part was 22.6% ± 8.4% in PD and 1.1% ± 0.5% in the control ($P < 0.01$). The difference was statistically significant in all parts of SN. Among PD patients, the proportion of intensely stained neurons was significantly higher in the medial part compared to that in the central part ($P < 0.05$); no significant difference was noted between the medial part and the lateral part or between the central part and the lateral part. When three parts of SN were combined, the proportion of intensely stained neurons was 28.5% ± 10.4% in PD and 1.1% ± 2.0% in the control ($P < 0.01$). When the proportions of weakly stained and intensely stained neurons were combined, the proportion of positively stained neurons was 64.5% ± 11.8% in the medial part of PD and 6.5% ± 2.9 in the control ($P < 0.01$); in the central part, the proportion of positively stained neurons was 47.3% ± 7.2% in PD and 8.7% ± 2.6% in the control ($P < 0.01$); that in the lateral part was 45.5% ± 14.8% in PD and 7.4% ± 2.4% in the control ($P < 0.01$). The differences were highly significant; the difference between the medial part and the central part was again statistically significant ($P < 0.05$). Thus, the medial part of SN contained the highest proportion of positively stained neurons.

The highest proportion of the intensely stained neurons (82.9%) was observed in the medial part of patient 1 with MSA. In the control subjects, no difference in the distribution of positively stained neurons was observed among the three

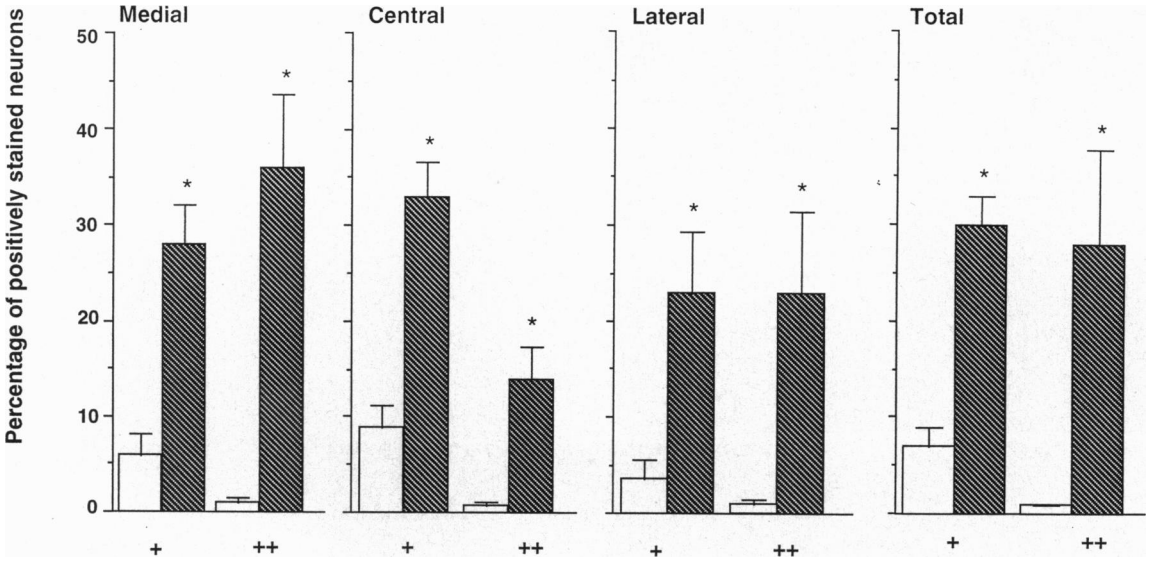

FIG. 3. Proportion of positively stained neurons with G_3HG_3 in three parts of the SN. Ordinate represents percentage of positively stained melanized neurons in three subregions of SN. Open columns, control subjects; hatched columns, patients with PD; +, weakly stained for G_3HG_3; ++, intensely stained for G_3HG_3. Medial, medial part of the SN; central, central part of the SN; lateral, lateral part of the SN; total, three parts combined. Data are means ± SE. *, $P < 0.01$ against control.

parts of the SN. In PD, no correlation was noted between the proportion of positively stained neurons and age of patients, age at onset, sex, disease duration, and period of levodopa therapy. In the oculomotor nucleus of the control subjects, the proportion of positively stained neurons increased exponentially with the age (Fig. 4); however, this correlation did not quite reach statistical significance ($r = 0.796$).

DISCUSSION

Recently, we reported that HNE-modified proteins determined by immunohistochemical procedures were identical to aldehydes detected by cold Schiff's reagent (21). Thus, HNE is considered to be the most reliable index of lipid peroxidation. We showed that a significantly higher proportion of nigral melanized neurons was positively stained for HNE-modified proteins in PD, and in MSA, indicating a significant increase in lipid peroxidation in these disorders. In the control patients, only small proportions of nigral neurons were positively stained and the proportion of intensely stained neurons was far less than that in the PD patients. Glia cells were not positively immunostained except for weak staining of a few glia cells in one patient each of PD, MSA, and SND, indicating that lipid peroxidation is taking place mostly within nigral neurons in PD.

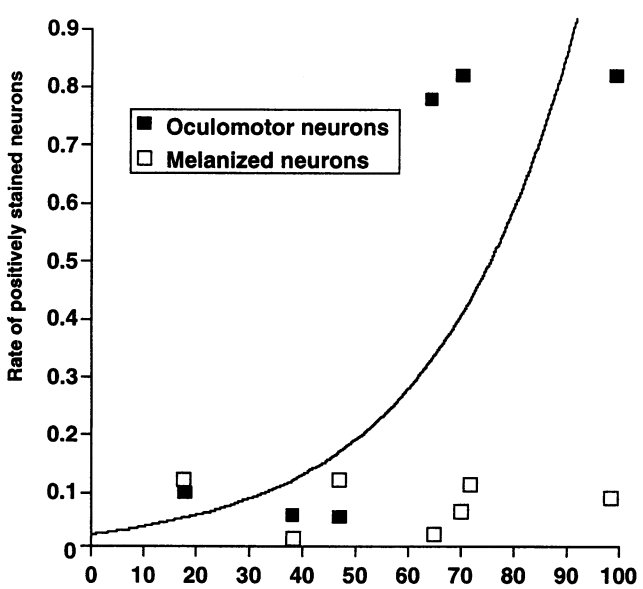

FIG. 4. Relationship between age and rate of positively stained neurons for G_3HG_3 (positively stained neurons). Ordinate represents rate of positive neurons to total nerve cells and abscissa shows age of control subjects. Solid squares, oculomotor neurons with Edinger–Westphal nucleus of the control subjects; open circles, melanized neurons. Exponential curve shows relationship between age and rate of positively stained oculomotor neurons.

Among the three parts of SN, the medial part contained the highest proportion of positively and intensely stained neurons. This is a somewhat unexpected finding because usually the lateral portion of the SN is the most severely damaged in PD (23), as was the case with our patients as judged by histological evaluation. This apparent discrepancy might be explained by the consideration that neurons in the lateral part of SN in PD frequently exhibit severe degenerative changes, including a marked loss of neuromelanins; the neurons are often shrunken with attendant loss of functional integrity. If during the progression of the disease there is a more rapid loss of polyunsaturated fatty acids from neurons in the lateral part, then the amount of HNE that could be produced by peroxidation would be diminished in the lateral region relative to that occurring in the more slowly degenerating medial part; thus, the level of polyunsaturated fatty acids in the medial part would be higher and the potential for HNE formation would be greater.

The possibility that the treatment of PD and MSA patients with levodopa might influence the intensity of immunostaining should also be considered. Slightly damaged medially located neurons might take up more levodopa than the more severely damaged laterally located neurons. In view of the fact that H_2O_2 formed during the oxidation of levodopa by monoamine oxidase and by autoxidation could give rise to hydroxyl radicals by the Fenton reaction (24), it has been suggested that levodopa therapy may accelerate progression of the disease (25), but this has not been confirmed in patients with PD (26). In our studies, there was no clear correlation between the proportion or the intensity of positive immunostaining and the approximate amount of levodopa taken by our patients. For instance, PD patient 7 had been treated with levodopa for only

6 months, while the other PD patients had been treated with levodopa for 5 years or more; the proportion of intensely stained neurons in patient 7 was the highest among the seven patients with PD. Therefore, it seems unlikely that levodopa treatment is the cause of positive immunostaining for HNE-modified proteins.

Most of the structures in the midbrain other than the SN were not positively stained for HNE-modified proteins in the PD patients and the control subjects; the only exception was the oculomotor nucleus. The oculomotor neurons showed weak staining in the PD patients and in the aged control subjects. In the control subjects, the number of positively stained neurons did not increase during the first 50 years, but between 64 and 99 years the rate increased exponentially, although the number of control subjects was small (Fig. 4). The result parallels the previous observations that the level of oxidized protein in the human brain and other animals increases exponentially (27, 28); there is in fact almost no change during the first 50 years, but between ages 55 to 99 the rate increases very rapidly. The reason why the oculomotor neurons showed positive staining may be due in part to its high oxidative respiration; in our previous report using immunohistochemical studies on mitochondrial electron transfer complexes, the oculomotor neurons showed intense immunostaining for complexes I, II, III, and IV as well as the SN among the midbrain structures (12).

From the results presented here, it appears to be firmly established that lipid peroxidation is increased within nigral neurons. This could seriously disrupt many important membrane functions such as oxidative phosphorylation, signal transduction, and regulation of electron, iron, and metabolite transport and thus contribute to nigral neurodegeneration in PD. Future research to develop drugs to overcome lipid peroxidation within nigral neurons appears to be very important, and such drugs may turn out to be a neuroprotective method of treatment for PD.

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