Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 5976–5980, August 1987
Neurobiology

Neuronal loss in the pedunculopontine tegmental nucleus in Parkinson disease and in progressive supranuclear palsy

(cholinergic neuron/NADPH diaphorase/basal ganglia/motor system/dementia)

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Communicated by Emilio Bizzi, May 4, 1987 (received for review March 3, 1987)

ABSTRACT **In the brains of humans and other mammals, there are two principal groups of cholinergic nuclei aside from those forming the cranial motor nuclei. One group lies in the forebrain and includes the nucleus basalis of Meynert. The second group lies in the hindbrain and includes the nucleus tegmenti pedunculopontinus (NPP), identified by Mesulam *et al.* [Mesulam, M.-M., Mufson, E. J., Wainer, B. H. & Levey, A. I. (1983) *Neuroscience* 10, 1185–1201] as cholinergic cell group Ch5. The basal forebrain cholinergic cell groups, which innervate widespread areas of the neocortex, undergo degeneration in Alzheimer disease and also in parkinsonism associated with dementia. We here report that the hindbrain NPP Ch5 cell group, thought to innervate many nuclei of the extrapyramidal motor system, the superior colliculus, and the substantia innominata, undergoes degeneration in idiopathic Parkinson disease and in the parkinsonian syndrome of progressive supranuclear palsy. These findings strongly suggest that degeneration in the brainstem in Parkinson disease is not confined to catecholamine-containing neurons, but that cholinergic neurons of the NPP are also vulnerable. The findings further raise the possibility that certain symptoms of Parkinson disease and progressive supranuclear palsy have their genesis in pathology of these cholinergic neurons.**

The cardinal neuropathologic characteristics of Parkinson disease (PD) are massive cell loss in the dopamine-containing substantia nigra pars compacta (1, 2), with variable degeneration in other aminergic nuclei of the brainstem, and the appearance of intracellular Lewy bodies in neurons of both the hindbrain and forebrain (3). It is now recognized that in some if not most parkinsonian brains there is further a cholinergic defect and loss of neurons in the nucleus basalis of Meynert, the principal cholinergic nucleus of the forebrain (4–6). A matter of particularly active study in the past 5 years has been the possibility that cell loss in the nucleus basalis could underlie the dementia exhibited by some parkinsonian patients (5, 6).

A second major set of cholinergic nuclei exists in the hindbrain (7–11), and one of these nuclei, the Ch5 group of Mesulam *et al.* (7), corresponds in location to one of the principal nuclei associated with the basal ganglia: the nucleus tegmenti pedunculopontinus (NPP) (11–13). Given the close association of the NPP with the extrapyramidal motor system (8, 14–18), we undertook a study of the NPP in autopsy material from the brains of persons who died with a diagnosis of idiopathic PD. We also determined the status of the nucleus in progressive supranuclear palsy (PSP), a parkinsonian syndrome associated with severe intellectual impairment (19). To identify the neurons of the NPP, we relied on histochemical stains for acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.7) and NADPH diaphorase [NADPH:(acceptor)oxidoreductase, EC 1.6.99.1], enzymes that have been shown in experimental animals to be colocalized in NPP neurons defined as cholinergic by their expression of choline acetyltransferase (ChoAcTase; acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6)-like immunoreactivity (9, 20, 21).

METHODS

Histochemistry. Observations were made on postmortem specimens from the brains of four control individuals who died without known neurologic or psychiatric deficit, six patients who had suffered from PD, and three patients who had suffered from PSP. The diagnoses were confirmed postmortem on the basis of neuropathological examination and retrospective examination of clinical records. The mean ages were 85 ± 3 years (range 83–90 years) for the controls, 73 ± 11 years (range 64–85) for the PD patients, and 73 ± 10 years (range 60–82) for the patients with PSP. Mean postmortem delays before fixation (range 3–51 hr) were similar for the three groups [control, 10 ± 7 hr (range 3–51); PD, 21 ± 16 hr (range 4–21); PSP, 18 ± 7 hr (range 6–26)]. All PD patients were under L-dopa therapy and showed moderate to severe akinesia rigidity, axial rigidity, and postural instability and (except for one case) moderate or pronounced tremor and intellectual impairment (22).

Hemisected (or, in one case, intact) brainstems were fixed in 4% paraformaldehyde and 0–15% picric acid, as described elsewhere (23). Free-floating 40-μm frozen transverse sections were processed for histochemical detection of AcChoEase activity (23) or NADPH diaphorase activity (24) so as to form regularly alternating sets with spacing between sections stained for the same enzyme at 720 μm (eight brains); 200 and 520 μm, alternating (three brains); or 1440 μm (two brains). Cell counts were performed on a minimum of five sections per brain at ×100 magnification with the aid of a semiautomatic computer-assisted system that allowed marking each neuron once and only once. For all but two cases, NADPH diaphorase-positive neurons were counted because AcChoEase-positive neurons were usually difficult to identify in the AcChoEase-rich core (see *Results*). Correspondence of the AcChoEase- and NADPH diaphorase-containing neuronal populations was confirmed in one control brain by counting, in 10 pairs of adjacent sections, the number of AcChoEase- or NADPH diaphorase-positive neurons; the numbers did not differ statistically. By this method we estimated the total number of neurons so as to avoid sampling problems associated with variations in planes of section (see Fig. 3).

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Abbreviations: AcChoEase, acetyl cholinesterase; ChoAcTase, choline acetyltransferase; NPP, nucleus tegmenti pedunculopontinus; PD, Parkinson disease; PSP, progressive supranuclear palsy.

Cell counts were obtained within an approximately rectangular region (see Fig. 3*A*) delimited for each section so as to include the scattered cells and core of the NPP region but to exclude the central gray substance, substantia nigra pars compacta, and parabigeminal nucleus. Estimates of the total number of NADPH diaphorase-positive NPP neurons, obtained as described in the legend to Fig. 3, were compared for the three populations by a two-tailed Mann–Whitney *U* test. There was no evidence for a difference in postmortem stability of NADPH diaphorase and AcChoEase. With the computer system we used, it was not possible to enumerate separately the neurons in the dense core and the periphery of the NPP.

Macroscopic measurements of the AcChoEase-rich dense core were made at ×12 to test for specific shrinkage of the core in the frontal plane. The rostrocaudal length of the core was estimated directly from the number of AcChoEase-stained sections in which it appeared, and indirectly, to control for plane of section, from the plots of numbers of neurons versus the distance between the sections (see Fig. 3*C*). Shrinkage of the whole brainstem was estimated in the frontal plane. Means for the macroscopic measurements were compared by two-tailed Student's *t* tests.

Biochemistry. Assays for ChoAcTase activity were carried out on samples dissected from 14 control brains (mean age 86 ± 9 years), 13 PD brains (mean age 73 ± 9 years), and 4 PSP brains (mean age 71 ± 9 years). Diagnosis was confirmed as described above; postmortem delays (4–28 hr) were similar for the three groups. Dementia was diagnosed in 7 PD cases. Two PD patients lacked resting tremor, 10 evidenced resting tremor, and for 1 patient the history was not clear on this point. Brains were prepared as described elsewhere (6), and the regions containing the NPP were dissected out by comparing the slabs to AcChoEase-stained sections. Weighed aliquots for each brain were then homogenized and analyzed by radioenzymatic assay as described (6). Means for the PD samples with and without dementia and for the PSP samples were compared to the control means by two-tailed Student's *t* tests.

RESULTS

NPP Region in Control Brains. As shown in Fig. 1*A*, the NPP was readily identifiable in control brains by the high concentration of AcChoEase-positive neuropil and of AcChoEase-positive and NADPH diaphorase-positive neurons in a sickle-shaped zone of the dorsolateral tegmentum corresponding to the region originally identified in the human by Olszewski and Baxter (12) as the subnucleus compactus of the NPP (TPc). Scattered enzyme-positive neurons appeared in the surrounding tegmentum in a larger region apparently corresponding in part to the NPP subnucleus dissipatus (TPd) identified by Olszewski and Baxter. The NPP region taken for analysis here included both the compact sickle-shaped core (*cf.* plate XXXI from ref. 12) and the zone of diffusely distributed NADPH diaphorase-positive and AcChoEase-positive neurons. This definition is in accord with that given by Mesulam *et al.* (9) for the cholinergic Ch5 cell group in the macaque brain and with histochemical observations in baboon (10) and human (11) brain. The NPP so defined extended rostrocaudally from the level of the caudal part of the parabigeminal nucleus [Ch8 of Mufson *et al.* (25)], identifiable by its AcChoEase-rich neuropil (25), to the level of the rostral part of the locus coeruleus. Many of the neurons in both the compact and the diffuse subdivisions of the NPP were large. Major and minor axes for the AcChoEase-stained neurons were 100–125 μm and 30–70 μm, and for the NADPH diaphorase-positive neurons 60–80 μm and 30–50 μm (see Fig. 3*B*).

Comparison of the Pedunculopontine Region in PD, PSP, and Control Brains. The central core of the NPP was sharply reduced in size in four of the six PD brains and in each of the three PSP brains. The difference between the control and pathologic specimens was immediately evident at low mag-

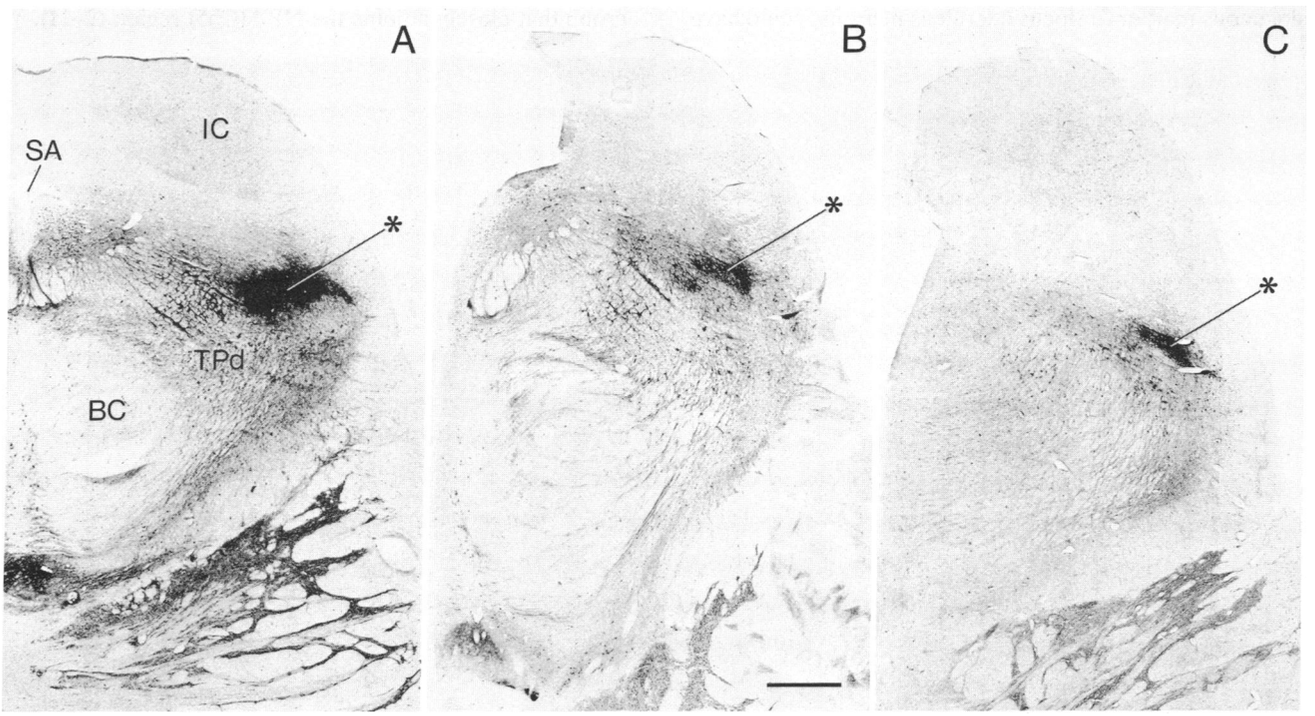

FIG. 1. Transverse section at the level of the NPP, stained for AcChoEase. (*A*) Control brain. (*B*) PD brain. (*C*) PSP brain. Asterisks in each panel indicate the dense core of the NPP, corresponding to the NPP subnucleus compactus identified by Olszewski and Baxter (12). Around this dense zone is the subnucleus dissipatus of the NPP (TPd, ref. 12). SA, sylvian aqueduct; IC, inferior colliculus; BC, brachium conjunctivum. (Bar = 2 mm.)

nification in the AcChoEase-stained sections, both because the region of dense neuropil staining appeared shrunken in the PD and PSP brains and because it had lost its characteristic sickle shape (Fig. 1). The loss of intense AcChoEase staining of the neuropil was accompanied by a loss of AcChoEase-positive and NADPH diaphorase-positive neurons. In Fig. 2, this hypocellular condition is shown for NADPH diaphorase-stained neurons in a severely affected PD brain (Fig. 2*D*), a mildly affected PD brain (Fig. 2*C*), and a typical PSP brain (Fig. 2*B*).

Quantitative estimates of the total numbers of neurons in the NPP confirmed the visual impression of diminished cell numbers in both the PD and PSP groups (Figs. 3 and 4). Relative to control values as 100%, the means were 43% for the PD brains and 21% for the PSP brains. No significant differences between the controls and patients appeared in analyses of the means according to age at onset of disease, age at death, postmortem delay, motor symptoms, or intellectual impairment.

The cross-sectional width of the AcChoEase-rich core of the NPP was significantly reduced in both the PSP and the PD groups. The mean width of the core was 2600 ± 500 μm for the controls, 1600 ± 760 μm for the PD brains ($P < 0.01$), and 1300 ± 300 μm for the PSP brains ($P < 0.001$). The measurements indicated shrinkage of the core away from the lateral edge of the brain in both the affected PD cases ($P < 0.01$) and the PSP group ($P < 0.02$). Interestingly, however, shrinkage away from the midline was only marked for the affected PD group ($P < 0.01$), despite the severe narrowing of the nucleus in PSP. Neither of the two measures of the anteroposterior extent of the dense core suggested shrinkage along this axis.

Observations on Other Cholinergic Nuclei. The region of the dorsolateral tegmental nucleus (cell group Ch6) was specifically excluded from quantitative analysis because it was not possible to make a definitive distinction between neurons of Ch6 and neurons in the adjoining locus coeruleus; the enzyme staining was sometimes so intense that neuromelanin, the endogenous marker for locus coeruleus neurons, could have been masked. By visual inspection, however, there was no difference between the control brains and the affected PD and the PSP brains, even when shrinkage of the neighboring NPP core was obvious. Similarly, there was no apparent loss of neurons in the cranial motor nuclei studied (III and IV). We did not observe AcChoEase staining of parabigeminal neurons, perhaps because of the dense neuropil staining, but AcChoEase-positive neuropil was present in the parabigeminal nucleus in brains of all three types.

Biochemical Analysis. Fig. 4*B* shows that in the midbrain specimens taken in the NPP region, ChoAcTase activity was decreased from a mean value of 6.59 nmol per hr per mg in the control samples to 4.67 nmol per hr per mg in the PD brains and 2.50 nmol per hr per mg in the PSP brains. These differences from control values were significant for the PSP but not for the PD groups or for demented and nondemented PD subgroups analyzed separately. The values for the PD patients without tremor were not different from the values for those with tremor.

DISCUSSION

The anatomical findings reported here demonstrate focal degeneration in the NPP region in idiopathic PD and in PSP and strongly suggest that in both of these parkinsonian disorders neuronal degeneration involves cholinergic as well as catecholaminergic cells of the brainstem. For technical reasons, it was not possible to make an unequivocal identification of the degenerating population of AcChoEase-positive and NADPH diaphorase-positive neurons in the NPP as cholinergic. However, the similar distributions of AcChoEase-positive and ChoAcTase-positive neurons in the human NPP region (11) and the colocalization of AcChoEase and NADPH diaphorase in the ChoAcTase-positive neurons of this region in baboon (10), monkey (9), and rat (20, 21) clearly support this suggestion. No striking differences between parkinsonian and control brains were found for other cholinergic cell groups of the brainstem, including the Ch6 cell group that closely adjoins the NPP (Ch5) region (9–11). We

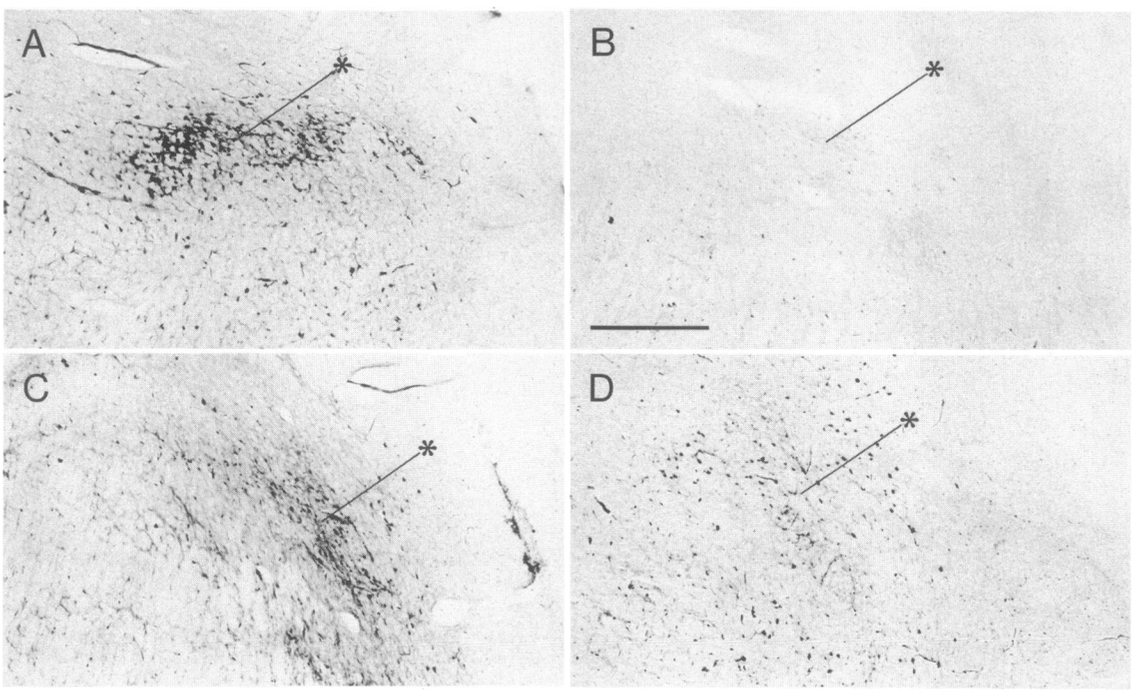

FIG. 2. Comparison of NADPH diaphorase staining of neurons in NPP. (*A*) Control. (*B*) PSP. (*C*) PD with moderate cell loss. (*D*) PD with severe cell loss. Dense core of NPP indicated by asterisks. Section shown in *A* is adjacent to the AcChoEase-stained section shown in Fig. 1*A*. AcChoEase-rich dense core of NPP corresponds to zone of densely packed NADPH diaphorase-positive neurons. (Bar = 1 mm.)

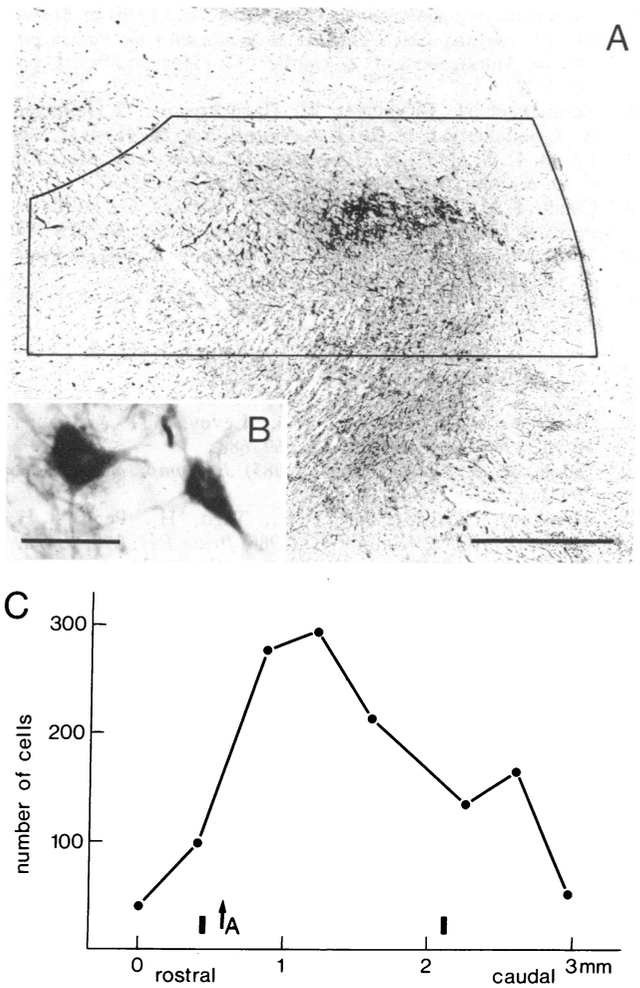

FIG. 3. Illustration of conventions followed in plotting and counting neurons. (*A*) NPP region of a control brain, in a transverse section stained for NADPH diaphorase. Solid contour indicates the region within which neurons were counted. Medial, lateral, dorsal, and ventral limits were determined for each section at the limit of NADPH diaphorase-positive neurons visible. Curved dorsomedial border was drawn parallel to the curve of the central gray substance at the location of the first neuromelanin-containing neurons encountered. (Bar = 2 mm.) (*B*) NADPH diaphorase-positive neurons from the same section. (Bar = 50 μm.) (*C*) Graph of the number of NADPH diaphorase-positive neurons in eight sections from a control brain plotted against the distance along the rostrocaudal axis of the nucleus. Surface area under the curve is proportional to the total number of neurons in the nucleus and was used for each brain as an estimate of the total neuronal population of the NPP. The level of 50 neurons was taken as minimum population for counting. Arrow indicates the level of the section shown in *A*. Vertical bars denote limits of AcChoEase-rich dense core.

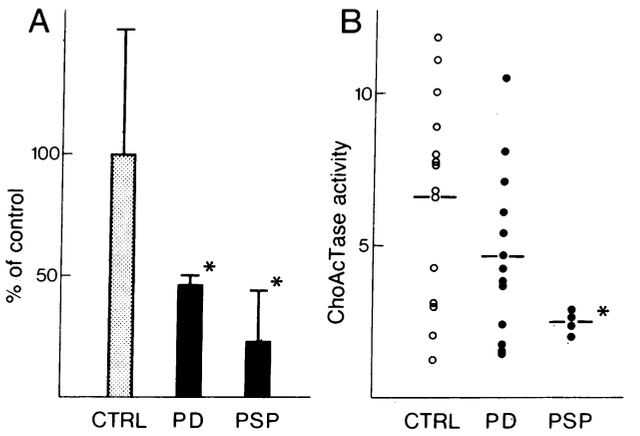

FIG. 4. (*A*) Estimates of the total number of NADPH diaphorase-positive neurons in control (taken as 100%), PD, and PSP brains. The relative numbers of neurons were obtained from curves as shown in Fig. 3*C*. Values for control, PD, and PSP brains were compared by a two-tailed Mann–Whitney *U* test. Asterisks indicate $P < 0.025$. (*B*) ChoAcTase activity (nmol per hr per mg of protein) in NPP samples from control, PD, and PSP brains. The means (horizontal bars) ± SEM were 6.59 ± 0.91 for control brains, 4.67 ± 2.63 for PD brains, and 2.50 ± 0.20 for PSP brains. The mean value for the PSP group is significantly different from the mean for the controls ($P < 0.05$). The decrease observed for the PD group is not significant ($P < 0.01$; see *Discussion*).

conclude that the defect described here may be specific for the Ch5 cell group among cholinergic nuclei of the brainstem.

The depopulation of the NPP was severe in each of the PSP brains. Taken together with the brief report by Zweig *et al.* (26), this suggests that neuronal loss in the NPP may be a standard feature of this parkinsonian disorder. There was greater variability in the PD brains: in four of the six there was moderate or severe cell loss in the NPP, but in two cases neuronal loss was marginal. This raises the possibility that there are subtypes of the disease with greater or lesser loss of neurons in the NPP. Similar variability has been reported for cell loss found in the nucleus basalis of some PD brains (5). In the material available for study, we were unable to count separately the neurons in the core and in the diffuse part of the NPP. The pattern of cell loss in the PD and PSP brains, however, was not identical. Shrinkage of the nucleus appeared laterally for both the affected PD and the PSP groups, but a reduction in the medial part of the nucleus was detected only in the affected PD group.

For the forebrain cholinergic deficit in PD, the severity of degeneration has been correlated with the clinical symptom of dementia, as has the loss of ChoAcTase activity found in the cerebral cortex of PD brains (6). With respect to the loss of neurons in the NPP reported here, the connections and location of the NPP suggested that a correlation might be made with the motor symptoms of the disease. The NPP lies in the region of the mesencephalic motor area (27, 28), and work with experimental animals has suggested that the NPP region is interconnected with the basal ganglia and superior colliculus (8, 14–18, 29–31). It is unclear, however, to what degree the cholinergic neurons of the region participate in these connections (16, 18). For example, a distinction has been made in experimental animals between a medial part of the NPP region that received pallidal and nigral afferents and a lateral part of the NPP region that does not (32, 33). A prime possibility for retrospective clinical correlation was a relation to tremor, as first suggested by Papez (34) for a single case of idiopathic parkinsonism with a lesion in the dorsolateral reticular formation, and as later suggested (14, 15) on the basis of pathway anatomy. No such relation was apparent on analysis of the medical records, however, and the severe neuronal loss in the NPP in the PSP brains also argued against this possibility, as tremor is not a clinical feature of PSP. The PSP cases, by contrast, suggested that there might be a particular relation to oculomotor deficits, to axial rigidity, or to dementia, all prominent features of PSP patients. No information was available regarding eye movements in the PD group, but there was no apparent relation between the degree of cell loss in the NPP of the PD patients and axial rigidity, dementia, or any other clinical feature examined. What is clearly needed now is a prospective study with a larger patient population.

The severe deficit in each of the PSP brains is of great interest in view of the pattern of cholinergic deficit found in the forebrain in PSP. In contrast to the significant loss of ChoAcTase in the cerebral cortex in brains from demented

PD patients, the loss of ChoAcTase in the cortex of PSP patients (characteristically severely demented) is moderate and not found in the temporal cortex (19). However, there is a 70% reduction of ChoAcTase in the substantia innominata in PSP and marked defects also in the corpus striatum, amygdala, and subthalamic nucleus. This widespread loss of subcortical ChoAcTase could in part reflect loss of neurons in the NPP, which has major subcortical but only minor cortical output connections. The "subcortical dementia" characteristic of PSP, and attributed to extrapyramidal dysfunction (19, 35), might also bear some relation to NPP pathology. One possibility is that in the primate the NPP-Ch5 region projects to the substantia innominata (36, 37).

The biochemical findings provided convincing evidence for a cholinergic deficit in the brainstem of the PSP brains, but the mean for the PD brains, though lower than that of the controls, was not significantly different from the control value. One possible reason for the lack of significance is the very large standard deviations for the control and PD samples, perhaps reflecting difficulty in dissection. A second possibility is that ChoAcTase activity in the NPP may reflect not only cholinergic neurons intrinsic to the nucleus but also cholinergic afferents that might be more affected in PSP than in PD. It is important to emphasize that we have no way of determining whether *noncholinergic* neurons in the NPP region were also affected in the PD and PSP brains. More evidence on this point will be crucial in making definitive clinicopathologic correlations.

Aside from these clinically oriented issues, the present findings raise important questions regarding the mechanisms underlying neuronal loss in the NPP. First, these observations again raise the possibility that there are common etiologic factors underlying degeneration of certain catecholamine-containing and acetylcholine-containing cell groups in the brain. This issue was initially posed by the realization that loss of cholinergic forebrain neurons may appear in parkinsonian disorders and that catecholamine-containing cell groups may undergo degeneration in Alzheimer disease, although a primary defect may be in the cholinergic forebrain system. The commonality of etiologic factors need not be strictly on a molecular basis. For example, if, as reported (14–16, 38), the NPP projects to the pars compacta of the substantia nigra, loss of nigral neurons could induce a retrograde "dying back" of neurons in the NPP. It would be of great interest to study the NPP region in brains exposed to the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine).

A second issue concerns the nature of the trophic factors responsible for survival of different cholinergic neuronal populations. In the basal forebrain system, nerve growth factor receptors have been implicated in the growth and survival of cholinergic neurons. By contrast, nerve growth factor receptors have not been found in the cholinergic cell groups of the pontine tegmentum (39). We were unable to carry out a coordinate study of neuronal loss in the hindbrain and forebrain, but our results do demonstrate that a drastic reduction in survival of neurons may occur in one cholinergic nucleus of the brainstem with sparing of neurons in nearby cholinergic cell groups. This selective destruction suggests that a highly differentiated set of trophic molecules may be involved in regulating these cell groups not only during development but also in the evolution of neuronal pathology.

We thank Drs. Y. Agid, J. J. Hauw, and P. Cervera for their help and suggestions; N. Le Balch for technical assistance; and H. F. Hall, who is responsible for the photography. This work was supported by the Seaver Institute, by National Institutes of Health Grant R01 EY02866-05, by a grant from the Tourette Syndrome Association, by Institute National de la Santé et de la Recherche Medicale Unité 289, Paris, and by the McKnight Foundation.

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