Neuronal Changes in the Substantia Nigra with Aging: A Golgi Study

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Abstract. In order to recognize substantia nigra neuronal changes occurring in aging, 20 human control brains from 13 males and 7 females with a mean age of 61 years (range 20 to 93 years) without neurological disease were examined using the Golgi method. A quantitative study of dendrites and dendritic spines was performed as well as a statistical analysis of obtained data. Parallel sections to the impregnated material were histologically and immunohistologically studied with the aim to identify possible neuronal cytoskeletal abnormalities. Results were compared to changes of substantia nigra reported in other conditions such as Parkinson's disease (PD) and methyl-4-phenylpyridine (MPTP) experimental toxicity. Three different substantia nigra neuronal types were observed. Morphological changes during aging consisted of distorted profile of the cell body and swelling and beading of dendritic branches. The quantitative assessment of changes observed in neuronal types showed a significant loss of dendrites and dendritic spines, especially in the oldest cases. These findings were similar to those previously described in other cerebral areas during aging, but a specific vulnerability of the largest substantia nigra neuronal type could be observed. Nodulations and beaded aspects of dendrites are reminiscent of changes previously described in MPTP toxicity. Dendritic varicosities found in the oldest cases have also been found in dendrites of large substantia nigra neurons in PD. Cytoskeletal abnormalities have been described in PD but were not found in the present study. Therefore, other pathophysiological mechanisms different from the cytoskeletal compromise occurring in some neurodegenerative diseases should be involved in aging.

Key Words: Aging; Golgi method; Substantia nigra.

INTRODUCTION

For some authors, a morphological feature of the substantia nigra in aging is neuronal loss (1–3), while this is disputed by others (4). The presence of intracytoplasmic inclusions such as Lewy bodies and its correlation with age has also been addressed (5). Several authors have described the anatomy of the substantia nigra and detailed cytological studies have been carried out (2, 6, 7). Golgi impregnation techniques are invaluable in demonstrating the cytoarchitecture of the central nervous system and are probably one of the best and most elegant ways of highlighting the three dimensional relationships of axons and dendrites (8–11). The value of this technique lies in its ability to impregnate the entire neuron, allowing a thorough study of individual neurons (11). This method has been successfully used to describe neuronal changes in pathological conditions affecting the substantia nigra (12–14) and other cerebral areas (15). Aging brains have also been studied at different anatomical sites using this technique (16–18). Neuronal changes of substantia nigra in aging have, however, not been addressed by this method. This study is aimed to fill this gap.

MATERIALS AND METHODS

The substantia nigra of 20 human control cases ranging in age from 20 to 93 years (7 women and 13 men with a mean age of 61.9 years) without a clinical history of neurological disease was examined using the rapid Golgi method (19). The mean postmortem delay was 10.9 hours (h). The cause of death was pneumonia or cardiac failure.

Cases were grouped according to age: group A (from 20 to 39 years old), four cases with a mean age of 26 years; group B (from 40 to 69 years), five cases with a mean age of 54; and group C (from 70 to 93 years), ten cases with a mean age of 79 years.

The substantia nigra was separated from the brainstem by cutting at a right angle to the long axis of the brainstem from the lower border of the superior colliculus to the point of origin of the third cranial nerve. A 16 mm thick block was obtained and divided parasagittally into two parts and transversely into four blocks. The second and third blocks (medial blocks) were fixed for 1 week in 3% potassium dichromate and 1% osmium tetroxide solution at a concentration rate of 20:6. After this period, blocks were washed in distilled water and impregnated in a 0.75% silver nitrate solution for 48 h. Tissues were then dehydrated in absolute alcohol, embedded in paraffin, and cut in 100 μm thick slices which were mounted in DPX.

Detailed tracings of neurons from impregnated material using the Golgi method were made using a drawing tube (20), and morphological details of several representative neurons were recorded on numerous photographs of adjacent fields. All impregnated neurons, primary and secondary dendrites, as well as dendritic spines (at the first and second 100 μm from the cell body) in each age group and neuronal type were quantified. Results were statistically analyzed using the Primer program (one-way statistical variance).

In order to identify neuronal cytoskeletal abnormalities, 7 μm sections obtained from parallel paraffin-embedded blocks to the impregnated material were stained with hematoxylin and eosin (H&E) and a modified Bielschowsky's method (19). Other sections were reacted with a panel of monoclonal antibodies which included two phosphorylated (150 kD and 210 kD) and two

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with fusiform shape and three or four long dendrites with few branches; spines were few. This type of neuron predominates in the pars reticularis of the substantia nigra and was scarce in the pars compacta. Type III neurons (16 ± 4 μm) had the smallest cell body, were round without apparent polarity, and had five or more dendrites radiating in all directions from the cell body. Spines were occasionally observed. This cell type was found in the pars reticularis of the substantia nigra and was the most difficult to find.

Significant abnormalities could be observed in neurons of cases from group C as compared to neurons from group A. Nerve cell bodies lost their normal profile, were swollen and showed proliferation of spine-like protrusions. Dendrites became shorter and branches were fewer than those from group A neurons. Nodulations and distortion of dendritic profile could be observed. Beading arising from dendrite shafts was also observed. Severe loss of spines was found and the remaining spines were thinner and elongated. Abnormalities increased with age and were more evident in type I (Fig. 2) and type II (Fig. 3) neurons from the oldest cases. Spines from type I neurons (Figs. 4, 5) in group C showed marked changes consisting of thinness, elongation and, in some cases, distortion. Changes in type III neurons (Fig. 6) consisted of loss of normal shape of the cell body and dendrite branches.

When counting the number of impregnated neurons, it was observed that the best impregnated group was the youngest one, especially type I neurons. Silver impregnation progressively decreased in elderly groups. The presence of changes involving cell bodies and dendritic trees from all neuronal types was not uniform in all age groups. They were observed in age groups B and C, and were more frequently found in age group C, showing the following percentages: type I, 50%; type II, 40%; and type III, 30%. Group B showed a lower percentage of affected neurons: type I, 5%; type II, 5%; and type III, 3.06%. Quantitative assessment of changes observed in neuronal types showed a significant loss of dendrites in type I neurons. This neuronal type also showed a significant loss of spines. These features were more evident when groups A and C were compared. Table I summarizes quantitative results and the statistical analysis.

No neuronal cytoskeletal abnormalities (neurofibrillary tangles, Lewy bodies, or other cytoplasmic inclusions) were observed in all studied cases (manuscript in preparation). Few senile plaques were observed in the neocortex in cases from group C. The diagnosis of Alzheimer's disease or Parkinson's disease was excluded in all cases according to the NINCDS-ADRDA (22) and Parkinson's Disease (23) Society Brain Bank diagnostic criteria, respectively.

Fig. 1. Microdrawing of different neuronal types of human substantia nigra (A: type I, B: type II, C: type III). Cells are grouped according to age. Note that neurons from the oldest group show swelling and distortion of the cell body profile with spine-like protrusions (arrows). Dendrites are fewer, shorter, and have some nodulations (arrowheads). Changes are conspicuous in cell types I and II and less evident in cell type III (Camera lucida, ×60).

non-phosphorylated (200 kD and 210 kD) neurofilament subunits (from Affinity; diluted 1:1,000) and antiserum for tau (45–62 kD, from Sigma; diluted 1:400) and ubiquitin (from Dako; 1:200). An analysis of the main histological features using the same histological and immunohistological stainings was made on sections from neocortex (including Brodmann areas A9, A4, A40, and A22), anterior cingulate gyrus and hippocampus with the aim of excluding cases with Alzheimer's and/or Parkinson's diseases.
Fig. 2. Substantia nigra type I neurons from 27 (A) and 83 (B) year old men. Compare the cell body and dendritic profiles as well as thickness and length of dendrites. Distorted profiles of the cell body and dendrites (arrows) are conspicuous in B. The number of spines is also reduced in B (arrowheads), compared with the homogeneous profile of spines in A (arrows). (Rapid Golgi method, ×300.)

### TABLE 1
Quantitative Findings of Dendritic Morphology in the Human Substantia Nigra

<table>
<thead>
<tr>
<th>Neuronal types</th>
<th>Age groups</th>
<th>p values</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imp. N.</td>
<td></td>
<td>31.33 ± 6.43</td>
</tr>
<tr>
<td>Den. 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>5.00 ± 0.89</td>
</tr>
<tr>
<td>Den. 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>6.67 ± 0.03</td>
</tr>
<tr>
<td>Sp. A</td>
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<td>20.67 ± 7.45</td>
</tr>
<tr>
<td>Sp. B</td>
<td></td>
<td>20.33 ± 7.50</td>
</tr>
<tr>
<td>II</td>
<td></td>
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</tr>
<tr>
<td>Imp. N</td>
<td></td>
<td>12.33 ± 10.12</td>
</tr>
<tr>
<td>Den. 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>3.33 ± 0.52</td>
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<tr>
<td>Den. 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>5.67 ± 1.86</td>
</tr>
<tr>
<td>Sp. A</td>
<td></td>
<td>21.00 ± 3.22</td>
</tr>
<tr>
<td>Sp. B</td>
<td></td>
<td>16.67 ± 2.38</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imp. N</td>
<td></td>
<td>8.67 ± 4.04</td>
</tr>
<tr>
<td>Den. 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>4.33 ± 0.52</td>
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<tr>
<td>Den. 2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>7.67 ± 0.52</td>
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</table>

Imp. N.: number of impregnated neurons, ±: standard deviation, Den. 1<sup>a</sup>: number of primary dendrites, Den. 2<sup>b</sup>: number of secondary dendrites, Sp. A: number of dendritic spines at the first 100 μm from the cell body, Sp. B: number of dendritic spines at the second 100 μm from the cell body, p: statistical significance.
DISCUSSION

Several authors have studied the aging phenomenon and have described morphological changes in the central nervous system using the Golgi method in various anatomical regions, but a study of silver-impregnated substantia nigra neurons related to aging has not been reported.

In our series, distorted profile of the cell body, loss of dendrites and spines, and swelling and beading of dendritic branches were common changes in neurons from the oldest group (from 70 to 93 years). These findings are similar to those observed by other authors who described morphological abnormalities related to human aging in cortical and hippocampal neurons (16–18). Machado-Salas et al (24) described similar changes in the spinal cord and lower brainstem of the aging mouse. In spite of common findings to those previously described, we found that different types of neurons were involved to a varying degree. The quantitative study demonstrated significant changes in type I and type II neurons, with the loss of dendritic spines the most significant feature. Type I neurons were most affected which may imply that they are especially vulnerable as years go by. The loss of dendritic spines was marked in distal portions of dendrites. Similar features have also been observed by other authors in cortical neurons (16–18). In agreement with these authors, the salient feature which emerges from this Golgi study of aging substantia nigra is that the dendrite system appears to follow a sequential pattern of deterioration which starts with loss of dendritic spines with a proximal progression followed by a decreased number of dendrites.

A distorted profile of the cell body and dendrites appears to be the pattern of neuronal aging in the different cell types. Some neuronal bodies were enlarged and some dendrites showed a tortuous profile; these features may be related either to neuronal atrophy or hypertrophy which have been recognized as changes of senescence (25). Finch (25) suggests that the accumulation of mel-
anin and lipofuscin and the presence of vacuoles are strongly related to the displacement of cell cytoplasma and considered localized age-related changes. Thinning of dendrites and elongated dendritic spines may be signs of neuronal atrophy, which also occurs in aging (25) and is probably due to loss of cytoskeletal components.

We used the rapid Golgi method with fresh impregnation. Previous studies (12, 21) with the Golgi method have used the Braitenberg’s variant with a previous long formalin-fixation time. These methodological differences may explain some differences in findings of normal morphology of human substantia nigra neurons. Our results in the oldest cases are similar to those showed by Braak and Braak (21) in control type I substantia nigra neurons. For these authors, dendrites in type I neurons were scanty and elongated with thin spines. However, we found differences between young and old cases. Braak and Braak (21) did not report the age of cases where such dendrites

Fig. 4. Dendrite of a substantia nigra type I neuron from a young man, 27 years old (A), showing a homogeneous profile of spines (arrows) compared with a dendrite of the same type of neuron from an 85 year old man (B). In this case spines are thin, elongated and distorted (arrows). (Rapid Golgi method, x400.)
were described. Thus, according to our results, those features should correspond to the oldest cases, our younger ones having dendrites with smaller spines and homogeneous dendritic profile.

The Golgi method allowed detection of various aspects of neuronal development (16–29), pathological conditions (30–32) and senescence (16–18, 33). In metabolic disorders, neurons showed swelling of perikarya and torpedo-like axonal and dendritic swellings which have been associated with progressive accumulation of uncatabolized substrates (34, 35). Neuronal loss cannot be estimated with the Golgi method due to a feature of silver impregnation which is not uniform and is related to unknown factors. Ramon y Cajal and De Castro (19) mentioned this aspect and observed that young tissues were more easily impregnated. They suggested that factors related to myelination and increasing accumulation of cellular pigments should modify the cellular affinity for silver. Our results are in agreement with such observations.

Fig. 5. Microdrawing of neuronal type I dendrites from cases from group A (22 years old), B (46 years old) and C (85 years old). Note the progressive distortion and loss of spines (Camera lucida, ×600).

Fig. 6. Type III neurons from the substantia nigra of 22 (A) and 93 (B) year old men. The profile of neurons from the younger case (A) consists of a small cellular body with thin dendrites radiating in several directions. Note that in the older case (B) dendrites are fewer and some of them are distorted (arrows). (Rapid Golgi method, ×900 (A), ×800 (B)).
There are numerous reports on dendrite pathology in neurodegenerative disorders suggesting that some changes are due to cytoskeletal abnormalities (12, 32, 36). Patt et al (12) described dendrite changes in type I substantia nigra neurons in Parkinson’s disease which were attributed to Lewy body-like dendritic inclusions. In our cases, dendrites showed distortion and a swollen profile, including a beaded aspect which could suggest cytoskeletal or other abnormalities different from Lewy bodies which may affect the afferent pathway of large pigmented cells. However, neuronal cytoskeletal abnormalities involving neurofilaments, microtubule-associated protein and the ubiquitin–proteolytic pathway could not be demonstrated.

We have described neuronal changes in the methyl-4-phenylpyridine experimental toxicity (MPTP)-treated mice (14), consisting of swelling and distortion of cellular bodies, discontinuous thickness and dendritic nodulations. However, immunohistological studies did not show cytoskeletal abnormalities (13). Some changes reported by these authors were similar to those observed by us in aging such as nodulations and a beaded aspect of dendrites. However, in MPTP-treated mice such changes are more conspicuous.

In the present study, as mentioned before, changes are marked in type I neurons. Braak and Braak (21), using silver deimpregnation techniques, demonstrated that these neurons correspond to large melanin-containing ones. The predominant compromise of this neuronal type could be due to the greater cell surface and size which may render morphological changes more evident or to a selective vulnerability of this neuronal type which has been related to the melanin content and its increase during aging (37). Neuramelin granules may also confer vulnerability to nigral neurons by accounting for a specific site of iron accumulation and reduction (38). The proposition that free radicals may be an important factor in aging remains to be rigorously proven (39–42). The neuronal melanin content in relation to changes observed in the present study should be studied further.

Finally, with the Golgi method we demonstrated neuronal morphological changes in aging. Some of them showed degenerative features and others were similar to those caused by MPTP. A mechanism akin to MPTP toxicity may be detrimental to substantia nigra neurons during aging. A cytoskeletal compromise in aging was not found in the present study. Thus, other pathophysiological mechanisms occurring in some neurodegenerative disorders such as Parkinson’s disease must be involved in aging.

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