Loss of C1 and C3 Epinephrine-Synthesizing Neurons in the Medulla Oblongata in Parkinson's Disease

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We used immunohistochemical analysis to determine whether medulla oblongata neurons containing phenylethanolamine *N*-methyltransferase (PNMT) are affected in patients who died with idiopathic Parkinson's disease (n = 7) compared with age-matched control subjects who died with nonneurological diseases (n = 8). Transverse sections (50 μm) of medulla were prepared either for conventional neuropathological examination or for the immunohistochemical demonstration of PNMT. Immunopositive neurons at approximately 30 rostrocaudal levels, evenly spaced throughout the whole medulla, were mapped and cells in each section were counted with a camera lucida system linked to a computer. In the ventrolateral medulla, from the level of the obex to 11 mm rostral to the obex where the C1 group of neurons is located, there were 7,631 ± 844 PNMT-positive neurons in control brains and 3,604 ± 1,051 in brains affected by Parkinson's disease (47% of control). Many PNMT-positive neurons contained Lewy bodies. We observed a previously undescribed midline (C3) group of PNMT-positive neurons in normal brains, and this group was also severely affected (12% of control) in parkinsonian brains. Neither the C2 group nor the small PNMT-positive neurons in the nucleus tractus solitarii were significantly reduced in numbers but there was a reduction in the numbers of melanin-pigmented cells in both the ventrolateral (50% of control) and the dorsomedial (79% of control) region. Our results demonstrate a selective loss of C1 and C3 PNMT-positive neurons, providing the first quantitative evidence for damage to these presumed brainstem sympathetic premotor neurons in Parkinson's disease. These changes may underlie some of the autonomic symptoms occurring in this condition.

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The medulla oblongata contains groups of catecholamine-synthesizing neurons, originally described using aldehyde fluorescence histochemical procedures [1]. Subsequent immunohistochemical studies in animals have established ventrolateral A1 and C1 cell groups, dorsomedial A2 and C2 groups, a rostral midline C3 group, and an additional unnumbered compact group in the dorsolateral portion of the nucleus tractus solitarii (nTS). The C1, C2, and C3 neurons, as well as the compact dorsolateral nTS cells, contain phenylethanolamine *N*-methyltransferase (PNMT), the enzyme usually present in cells that synthesize epinephrine [2, 3]. Several immunohistochemical studies have mapped PNMT-positive neurons in the human medulla [4–7]. The distribution of cells is generally similar to that observed in animals except that no C3 group has been observed in humans.

In idiopathic Parkinson's disease (PD) the medullary distribution of Lewy bodies is similar to the distribution of the catecholamine-synthesizing neurons [8]. However, there is surprisingly little documentation that Lewy bodies occur in neurons containing melanin pigment. Three studies have provided conflicting quantitative data concerning the loss of medullary catecholamine neurons in PD. An earlier study from our laboratory [9], based on single sections from three rostrocaudal levels of the medulla in 4 patients with PD, found that the number of tyrosine hydroxylase (TH)–positive neurons was not significantly reduced in PD patients, whereas PNMT-positive cells were reduced to 41% of control levels. Malessa and colleagues [10] studied 3 patients with PD. They divided TH-positive neurons into ventrolateral (A1 and C1) groups and dorsomedial (A2 and C2) groups, and found no difference in these groups between control and PD patients. Only a limited rostrocaudal portion of the A2

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and C2 group was examined. Saper and colleagues [11] studied a larger group of patients with PD and found reduced numbers of TH-positive A2 and C2 cells, but normal numbers of A1 and C1 cells. The importance of examining the full rostrocaudal extent of the immunopositive neurons was emphasized, as was the necessity to compare defined subgroups of the catecholamine-containing cells.

We have now followed these recommendations in a detailed study of the different subgroups of PNMT-positive neurons in 7 patients with PD and 8 normal control subjects. We obtained serial sections from the complete rostrocaudal extent of the medulla oblongata (approximately 23 mm) and stained every 15th section with an antibody that is particularly suitable for the detection of PNMT in the primate brain [4].

Materials and Methods

Patients

Seven brains were obtained from patients with clinical histories consistent with PD, and 8 from patients without apparent neurological diseases. For all the patients with PD, the clinical diagnosis was confirmed by neuropathological demonstration of cell loss and the presence of intracellular Lewy bodies in the substantia nigra and locus ceruleus. No significant neuropathological changes were found in the normal brains. The PD patients had been receiving dopamine replacement therapy until at least a few months before death. The control patients were matched for age at death, and time between death and brain fixation (Table 1). All the PD brains, and all the normal brains except for N21, have been used in previous studies [7, 9, 12, 13]. A summary of the clinical details for the patients is given in Table 1. None of the patients, normal or with PD, had obvious, marked autonomic disorders.

Histochemistry

Brains were removed from the skull and fixed, within 24 hours after death, by perfusion of formaldehyde and picric acid solution through the carotid and vertebral arteries, as previously described [7]. Brainstems were cut into blocks in the coronal plane. One block contained the complete medulla oblongata, extending 23 mm from below the pyramidal decussation to the lower pons. Coronal frozen sections (50 μm) were cut through the entire block, and collected serially in 15 containers. After four 10-minute washes in 50% alcohol, the sections were stored in 0.1 M Tris buffer (pH 7.4) containing 0.1% sodium azide at 4°C. Separate series of sections were immunostained with either a rabbit antiserum directed against bovine PNMT, diluted 1 in 10,000 [4]. Some separate sections were immunostained with an antiserum to TH as previously described [7]. Sections were processed with avidin-biotin-peroxidase and visualized with diaminobenzidine–hydrogen peroxide, with or without nickel enhancement. Other section series were stained with cresyl violet, hematoxylin and eosin, or Bodian-silver stain. Detailed histological staining procedures are given by Halliday and colleagues [7]. Few sections were lost during cutting and processing, so that sections from different individuals could be matched, level by level.

Analysis

PNMT-positive neurons were mapped and counted (×250 magnification) on every 15th medullary section, with the Magellan computer image analysis system (Paul Halasz, Sydney,

Table 1. Details on Normal (N) Patients and Patients with Parkinson's Disease (PD)

Patient No.	Sex	Age at Death (yr)	Postmortem Delay (hr)	Cause of Death	Duration of PD (yr)
N10	F	79	4	Cancer	
N12	M	80	20	Airway disease	
N13	M	70	10	Cancer	
N14	F	72	7	Myocardial infarction	
N15	F	59	6	Myocardial infarction	
N16	F	87	22	Bowel obstruction	
N19	F	61	8	Diabetes	
N21	M	88	19	Renal failure	
Mean ± SEM		75 ± 4	12 ± 3		
PD2	F	71	19	Pneumonia	21
PD3	M	73	18	Pneumonia	5
PD5	F	77	5	Myocardial infarction	?
PD6	M	78	3	Pneumonia	23
PD8	M	88	3	Inanition	20
PD10	M	79	12	Inanition	21
PD12	M	72	4	Inanition	20
Mean ± SEM		77 ± 2	9 ± 3		18 ± 3
Student's *t*		$p > 0.05$	$p > 0.05$		

SEM = standard error of mean.

Australia) [12]. A three-dimensional map of the distribution of PNMT-positive neurons was reconstructed to facilitate the definition of cell groups. The number of neurons on each side of each section was compared within each patient for each cell group. There was no significant difference between the numbers of ipsilateral and contralateral PNMT-positive cells ($p > 0.05$, unpaired Student's t test), and the number of PNMT-positive neurons on both sides in individual brains was highly correlated ($r > 0.75$, $p < 0.01$). Cell counts from both sides were therefore pooled, and multiplied by 15 to give the total number for each PNMT-positive cell group. Abercrombie's [14] factor was 0.68 for the C1 cell group, 0.7 for the C2 group, and 0.72 for the C3 group and for the PNMT-positive cells in the nTS. However, we chose not to correct for split cell counting. The size of PNMT-positive neurons was not significantly different between patients with PD and control subjects.

Neurons containing melanin pigment were mapped in the sections stained for PNMT. In the immunostained sections with nickel enhancement, the immunoreactive products appeared dark blue, whereas the pigment was yellow-brown, and refractile under the darkfield, enabling us to determine whether particular PNMT-positive cells were pigmented. We also counted pigmented cells on adjacent sections stained with cresyl violet, and the number was very close to that estimated from immunostained sections. The small pigmented cells in the area postrema, as previously noted [11], were not readily recognizable without melanin enhancement staining, and they were not counted in the present study.

Comparisons between cell counts in PD and normal brains were performed using analysis of variance and Fisher's protected least significant difference test. Linear regression and analysis of covariance were used to examine the effects of variables such as age at death on the cell counts in PD and normal brains.

Results

PNMT-Positive Neurons and Pigmented Neurons in the Normal Medulla

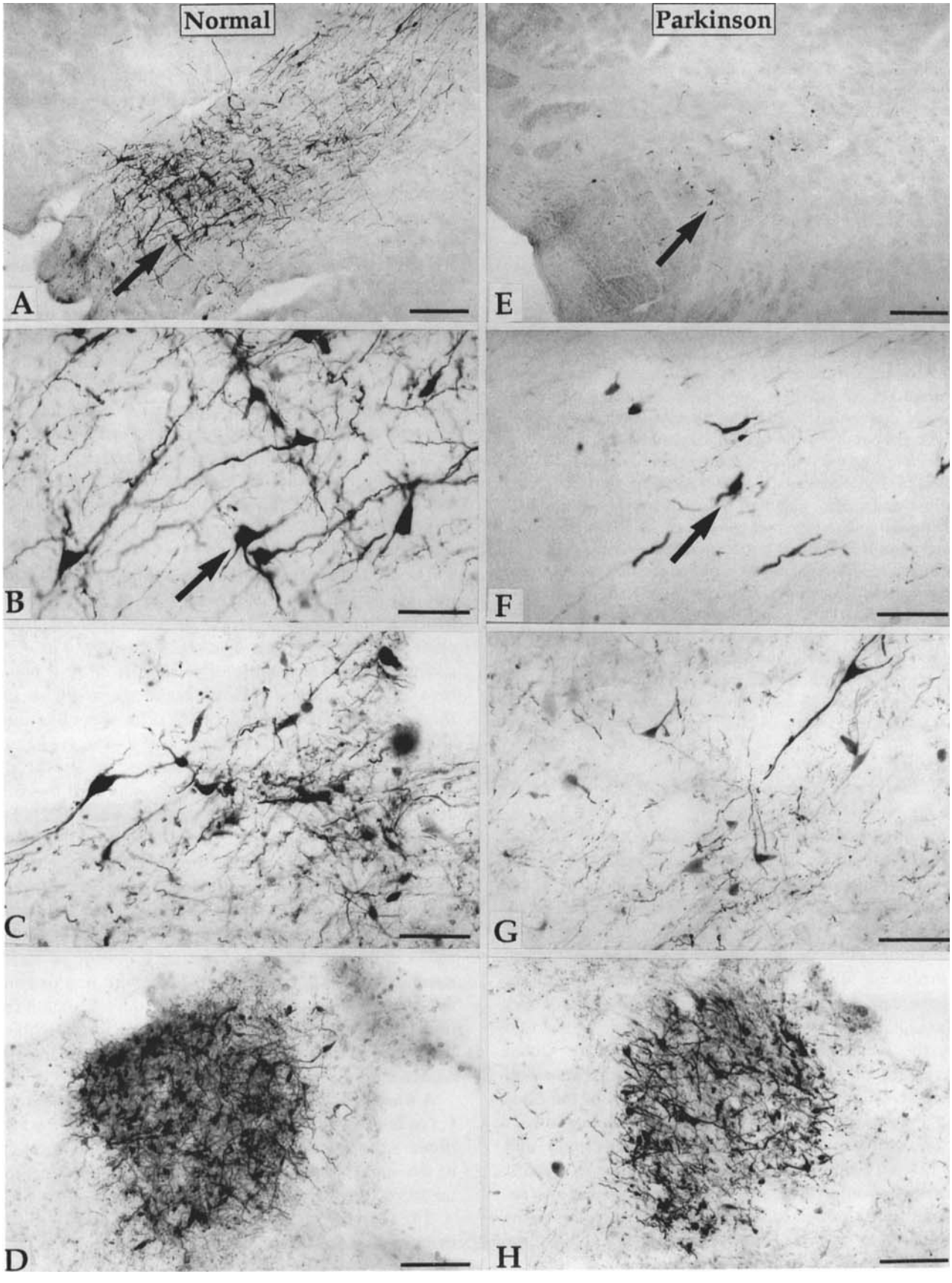
Our immunohistochemical procedure clearly defined PNMT-containing perikarya, sometimes with extensive immunopositive dendrites (Fig 1A–1D). There was little background staining and it was virtually always easy to determine whether or not a cell was immunopositive or immunonegative. The anatomical distribution of PNMT-positive neurons, as well as pigmented neurons, is summarized in Figures 2A through 2D, and enlargements of the areas enclosed by the rectangles in Figures 2B and 2D are shown in Figure 3. PNMT-positive neurons were found in sections extending rostrally from the level of the obex for approximately 11 mm (Fig 4). Pigmented cells were found caudal to the obex (not shown in the figures) and as far as 11 mm rostral to the obex, with the greatest concentration of these neurons being found in the region from the obex to 4 or 5 mm rostral to this point (see Fig 4).

PNMT-positive neurons were located in three medullary regions, and they aggregated into four distinct cell groups. The largest group, corresponding to the C1 cells, was located in the ventrolateral medulla (see Figs 1A, 1B, 2A–2D). This longitudinal cell column extended about 11 mm from the level of the obex to the rostral pole of the hypoglossal nucleus, with peak numbers appearing in the middle one-third of the column (see Fig 4A). The cells were medium-sized (18–30 μm) and triangular or multipolar in shape, with two or three prominent dendrites, generally oriented in a dorsomedial-ventrolateral direction (see Fig 1B). Pigmented cells were found in the vicinity of C1 cells in most sections, except at the rostral extent of the PNMT-positive cells where pigmented cells were rare. Occasional C1 PNMT-positive cells were also pigmented. The C1 PNMT-positive neurons surrounded the nucleus ambiguus and stretched in a narrow band from the ventrolateral medulla toward the dorsomedial medulla (see Figs 2B–2D) and there was not always a clear demarcation from the C2 group. We defined the C1 cells as those occurring ventrolateral to the major PNMT fiber bundle, designated the "principal tegmental adrenergic bundle" by Arango and colleagues [6], and shown as the hatched area in Figures 2 and 3. Using this definition there were 7,631 ± 844 C1 neurons in the normal medulla.

The C2 cell group extended for approximately 9 mm (see Fig 4C) in the dorsomedial part of the medulla, generally in the ventral aspect of the dorsal vagal nucleus and the nTS (see Figs 1C, 2B–2D). The C2 group contained a mixture of small (10–20 μm) neurons as well as larger cells similar in size to those in the C1 group. About 20% of C2 cells, especially the large ones, were also pigmented. There were 1,470 ± 505 C2 neurons in the normal medulla.

In the dorsomedial medulla, dorsolateral to the C2 cells, there was a compact group of small PNMT-positive neurons within the dorsolateral portion of the nTS. This group had a limited rostrocaudal extension (4–5 mm) and was best represented just rostral to the area postrema (see Figs 1D, 2D, 3B). As first described by Kitahama and coauthors [4], these PNMT-positive cells clearly differed from C2 cells by virtue of their small ovoid cell bodies (10–20 μm), with one or two dendrites circumscribed within the dorsolateral nTS (see Fig 1D). No pigmented cells were found within this group. There were 4,883 ± 317 neurons in this compact group in the normal medulla.

A small group of PNMT-positive cells, equivalent to C3 cells described in the rat [2, 3], but not previously observed in the primate or human brain, was located in the raphe region approximately 10 mm rostral to the obex (see Figs 2A, 2B; Fig 5B). There were 516 ± 137 C3 neurons in the normal medulla. TH-positive neurons were also found in this region (Fig 5A). The cells were small to medium-sized, and oval or elongated in shape, with dendrites directed dorsoventrally


Normal
Parkinson
A
E
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(see Fig 5B). No pigmented cells were found in this C3 region.

PNMT-Positive Neurons in Parkinson's Disease

In PD brains we observed degenerative changes in PNMT-positive cells in C1, C2, and C3 groups, but not in the compact nTS group. Dendrites were swollen, and perikarya were sometimes fragmented (see Figs 6A, 6D, 6F). Lewy bodies were observed in C1, C2, and C3 PNMT-positive cells (see Figs 6B, 6C, 6E).

The number of PNMT-positive cells in C1 and C3 groups was significantly reduced in PD, as shown in Figures 2E through 2H, Figures 4A and 4B, and Table 2. The number of PNMT-positive neurons in the C1 group in PD brains, considered as a whole, was reduced to 47% of the control value. The loss was fairly uniform throughout the rostrocaudal extent of the C1 group (see Fig 4A). Six of the 7 PD patients showed loss of C1 neurons. In the patient with normal C1 cell numbers (Patient PD6) there were degenerative neuropathological changes and Lewy bodies in many of the surviving C1 cells. In the C3 group of cells from PD patients, the number of PNMT-positive cells was reduced to 12% of control values, with the loss particularly obvious approximately 10 cm rostral to the obex, where the C3 cells were concentrated (see Figs 4B, 5C).

In our control group there was a large individual variation in the number of PNMT-positive C2 neurons and we found no significant difference between control and PD patients. However, Lewy bodies were clearly present in some C2 cells in the PD brains and some C2 neurons were fragmented (see Fig 6D). In contrast, no neuropathological changes or Lewy bodies were observed in the compact group of PNMT-positive cells in the dorsolateral nTS (see Fig 1H) and the number of PNMT-positive neurons was similar in the PD and the control group. The number of melanin-pigmented cells in both the C1 and the C2 regions was significantly reduced (see Table 2), and Lewy bodies were seen in some pigmented neurons in these regions.

For the different subregions there was no relationship between the number of PNMT-positive or pigmented neurons and age at death in either control or PD patients. Nor was there a relationship between the number of PNMT-positive or pigmented cells and the duration of symptoms in the patients with PD.

Computer reconstructions showing pigmented and PNMT-positive cells in all medullary groups in 1 control patient (Patient N16) and 1 PD patient (Patient PD12) are illustrated in Figure 7.

Fig 1. Immunohistochemical staining of phenylethanolamine N-methyltransferase (PNMT)–positive cell groups in the medulla in normal brains (A–D) and PD brains (E–H). (A, E) C1 neurons. Bar = 500 μm. (B, F) Enlargements of the regions pointed by the arrows *in A and E. Bar = 100 μm. (C, G) C2 neurons. Bar = 100 μm. (D, H) The compact PNMT-positive neuron group of the nucleus tractus solitarii. Bar = 200 μm.*

Discussion

The present study demonstrated severe loss of PNMT-positive neurons in the rostral ventrolateral medulla (C1 group) and in the rostral midline medullary raphe (C3 group) in patients with PD. The presence of intracellular Lewy bodies in surviving PNMT-positive neurons indicates that these groups were affected by the primary disease process. Although there was no statistical loss of C2 cells, some of these neurons exhibited the neuropathological changes of PD, including Lewy bodies. A previous biochemical study found normal levels of PNMT activity in the C2 area [15]. In contrast, the compact group of PNMT-positive cells in the dorsolateral nTS appeared not to be affected at all in PD patients. Our findings confirm our previous observation based on a limited sample of PNMT-positive cells in PD [9].

Ours is the first detailed evidence that medullary PNMT-positive neurons are affected by the primary disease process in PD. Two recent quantitative studies of the medulla in PD found either no loss of catecholamine cells [10] or a loss limited to the A2 or C2 group around the level of the area postrema [11]. The authors did not comment on whether Lewy bodies occurred in neurons identified as catecholamine synthesizing in the medulla in PD. The studies used antisera against TH, an enzyme present in all catecholamine neurons, in contrast to PNMT-positive cells, which is present in only a portion of total medullary TH-positive neurons [4–7]. We previously reported normal TH-positive cell counts but significantly decreased PNMT-positive cell numbers in the PD medulla [9]. However, our previous study was based on a limited number of medullary sections and we did not subdivide the immunopositive neurons into the various regional groups. This presumably accounts for our failure to detect decreased numbers of TH-positive cells in the C1 area.

Lewy noted the presence of the inclusion bodies, now named after him, in the dorsal motor nucleus of the vagus (see [16] for review) but it is not clear whether the involved neurons contained melanin or whether they belonged to the nonmelanized preganglionic neurons of the dorsal motor nucleus of the vagus, also severely affected in PD [9, 13, 17]. Other reports of Lewy bodies in the medulla also failed to specify whether or not they actually occur in catecholamine neurons [8, 18].

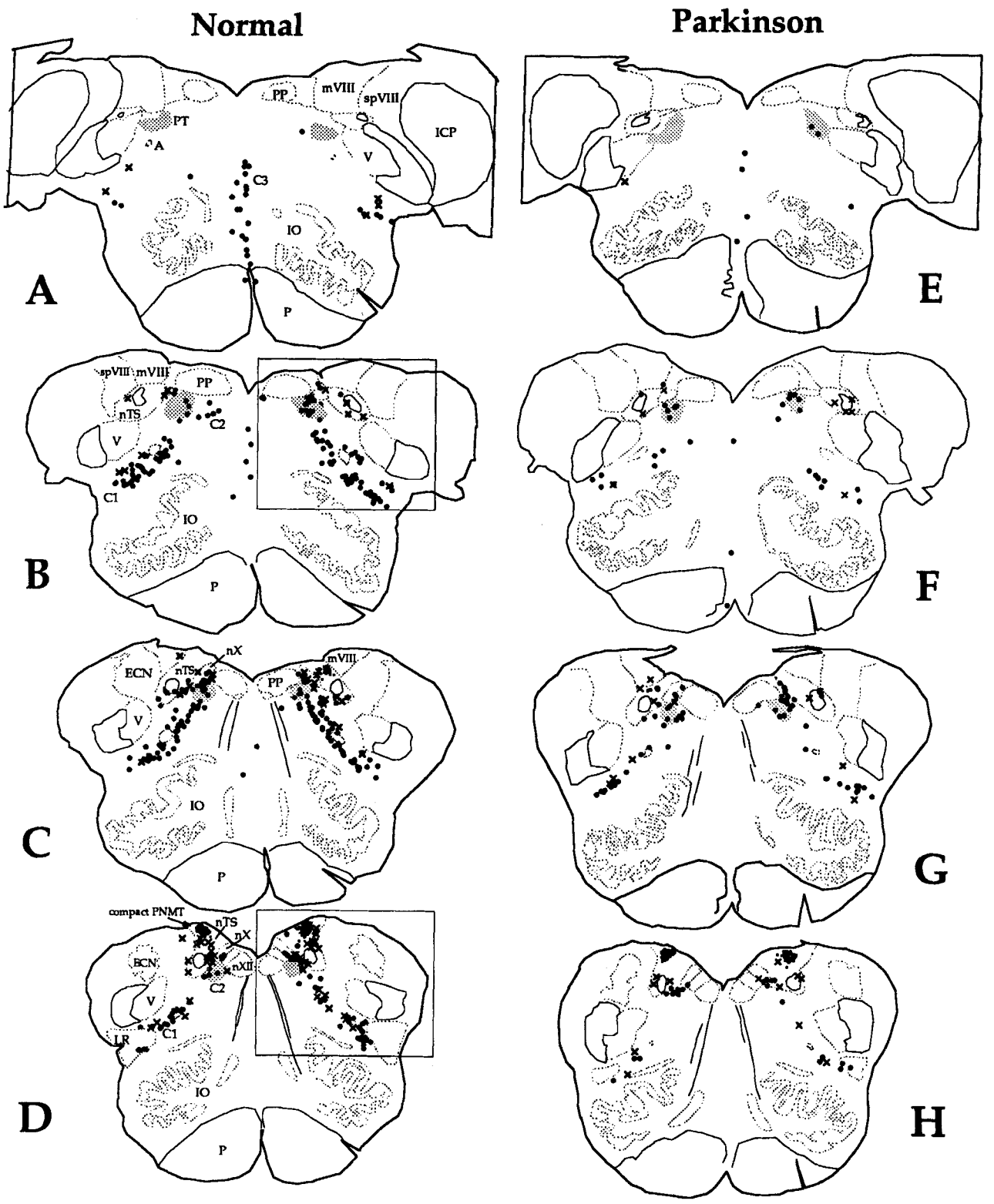

Fig 2. Distribution of phenylethanolamine N*-methyltransferase* (PNMT)*–positive and pigmented neurons in the medulla in normal brains (A–D) and PD brains (E–H). The upper-right quarters of B and D are enlarged and shown in Figure 3. The* solid circles *indicate C1, C2, or C3 PNMT-positive neurons; the* small solid squares, *PNMT-positive neurons of compact group of the nTS; and the* crosses, *pigmented neurons. A = nucleus ambiguus; ECN = external cuneate nucleus; IO = inferior olivary nuclei; ICP = inferior cerebellar peduncle; LR = lateral reticular nucleus; mVIII = medial vestibular nucleus; nTS = nucleus tractus solitarii; nX = dorsal motor vagal nucleus; nXII = hypoglossal nucleus; P = pyramidal tract; PP = prepositus hypoglossal nucleus; PT = principal tegmental adrenergic bundle; spVIII = spinal vestibular nucleus; V = spinal trigeminal nucleus.*

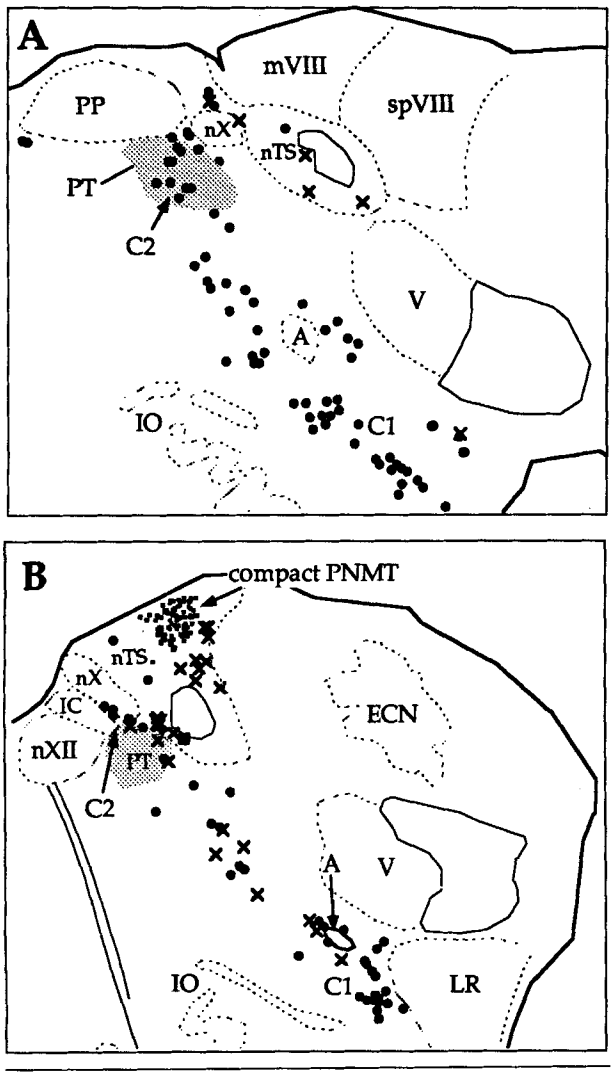

Fig 3. Detailed distribution of phenylethanolamine N-*methyltransferase* (PNMT) *C1, C2, and compact nucleus tractus solitarii PNMT-positive neurons and of pigmented neurons in the boxed regions in Figures 2B and 2C. Symbols and abbreviations are the same as in Figure 2. IC = intercalated nucleus.*

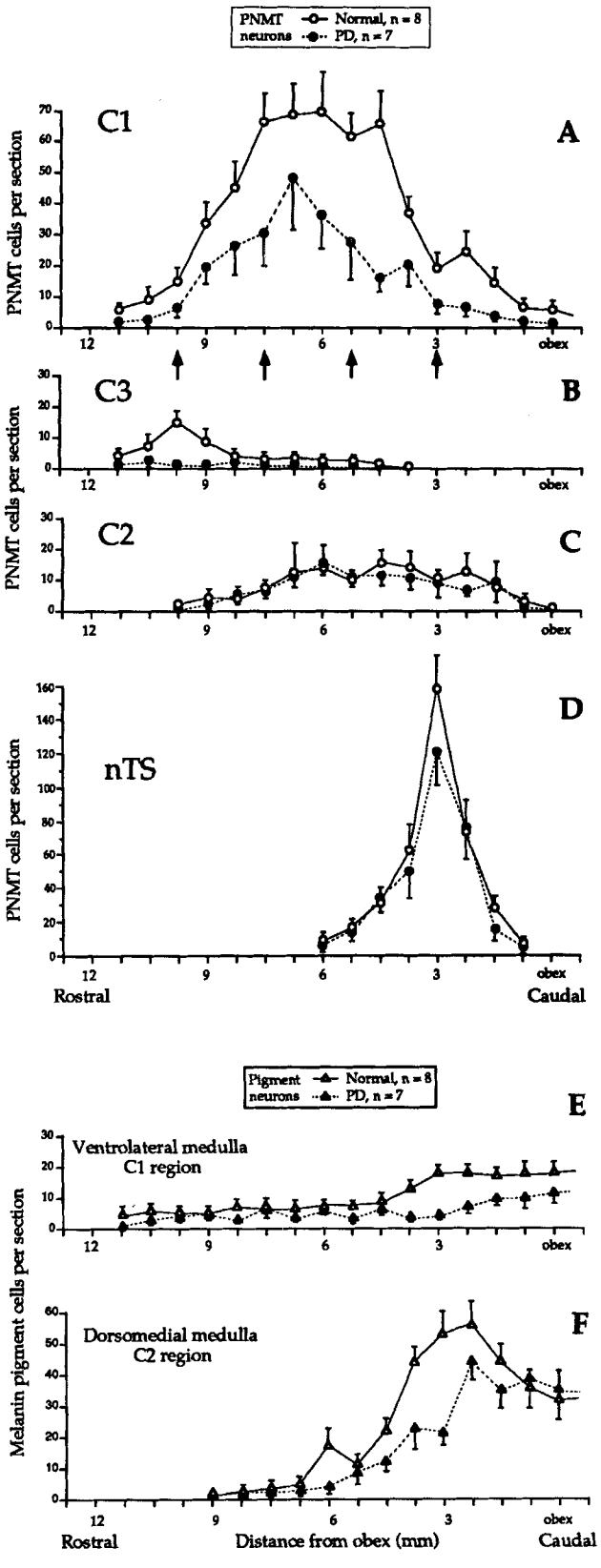

Fig 4. Distribution of the bilateral number per 50-μm-thick section for each phenylethanolamine N-*methyltransferase* (PNMT)*–positive and pigmented neuron groups through the medulla. Pigmented cells that extended further caudally below the obex are not included. The number under the x axis is the section distance (in millimeters) from the level of the obex. It is calculated by multiplying the section thickness (50 μm) by 15. The sections indicated by* arrows *in A represent the approximate levels of the four sections used to construct the map in Figure 2. nTS = nucleus tractus solitarii.*

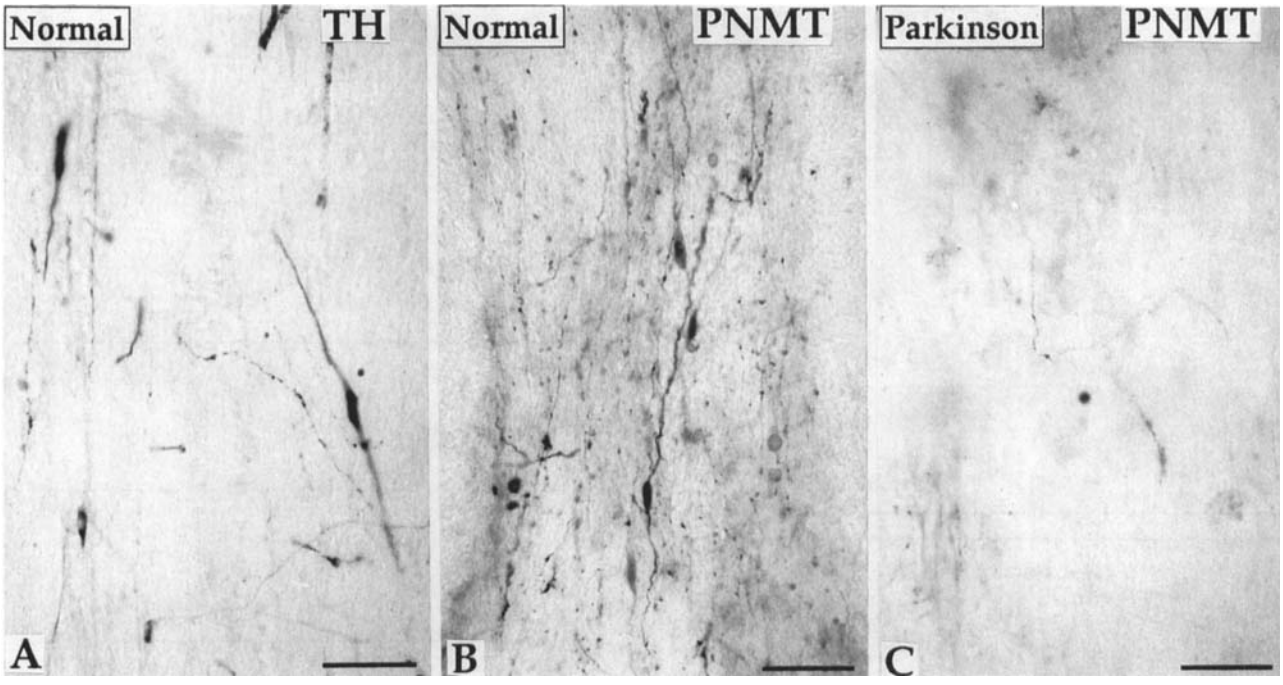

Fig 5. Immunohistochemical staining of C3 cells in the medulla. (A) Midline tyrosine hydroxylase (TH)*–positive neurons in the medulla of a normal control. C3 phenylethanolamine* N*-methyltransferase* (PNMT)*–positive neurons in the medulla of normal control (B) and PD brains (C). Bar = 150 μm.*

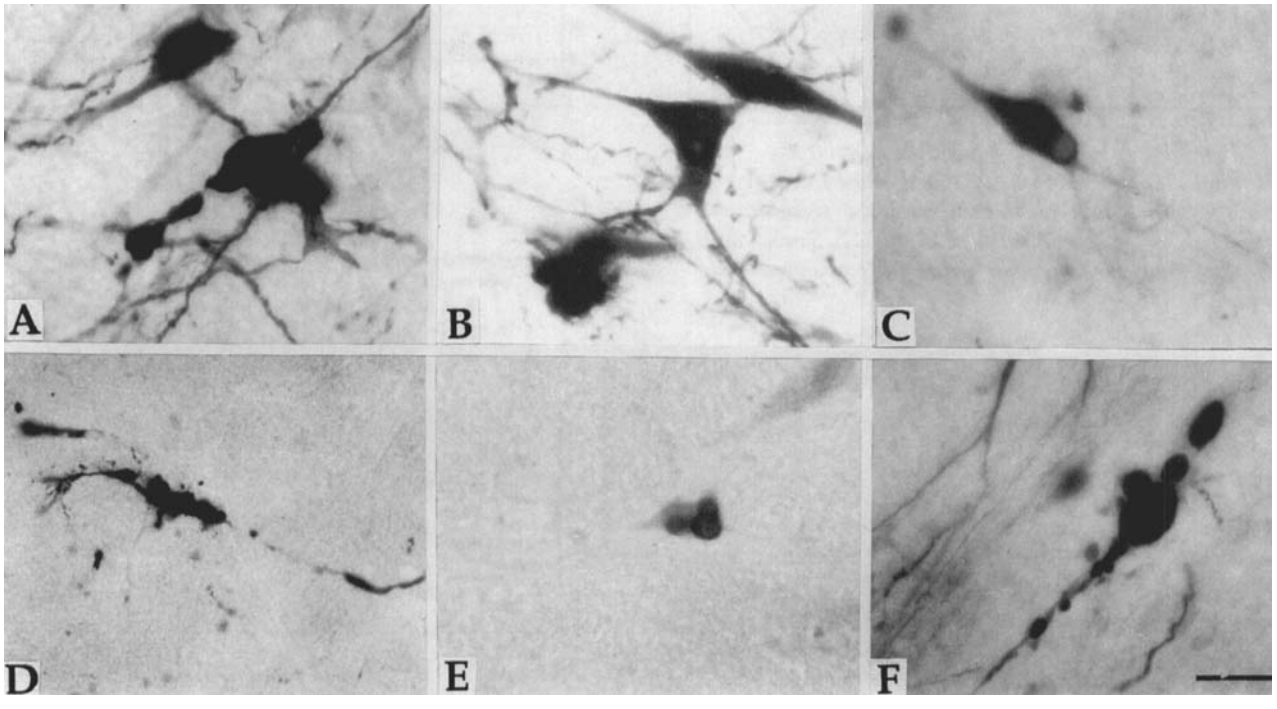

Fig 6. Pathological changes in phenylethanolamine N*-methyltransferase (PNMT)–positive neurons in PD patients. (A, F) Swollen and fragmented neuronal processes of C1 neurons. (D) A fragmented C2 neuron. Lewy body–containing C1 (B, C) and C3 (E) neurons. Bar = 50 μm for all photographs.*

Table 2. Total Number of PNMT-Positive and Pigmented Neurons in the Medulla of Normal (N) Patients and Patients with Parkinson's Disease (PD)

	PNMT				Pigmented	
Patient No.	C1	C3	C2	nTS	C1 Region	C2 Region
N10	5,670	360	645	3,420	1,785	4,440
N12	6,465	360	405	5,745	3,150	3,285
N13	7,050	1,200	2,490	4,065	2,715	3,930
N14	6,150	405	1,110	5,745	2,220	4,830
N15	7,470	195	1,935	5,280	1,305	3,975
N16	12,165	1,050	4,410	5,670	1,890	4,695
N19	10,410	375	525	4,140	1,770	4,950
N21	5,670	180	240	4,995	3,060	5,280
Mean ± SEM	7,631 ± 844	516 ± 137	1,470 ± 505	4,883 ± 317	2,237 ± 237	4,423 ± 231
PD2	1,125	0	135	6,465	1,410	4,425
PD3	2,250	30	2,085	4,785	1,575	2,985
PD5	5,235	135	630	3,030	690	3,120
PD6	9,210	60	1,665	1,620	1,275	3,690
PD8	2,715	30	1,875	6,885	405	2,985
PD10	1,905	150	360	1,080	1,995	4,350
PD12	2,790	30	375	4,485	420	3,000
Mean ± SEM	3,604 ± 1,051	62 ± 22	1,018 ± 311	4,050 ± 850	1,110 ± 232	3,510 ± 246
p	< 0.01	< 0.01	> 0.05	> 0.05	< 0.01	< 0.05
% Normal	47	12	—	—	50	79

PNMT = phenylethanolamine *N*-methyltransferase; nTS = nucleus tractus solitarii; SEM = standard error of mean.

We found that very few PNMT-positive cells, including those in the affected C1 and C3 groups, contained visible pigment in either PD or control specimens. Nonpigmented cells in the pedunculopontine tegmental nucleus and in the dorsal motor nucleus of the vagus are also severely affected by the primary neuropathological process of PD [12, 13, 19–21]. These results do not support the hypothesis that neuronal vulnerability in PD is especially dependent on the presence of melanin in the neuron [10, 22]. In the present study we also noted a loss of pigmented cells in both the dorsomedial and the ventrolateral medulla. Since most pigmented cells did not contain PNMT, our results indicate that medullary catecholamine neurons other than PNMT-positive cells are also affected in PD.

Studies in experimental animals, including nonhuman primates, have established three major projection regions for the C1 PNMT-positive neurons. Many of the cells project to the intermediolateral columns of the spinal cord where they synapse directly on sympathetic preganglionic neurons [23–26]. Other C1 cells project to the hypothalamus [27, 28] and to the locus ceruleus [29]. Although no functional studies in humans are available, there is strong evidence from animals that activity of the projection to the spinal cord maintains peripheral sympathetic vasomotor tone, and mediates baroreceptor-vasomotor reflexes [30, 31]. There is little information concerning the function of the hypothalamic projection. The projection to the locus ceruleus may mediate the excitation of locus ceruleus neurons in response to various peripheral stimuli, including pain; a projection which may contribute to the "vigilance" role attributed to the locus ceruleus [29]. The C3 neurons also project to the spinal cord but no functional studies are available.

The loss of C1 PNMT-positive cells may be relevant to some of the autonomic symptoms that sometimes occur in PD (see [32] for review). Most of our patients died after a long period of inactivity and their autonomic status in the months before death was difficult to assess. None of the patients were noted to have major problems with postural hypotension during the earlier portions of their illness. It may be that even more severe C1 neuronal loss is necessary before cardiovascular autonomic symptoms develop. Neuronal loss has been reported in peripheral sympathetic ganglia in patients with PD and marked autonomic dysfunction, with lesser involvement of the intermediolateral column [33]. The intermediolateral column is more affected in patients with PD in association with multiple-system atrophy and postural hypotension [34, 35]. No studies of medullary catecholamine neurons are available for PD patients with the marked postural hypotension that sometimes accompanies the disease, with or without multiple-system atrophy.

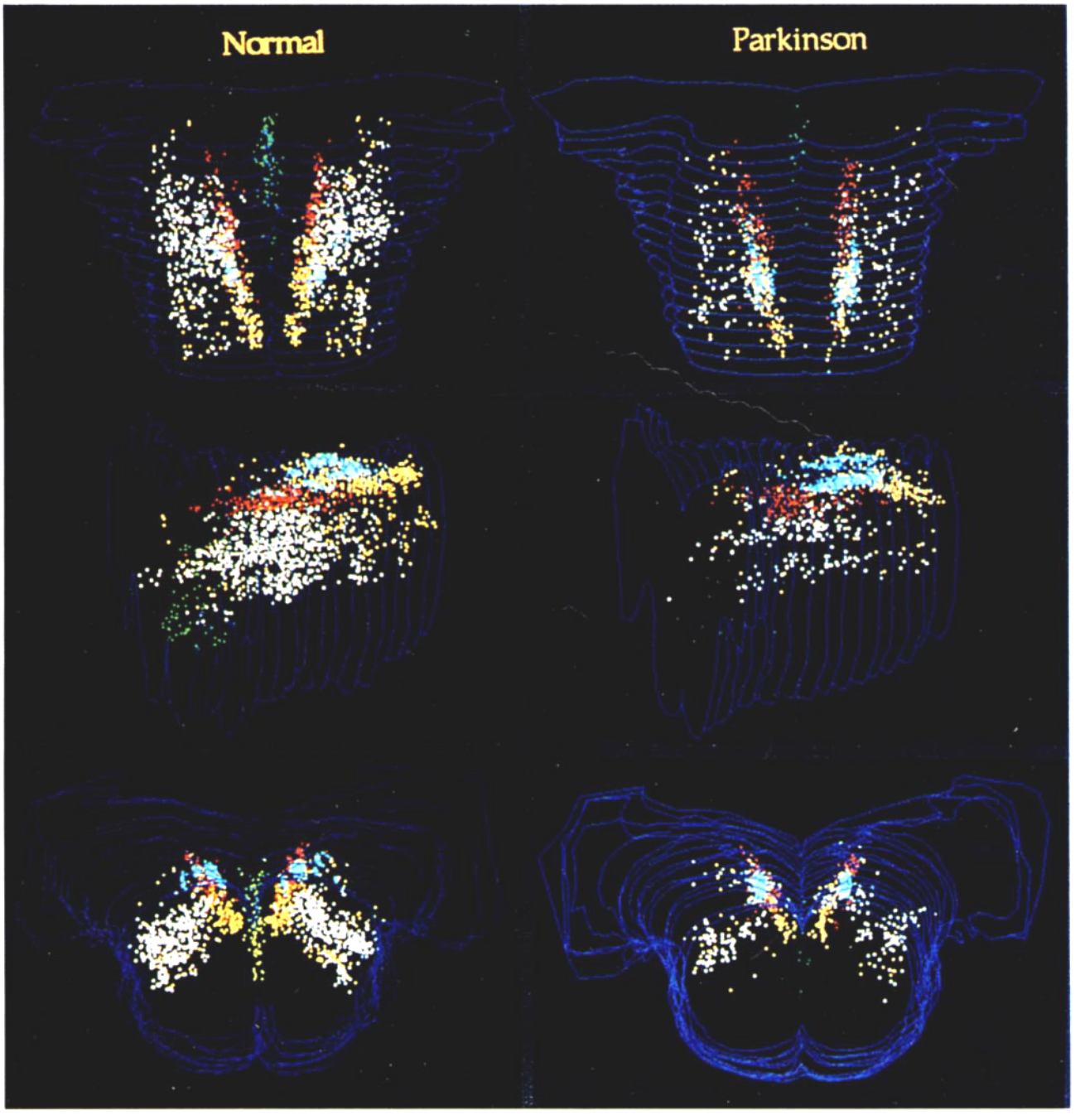

Fig 7. Three-dimensional reconstruction of the distribution of phenylethanolamine N*-methyltransferase (PNMT)–positive and pigmented neurons in normal* (left panel) *and PD medulla* (right panel). *Each* colored dot *represents one neuron.* White *indicates C1 neurons;* red, *C2 neurons;* green, *C3 neurons;* cyan blue, *compact nucleus tractus solitarii PNMT neurons;* yellow, *pigmented neurons. The* top images *show the ventral view;* middle, *the lateral view; and the* bottom *images show the caudal view of the medulla.*

The present study adds the C1 and C3 PNMT-positive groups to the growing list of brainstem nuclei affected by the primary process of PD. It may well be that the different presentations of PD reflect variations in the manner in which these vulnerable groups are affected in individual patients.

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