Loss of non-phosphorylated neurofilament immunoreactivity, with preservation of tyrosine hydroxylase, in surviving substantia nigra neurons in Parkinson’s disease

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Abstract

The distribution of neurofilament immunoreactivity in the substantia nigra was examined by immunohistochemistry in five patients dying with Parkinson’s disease and six control patients dying without neurological disease. In controls, pigmented neurons in the substantia nigra were intensively labelled by SMI32, a monoclonal antibody to non-phosphorylated neurofilament protein. In the substantia nigra from patients who had Parkinson’s disease, there was a pronounced reduction of SMI32 labelling intensity in surviving pigmented neurons. By contrast, tyrosine hydroxylase immunoreactivity in surviving pigmented neurons was normal. SMI32 labelling was normal in regions of the brainstem not affected by the neuropathological process of Parkinson’s disease. Findings with either antibodies to phosphorylated neurofilament, or enzymatic dephosphorylation followed by SMI32 labelling, indicated that loss of SMI32 immunostaining in Parkinson’s disease was not due to masking of the neurofilament epitopes by phosphorylation. Our results indicate that neurofilament proteins are particularly likely to be disrupted or destroyed by the neuropathological process of Parkinson’s disease. Nevertheless, the normal appearance of tyrosine hydroxylase indicates that protein synthesising systems may be intact in surviving neurons. Loss of neurofilament immunoreactivity may prove a sensitive neuropathological marker for characterisation of degenerating neurons in Parkinson’s disease.

Biochemical studies have confirmed that neurofilament proteins are a major constituent of the Lewy bodies found in the cortex of patients with diffuse Lewy body disease. The neuronal content of particular neurofilament proteins seems to be highly regulated, as shown by the differential distribution of such proteins in particular subsets of neurons, and by the propensity of the neurofilaments to modification via various metabolic pathways. The presence of neurofilaments may underlie the vulnerability of neurons in degenerative disease. Neurofilament content in particular subpopulations of cortical neurons has been shown to correlate with their propensity to form neurofibrillar tangles present in Alzheimer’s disease, and neurofilament immunohistochemistry has been used to identify the early pathological changes in neurons undergoing neurofibrillary degeneration in this disease.

When simple Nissl stains are used to identify neurons in brains obtained from patients dying with Parkinson’s disease, it is difficult to determine whether or not surviving neurons are themselves affected by the underlying disease process. In the present study we used neurofilament immunohistochemistry to examine the cytoskeletal profile of substantia nigra neurons in normal controls, and to characterise any alterations in neurofilaments that occur in surviving neurons in Parkinson’s disease.

Materials and methods

PATIENTS AND HISTOLOGICAL PROCEDURES

Brains were obtained at necropsy from five patients dying with Parkinson’s disease, and six control patients dying from non-neurological conditions (table 1). The patients with Parkinson’s disease had been on dopamine replacement therapy until at least a few months before death. No patient with Parkinson’s disease had a long history of dementia, but cognitive changes were present during the last months of life.

Brains were removed, generally within 24 hours of death (table 1), and fixed with aldehyde perfused through the carotid veins and vertebral arteries (see Hallday et al for details). The brainstem was separated from the forebrain at the level of the caudal limit of the mammillary bodies ventrally, and the rostral extent of the superior colliculus dorsally, and divided into two blocks at the mid-pons. The blocks were cut transversely into serial frozen
Table 1  Pre and postmortem data of controls (N) and patients with Parkinson’s disease (PD)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Postmortem delay (h)</th>
<th>Duration of PD (y)</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>N10 F</td>
<td>79</td>
<td>4</td>
<td>Cancer</td>
</tr>
<tr>
<td>N15 F</td>
<td>59</td>
<td>6</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>N16 F</td>
<td>87</td>
<td>22</td>
<td>Bowel obstruction</td>
</tr>
<tr>
<td>N19 F</td>
<td>61</td>
<td>8</td>
<td>Diabetes</td>
</tr>
<tr>
<td>N20 F</td>
<td>84</td>
<td>27</td>
<td>Peritonitis</td>
</tr>
<tr>
<td>N21 M</td>
<td>88</td>
<td>19</td>
<td>Renal failure</td>
</tr>
<tr>
<td>Mean (SEM)</td>
<td>76 (5)</td>
<td>14 (4)</td>
<td>-</td>
</tr>
</tbody>
</table>

Means for Parkinson’s disease v control were not significantly different (p>0.05).

sections (50 μm), and consecutive sections were serially collected in 15 containers. Separate section series were stained conventionally with haematoxylin and eosin, cresyl violet, Bielschowsky silver, and Weil fibres stains. Other series of sections were stained with antibodies to either tyrosine hydroxylase (TH) or to different forms of neurofilament proteins.

Monoclonal antibodies directed against neurofilament proteins were commercially obtained (Sternerberger Monoclonal Inc., Baltimore, USA), including SMI32 for non-phosphorylated neurofilaments and SMI31 and SMI34 for phosphorylated neurofilaments. All neurofilament antibodies were used at 1/1000 dilution. A rabbit anti-TH (Eugene Tech, NJ, USA), or a monoclonal antibody to TH (Incstar, MN, USA) were used at 1/2000 dilution. Immunoreactivity was visualised with appropriate biotinylated secondary antibodies and the avidin-biotin peroxidase complex (Vector), followed by the DAB-hydrogen peroxide reaction, with or without ammonium nickel sulphate.

In sections treated with nickel, immunoreactive products became dark blue and melanin pigments were yellow brown.

To further determine whether some neurofilaments had been phosphorylated in nerve cell bodies, sections from controls (N10, N19) and patients with Parkinson’s disease (PD8, PD11) were treated with phosphatase before immunostaining with SMI32. The sections were incubated for three hours, or overnight, at 35°C in a solution containing 140 μg/ml bovine intestinal alkaline phosphatase (type VII-T, Sigma) and 0-01 M phenylmethylsulphonyl fluoride in 0-1 M Tris buffer (pH 8-0).

All brains from patients with Parkinson’s disease satisfied the conventional neuropathological criteria for idiopathic Parkinson’s disease (pronounced loss of pigmented neurons in the substantia nigra and locus coeruleus, and the presence of Lewy bodies in these regions). Neuropathological changes that occur in Alzheimer’s disease were not found. No significant neuropathological changes were found in control brains. There was no significant difference between Parkinson’s disease and control groups with respect to age or postmortem delay time. No obvious effects of postmortem delay on the immunohistochemical staining were noticed.

Analysis

For quantitative morphological analysis we used a computer image system as previously described. Outlines of sections, major nuclei, and fibre bundles were mapped under 5 × magnification and used to match section levels between individual cases. Cells were counted under 250 × magnification. In both control and Parkinson’s disease groups the substantia nigra was measured rostrocaudally approximately 9-5 (0-5) mm (mean (SD), control, n = 3; Parkinson’s disease, n = 3), from the caudal portion of the subthalamic nucleus to the caudal limit of the trochlear nucleus. Both these nuclei contained characteristic SMI32 positive neurons.

The number of melanin pigmented neurons varies through the rostrocaudal substantia nigra. In the present study cell counting was made in sections at three carefully defined rostrocaudal substantia nigra levels. The rostral section was at the rostral limit of the oculomotor nucleus and the medial geniculate nucleus. At this level, no subthalamic neurons were present. The middle section was at the level where the medial geniculate nucleus was best represented in SMI32 immunostained sections. At the middle level, the boundaries of the paranglial nucleus and the substantia nigra were separated by the fibre tracks of the oculomotor neurons. The caudal section was at the caudal border of the red nucleus and the trochlear nucleus, where the superior cerebellar peduncles just separate from the decussation and form isolated fibre bundles. The three section levels were approximately 3 mm apart, spanning the rostral two thirds of the substantia nigra. The caudal one third of the substantia nigra contains fewer pigmented neurons, and it is sometimes difficult to distinguish between these cells and pigmented cells of the A8 group. Sections from the caudal one third were examined, but not quantitatively analysed.

For three control cases (N10, N19, N21) and all Parkinson’s disease cases, we also counted, at the middle level of the substantia nigra, the total number of pigmented or TH positive pigmented neurons in sections adjacent to the sections stained with SMI32. The number of total pigmented cells in sections stained for TH was not significantly different from that in sections stained with SMI32 (p > 0-05; table 2, middle column). The cell numbers in each side were not significantly different (p > 0-05). Occasionally one side of the section was damaged. Therefore cell counts are expressed as number per unilateral section. Statistical differences were assessed by unpaired Student’s t tests and by linear regression.

Results

In brains from both control and Parkinson’s disease groups SMI32 antibody labelled
neuronal cell bodies and dendrites in particular midbrain nuclei including the substantia nigra, the superior colliculus, the oculomotor and the interstitial nuclei, and the red nucleus, consistent with the known cellular distribution of non-phosphorylated neurofilament proteins in animal brains. Figure 1(A), shows the distribution of SMI32 positive neurons at the level of the red nucleus in a control midbrain. The multiple well stained dendrites of these cells sometimes extend as far as 0.5 mm in individual sections (figs 1(B), 2(A)).
The perikarya of pigmented neurons, dominated by melanin particles, were stained less intensely for SMI32, than the dendrites. The intensity and pattern of SMI32 immunostaining was uniform throughout the rostrocaudal extent of the substantia nigra.

In Parkinson’s disease there was the expected extensive loss of pigmented neurons. There was also a considerable decrease in the intensity of SMI32 staining in surviving pigmented neurons, particularly in dendrites (figs 1(D), 2(B)). This was most appreciable in subregions of the substantia nigra where the density of surviving pigmented cells was, presumably by chance, comparable with normal controls (fig 2(A and B)).

SMI32 immunoreactivity appeared uneven among the remaining neurons. Some neurons contained faint reactivity in cell bodies, with one or two weakly stained dendrites traceable for only a short distance (figs 1(D), 2(B)). Other surviving neurons had hardly detectable SMI32 immunoreactivity, leaving behind bare pigment (fig 2(B, E, F)). Nevertheless, some pigmented neurons had perikaryal and dendritic SMI32 staining comparable with substantia nigra neurons in controls. Lewy bodies were found in both pigmented SMI32 negative and in pigmented SMI32 positive neurons. In both classes of neurons the Lewy bodies themselves appeared SMI32 positive (fig 2(E, F)). SMI32 immunoreactivity was well preserved in non-pigmented neurons in the pars reticulata of the substantia nigra. Similarly,
SMI32 staining of other normally SMI32 positive midbrain nuclei, including the red nucleus, was normal.

In the substantia nigra cases of Parkinson’s disease, most surviving pigmented neurons were intensely and normally stained for TH by both polyclonal or monoclonal antibodies, with well preserved dendrites similar to those seen in control brains (figs 1(E), 2(D)). There was no obvious difference in the intensity and pattern of TH immunostaining throughout the rostrocaudal extent of the substantia nigra.

Figure 3 summarises the distribution of pigmented neurons, positive or negative for SMI32 or TH, at three rostrocaudal substantia nigra levels in control cases. The SMI32 negative pigmented neurons were preferentially located in the dorsal substantia nigra. In addition, many SMI32 negative pigmented cells were seen in the paranigral nucleus and in the midline region (not shown in fig 3). SMI32 positive neurons containing no visible melamin pigment were found in the pars reticulata of the substantia nigra. Figure 4 shows the distribution of pigmented neurons containing or lacking SMI32 or TH, mapped at three corresponding rostrocaudal substantia nigra levels from the brain of a patient with Parkinson’s disease. By contrast with the normal nigra, pigmented neurons lacking SMI32-ir in Parkinson’s disease were numerous and widespread, contrasting with the relatively rare occurrence of TH negative neurons.

QUANTITATIVE ANALYSIS OF SMI32 AND TH CONTAINING NEURONS

For both controls and cases of Parkinson’s disease the unilateral number of total pigmented neurons was counted at the three defined rostrocaudal levels of the substantia nigra shown in figs 3 and 4. Fragmented particles of pigment were not counted as cells. There was considerable variability in the number of pigmented neurons at the different rostrocaudal levels (table 2) in control brains, ranging from around 1000 cells at the rostral level to around 1800 cells at the caudal level. In brains from patients with Parkinson’s disease there was an approximate 70% loss of pigmented neurons, fairly constant at each rostrocaudal level (table 2).

As documented in table 2, in control brains, 95% of pigmented substantia nigra neurons at the middle rostrocaudal level were TH positive. In Parkinson’s disease the corresponding value was 91%, not significantly different from controls (table 2). In control brains 95% of pigmented substantia neurons were SMI32 positive, with little variation at the different rostrocaudal levels (table 2). In brains from the Parkinson’s disease group the proportion of pigmented neurons that contained SMI32 was reduced to around 50% and ranged from 27% to 72% (table 2).

In both control and Parkinson’s disease groups, linear regression analysis showed no relation between age at death and number of total pigmented cells or SMI32 or TH positive pigmented cells. We examined the relation, in cases of Parkinson’s disease, between number of surviving pigmented neurons and number of SMI32 positive neurons. There was a highly significant linear regression between these variables. The regression line passed near the origin and the slope was 0-57, indicating that the proportion of surviving pigmented neurons containing SMI32 was constant for different rostrocaudal levels and for individual cases, regardless of the total number of surviving neurons at death of the patient.

EFFECTS OF PHOSPHATASE TREATMENT ON SMI32 STAINING IN THE SUBSTANTIA NIGRA

In normal controls, phosphatase treatment for three hours or overnight had no obvious effect on SMI32 perikaryal and dendritic staining, either in the substantia nigra, or in other SMI32 positive nuclei. After phosphatase treatment, however, numerous axons became immunoreactive for SMI32. This was expected as SMI32 reacts with non-phosphorylated neurofilaments. The stained axons included intra-axial fibres of the oculomotor
neurons. Increases in SMI32 axonal staining, including axons of the oculomotor nerve traversing the substantia nigra, were seen in Parkinson’s disease sections after phosphatase treatment (fig 2G), but the neuronal staining remained unchanged, with no increase in staining in the remaining pigmented neurons (fig 2G). This indicates that the loss of neurofilament staining in Parkinson’s disease did not simply reflect abnormal phosphorylation. We counted SMI32 positive pigmented cells in four sections from two midbrains from patients with Parkinson’s disease (PD8, PD11) treated with phosphatase. There was no significant difference in the percentage of SMI32 positive pigmented cells, compared with adjacent sections without phosphatase treatment.

SMI31 AND SMI34 STAINING IN THE SUBSTANTIA NIGRA
SMI31 and SMI34 gave similar staining patterns, labelling mainly axons in midbrain sec-

Figure 4  Distribution of pigmented neurons immunopositive (dot) or immunonegative (circle) for SMI32 (A, C, E), and for TH (B, D, F), at three rostrocaudal levels of the substantia nigra in a brain from a patient with Parkinson’s disease (PD) (PD11). Each dot or circle represents one pigmented neuron. Abbreviations and illustrated regions as for fig 3.

Discussion
In control brains the number of pigmented neurons at a given level of the substantia nigra was reasonably constant across subjects. About 95% of these pigmented neurons contained intense SMI32 immunoreactivity, in both perikarya and dendrites. About 95% of pigmented cells also contained TH. In brains from patients with Parkinson’s disease there was considerable variability in the number of surviving substantia nigra neurons. The proportion of pigmented cells that contained TH in the Parkinson’s disease substantia nigra was 91%, similar to controls. In Parkinson’s disease, however, only 53% of surviving pigmented neurons contained SMI32 immunoreactivity, and there was considerably reduced staining in pigmented cells that did contain SMI32. On the other hand, SMI32 immunoreactivity was preserved in midbrain nuclei less vulnerable to parkinsonian changes, including oculomotor neurons and neurons of the red nucleus. Immunoreactivity to SMI32 was also well preserved in the non-pigmented neurons situated in the pars reticulata of the substantia nigra. Neurofilament proteins in the pigmented neurons of the substantia nigra are thus more severely affected than are transmitter related proteins such as TH. The SMI32 negative pigmented neurons are presumably in a transitional degenerative phase, culminating in death of pigmented cells in Parkinson’s disease.

In patients with Parkinson’s disease the proportion of SMI32 positive neurons in surviving pigmented cells was as low as 27%. Somewhat surprisingly, there was a strong linear relation between the number of SMI32 positive pigmented neurons and the total number of pigmented neurons in individual cases of Parkinson’s disease (n = 13, r = 0.91, p < 0.01). Thus the proportion of pigmented cells containing SMI32 immunoreactivity (around 50%) was constant for each patient, regardless of the number of surviving pigmented cells. This finding would be easier to interpret if we knew what happens to SMI32 immunoreactivity early in the course of Parkinson’s disease. Our patients died after manifesting symptoms of Parkinson’s disease for over 20 years. If neurofilament proteins are particularly vulnerable in Parkinson’s disease, one might expect to see a pronounced loss of this protein early in the course of the disease, before neuronal death occurs.

The mechanism underlying the loss of SMI32 staining in Parkinson’s disease is not known. SMI32 binds to non-phosphorylated neurofilaments. The SMI32 binding sites are located in the tail domains of proteins of normal controls, with occasional positive cell bodies. Mesencephalic trigeminal neurons were consistently labelled by both antibodies. In cases of Parkinson’s disease, the axonal staining was similar, but no antibody positive pigmented cells were seen in the substantia nigra. Some Lewy bodies were stained positively by these antibodies (fig 2H).
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neurofilament-H and neurofilament-M subunits. These epitopes are sensitive to proteolysis, and can be masked from antibody binding by phosphorylation. This presumably reflects the neurofilament structure in which the neurofilament-H and neurofilament-M tail domains protrude from the neurofilament backbone, and constitutes the major target for post-transcriptional modification of the molecule by phosphorylation. Similar immunological properties are shared by other monoclonal antibodies raised against non-phosphorylated neurofilaments. Because Lewy bodies contain phosphorylated neurofilament epitopes, it has been suggested that phosphorylation of neurofilaments may occur in nerve cell bodies in Parkinson’s disease, and this may interfere with SMI32 antibody binding, falsely suggesting absence of the protein. In the present study we obtained evidence that the neurofilament protein was indeed absent, rather than being present but phosphorylated, by using antibodies against phosphorylated neurofilaments. No immunohistochemical reaction product for phosphorylated neurofilament (SMI31 and SMI34 antibodies) was seen in perikarya in regions affected in Parkinson’s disease, but axons traversing the substantia nigra region, including those of the oculomotor nerve, did contain phosphorylated neurofilaments. These axons become SMI32 positive after the sections were treated with phosphatase enzyme. These findings confirm that our methodology could detect phosphorylated neurofilaments if they were present in pigmented substantia nigra neurons or in the proximal axonal processes of these cells. Nevertheless, we acknowledge that our results still do not exclude the possibility that the neurofilaments are present in the Parkinson’s disease neurons, but structurally altered by some other process. Some non-lysosomal proteases, such as calcium-activated neutral proteases (calpains), are very active towards neurofilaments. The exposed neurofilament tails might be more accessible to these attacks, leading to the disappearance of immunohistochemical staining for both non-phosphorylated and phosphorylated forms. Global protein degradation is less likely, as TH proteins were well preserved in the pigmented neurons. Hill et al recently reported that pigmented neurons in the substantia nigra from patients with Parkinson’s disease contain lower levels of mRNA for neurofilament-H and neurofilament-L. Decreases in TH mRNA also occur in the surviving pigmented neurons in Parkinson’s disease. We tested several other monoclonal and polyclonal neurofilament antibodies directed against various forms of neurofilaments including phosphorylation independent ones (see table 1, Vickers et al), but did not obtain good staining with our material. Further study is needed to determine whether there is actual loss of neurofilament proteins in the pigmented neurons.

Lewy bodies can be found in patients with a minor loss of substantia nigra cells, and with normal dopamine concentrations in the caudate and putamen, with no clinical evidence of Parkinson’s disease. These patients may represent the presymptomatic stage of Parkinson’s disease. Lewy bodies are composed of neurofilaments or neurofilament fragments and their presence in presymptomatic Parkinson’s disease also suggests early involvement of neurofilaments in the disease process.

A number of neurological diseases, apart from idiopathic Parkinson’s disease, are accompanied by degeneration of substantia nigra neurons, including Steele-Richardson-Olszewski syndrome, striatonigral degeneration, and Alzheimer’s disease. Elucidation of neurofilament changes in these diseases may provide clues to the degeneration process in Parkinson’s disease. During the present study, we examined one patient with pathologically documented Steele-Richardson-Olszewski syndrome. This patient had severe (70%) loss of pigmented neurons in the substantia nigra but the proportion of surviving pigmented neurons which were SMI32-positive (97%), was similar to controls. In cross sections of human substantia nigra, some pigmented or non-pigmented neuronal groups are also affected by the processes underlying Parkinson’s disease pathology. These include the neurons in the midbrain ventral tegmental area, locus coeruleus, pedunculopontine tegmental nucleus, preganglionic dorsal motor vagal neurons, and catecholamine neurons in the pons and medulla. We are currently studying whether neurons in these regions contain neurofilaments, and whether these neurofilaments are altered in Parkinson’s disease.

We thank Ms Robyn Flook for valuable technical assistance. We thank Professor Marcello Costa for focusing our attention on cytoskeletal proteins. This work was supported by the National Health and Medical Research Council and the Australian Brain Foundation. JCV is an NH and MRC CJ Martin Research Fellow.

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