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Loss of cholinergic neurons in the pedunculopontine nucleus in Parkinson's disease is related to disability of the patients

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Abstract

We investigated neuronal number and size in the pars compacta of the pedunculopontine nucleus (PPN) in Parkinson's disease (PD). In PD, the number of Luxol fast blue (LFB) neurons was reduced by 27% from the mean control value ($p = 0.04$) and the cholinergic (choline acetyltransferase, ChAT-positive) neuron number was reduced by 36% ($p = 0.03$). In addition to neuronal loss, the remaining neurons in the PPN in PD were smaller than in controls. The profile area of LFB neurons was reduced by 14% ($p = 0.009$) and that of ChAT-positive neurons by 26% ($p = 0.001$).

There was more severe loss of ChAT-positive neurons with a more severe stage of the disease, evaluated by the modified Hoehn and Yahr scale ($r = -0.66$, $p = 0.03$). The neuron number decreased much more than could be expected on the basis of decrease in cell size alone.

Keywords: Choline acetyltransferase; Cholinergic system; Neuromorphometry; Neuron counts; Parkinson's disease; Pedunculopontine nucleus; PPN

1. Introduction

The main pathophysiological mechanism of Parkinson's disease (PD) is a progressive loss of dopaminergic neurons in the substantia nigra (SN), leading to deficiency of dopamine. Dopaminergic therapy is the most common drug treatment for PD, and alleviates many of the parkinsonian symptoms. However, deficits such as gait disturbances, postural instability, freezing, unexplained falls and sleep disturbances do not respond well or at all to dopaminergic therapy, and cannot be explained only by dysfunction of the nigrostriatal dopaminergic system [1–4]. Dopamine-resistant parkinsonian deficits of this kind are more frequently seen in patients with widespread degenerative brainstem disorders such as progressive supranuclear palsy (PSP) [5,6]. The pedunculopontine nucleus (PPN) is located in the dorsolateral part of the caudal mesencephalic tegmentum. PPN has reciprocal connections with the limbic system, basal ganglia nuclei and brainstem reticular formation [7]. There are connections to many thalamic nuclei, the globus pallidus, subthalamic nucleus, substantia nigra and ventral tegemental area [8–11]. In addition, the PPN is connected to the prefrontal and motor cortex [7,9]. In non-human primates, most of the large neurons and in humans 58% of the medium to large neurons in the PPN are cholinergic [12–14]. About 30–40% of the large cholinergic neurons also contain substance P [15,16].

In PD about 40–57% loss of large PPN neurons has been found [17–19], which is slightly less than the loss seen in PSP [18,20]. In these studies no immunocytochemical staining was used to separate cholinergic and non-cholinergic neurons. In addition, it is unclear at present whether the loss of PPN neurons is related to the clinical symptoms of PD patients, although abnormalities in gait and posture in addition to rigidity

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and akinesia may partly be due to neuronal loss or reduced neuronal activity in PPN [7]. Moreover, there is a high correlation between neuronal loss in PPN and that in the SN pars compacta [19]. The role of PPN in motor control is further supported by recent targeting of PPN for deep brain stimulation in PD [21–23].

The purpose of this study was to investigate the number and size of cholinergic and non-cholinergic PPN neurons in PD in relation to clinical disability. Our hypothesis was that neuronal loss in the PPN is associated with more severe stage of PD.

2. Patients and methods

2.1. Patients

Nine controls (4 M/5 W) and 11 PD patients (7 M/4 W) were studied. The mean age; SD of controls was 73; 14 and that of the PD patients 79.3; 4.4 years ($p = 0.17$). The duration of disease in the PD patients was 9.3; 3.4 years (mean; SD). The patients were distributed between the modified Hoehn and Yahr stages [24] as follows: stage 2.5, two patients; stage 3, four patients; stage 4, four patients and stage 5, one patient. The PD patients were diagnosed and followed-up until death in the Department of Neurology, University of Turku. All of the patients showed a good response to levodopa.

The brain samples were obtained from autopsies performed at the University of Turku. The use of tissue from deceased individuals was permitted by the National Board of Medicolegal Affairs (Finland). The brain was halved sagittally and the left half was fixed in 4% phosphate-buffered formalin. There was no difference either in the post-mortem times or in the fixation times between PD and control brains. In neuropathological examination PD patients showed characteristic abnormal findings: loss of pigmented neurons, and presence of Lewy bodies in the SN, confirming the clinical diagnosis of PD. Altogether samples from 16 defined brain areas were examined, after staining with hematoxylin–eosin and Bielschowsky's silver method to exclude any other relevant brain pathology in PD patients and controls [25]. In PD patients alpha-synuclein antibody was used to detect Lewy bodies for staging of pathology related to PD [26]. Six of the patients were at stage 3, three patients at stage 4 and one patient at stage 5. The cortical samples of one patient had been destroyed, but according to nigral and brainstem samples the patient was at least at stage 3. In addition, clinically, the controls had no evidence or history of symptoms of PD or other neuropsychiatric diseases.

The entire PPN was embedded in several paraffin blocks about 3–4 mm in thickness perpendicular to the longitudinal axis of the pons. The blocks were sectioned at a thickness setting of 11 μm. Every 40th section was stained with Luxol fast blue and the adjacent section was stained with a monoclonal antibody against choline acetyltransferase (ChAT) (Boehringer Mannheim GmbH).

2.2. Morphometric methods

The measurements were made at several levels through the rostrocaudal extent of the pars compacta of the PPN as described by Zweig et al. [20]. The counting area of the PPN on the section was outlined with a thin felt-tip pen as described earlier [27]. The comparability of levels and the outlining of the PPN were made in agreement by two investigators (M.R. and J.O.R.). When doing this morphometric study, the investigators were unaware of the subjects' history and their clinical data.

All samples were morphometrically analyzed using an image overlay drawing system applied by the Prodit morphometry program (Promis Computerized Inc., The Netherlands). In addition to the microscope (Leitz Orthoplan, Germany), the system included a personal computer (Compaq Deskpro 386/20e; Compaq Computer Corporation, Houston, TX), a video camera (JVC TK-870U; JVC, Japan) and a digitizer board (PIP-512B video digitizer board; Matrox Electronic Systems; Dorval, Quebec, Canada). The neurons were measured by outlining their profiles with a computer mouse on the monitor screen. Each measurement gave the number of neurons and the area (μm^2) of each neuron. The PPN was systematically scanned at a final magnification of 670× for every field throughout the entire PPN. The neurons greater than 20 μm in neuronal diameter were measured as described by Zweig et al. [20]. In order to obtain accurate data, the instrumentation was calibrated before each measurement series, and the measuring technique was applied in a uniform way throughout all samples. The above-mentioned procedure was performed for sections stained with LFB and an antibody against ChAT. The neuron number obtained from the LFB sections represents the number of all (large) neurons in the PPN, whereas the ChAT-positive neurons represent the cholinergic neurons. Thus by subtracting the ChAT-positive neuron count from that of the LFB sections, we obtained an estimate of the number of non-cholinergic neurons in the PPN.

2.3. Stereological evaluation

The results from single sections can be stereologically analyzed after making some basic assumptions. The assumption we made was that the cell bodies of the studied neurons were spherical. This assumption will not change the differences between PD and control samples, but will give a way to evaluate the average 3-D diameter of the cell bodies. As shown in Table 1, the profile areas varied between PD and control samples, and also between LFB and ChAT-positive cells. The following example shows the calculation of the mean diameter of LFB neuronal bodies after the basic assumption. The average area of the cell bodies was $1689 \pm 148\ \mu m^2$. If this area is circularized the diameter (d) of the circularized profile can be estimated from the formula $\pi r^2 = A \Rightarrow \pi(d/2)^2 = A \Rightarrow d^2 = 4/\pi A \Rightarrow d = \sqrt{4A/\pi}$, and d will get the value $\sqrt{4 \times 1689\ \mu m^2/\pi} = \sqrt{2151\ \mu m^2} = 46.4\ \mu m$.

The 3-dimensional sphere diameter (D) can be estimated from the average profile diameter (d) [28] by the formula $D = 4d/\pi$. Because in our example d is 46.4 μm, and D can be estimated as 59.1 μm. In a corresponding fashion, estimates for LFB cell diameter can be calculated in PD patients, and for ChAT-positive cells in controls and PD patients.

The influence of cell size changes on cell number can be approximated with the Abercrombie formula [28,29]. $Nv = Na/(D + t)$, where Nv = number of cells by volume, Na = number of cells by area, D = average diameter of the cells, and t = thickness of section. As the formula shows, differences in the number of cells by volume can be expected when D (the average diameter of the cells) changes. Table 1 shows number of neurons by cross section of PPN and these figures can be tested for various cell sizes. The formula shows that a decrease in cell size can be expected to cause an increase in the number of cells by volume (Nv). The figures given in Section 3 are based on the use of the three described formulae.

The differences in the number of neurons and their size in the PPN between controls and PD patients were evaluated by Student's t-test (two-tailed). The correlations between the number of neurons in the PPN and the clinical features in PD patients were made using Pearson's correlation coefficient.

3. Results

The distribution of LFB and ChAT-positive neurons in different levels of the PPN in controls and PD patients is shown

Table 1
The neuron profile counts and neuronal profile area in the pedunculopontine nucleus in controls and patients with PD

	Neuron profile number		Neuronal profile area (μm^2)	
	LFB	ChAT-positive	LFB	ChAT-positive
Control (mean (SD))	302 (90)	97 (31)	1689 (148)	2223 (390)
PD (mean (SD))	219 (80)	62 (34)	1446 (208)	1634 (292)
% Reduction from controls	27	36	14	26
p-Value	0.04	0.03	0.009	0.001

LFB = Luxol fast blue positive neuron profiles; and ChAT-positive = neuron profiles positive for choline acetyltransferase antibody.