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Striatal phosphoproteins in Parkinson disease and progressive supranuclear palsy

(dopamine/cAMP-dependent protein kinase/basal ganglia/tyrosine hydroxylase/dopamine- and cAMP-regulated phosphoprotein of M_r 32,000)

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ABSTRACT **This study was undertaken to evaluate the levels of cAMP-regulated phosphoproteins in the striatum of patients with neurodegenerative diseases of the dopaminergic system. Postmortem samples of caudate nucleus and putamen from 24 control subjects, 23 patients with Parkinson disease, and 13 patients with progressive supranuclear palsy were studied with immunoblotting techniques. The levels of tyrosine hydroxylase were reduced in patients with Parkinson disease (levels were 24% and 10% of controls in caudate nucleus and putamen, respectively) and with progressive supranuclear palsy (levels were 11% and 6% of controls in caudate nucleus and putamen, respectively). Five phosphoproteins, which are present in striatal neurons and are likely to play a role in the postsynaptic actions of dopamine, were measured. These included ARPP-16, ARPP-19, ARPP-21 (cAMP-regulated phosphoproteins of M_r 16,000, 19,000, and 21,000, respectively), DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of M_r 32,000), and phosphatase inhibitor I. The levels of these phosphoproteins were inversely correlated with postmortem delay. In brains of patients with Parkinson disease or progressive supranuclear palsy with postmortem delays comparable to those of controls, the levels of these proteins as well as those of synaptic (synapsin I and synaptophysin) and glial (glial fibrillary acidic protein and myelin basic protein) markers were not significantly modified. We conclude that the levels of several phosphoproteins involved in signal transduction in striatal neurons are not altered in Parkinson disease and progressive supranuclear palsy. This observation supports the view that the striatal output neurons are intact in both diseases.**

Parkinsonian syndromes are caused by an impairment of dopaminergic transmission in the neostriatum (1). In idiopathic Parkinson disease this impairment is due to a selective degeneration of the nigrostriatal dopaminergic neurons, and treatment by L-dopa dramatically improves the motor deficit during the first years after manifestation of the disease (1). In progressive supranuclear palsy [Steele, Richardson, Olszewski syndrome (2)], in addition to the lesions of the dopaminergic neurons, degeneration occurs in other regions of the brainstem and in the pallidum, a fact thought to account for the lack of efficiency of L-dopa in this disease (3). In both diseases there is no gross anatomical alteration in the striatum, although histological and biochemical evidence suggests a specific deficit of the cholinergic interneurons in progressive supranuclear palsy (4, 5). Little information is available concerning the status of the main target of the nigrostriatal pathway, the medium-sized spiny neurons. The question of functional and/or degenerative alterations in striatal dopaminoceptive neurons, secondary to degeneration of the dopaminergic neurons, is an important issue to address because the basic assumption of treatment by dopaminergic agonists is that striatal neurons are functionally intact and able to respond to dopamine.

Dopamine activates adenylate cyclase through D_1 receptors (6). Increased cAMP levels result in the activation of cAMP-dependent protein kinase, which phosphorylates a variety of proteins in nervous tissue, some of which are specifically enriched in the medium-sized spiny neurons of the neostriatum (refs. 7 and 8; E. Gustafson and P.G., unpublished observations). Among these proteins, DARPP-32 (*d*opamine- and c*A*MP-*r*egulated *p*hospho*p*rotein of M_r 32,000), ARPP-16, and ARPP-21 (c*A*MP-*r*egulated *p*hospho*p*roteins of M_r 16,000 and 21,000, respectively) have been purified (7, 9, 10). Protein phosphatase inhibitor I and ARPP-19, two phosphoproteins that are closely related to DARPP-32 (7) and ARPP-16 (9), respectively, but which are more widely distributed, are also present in striatonigral neurons (11, 12). All these proteins are phosphorylated by cAMP-dependent protein kinase and either have been shown to be (7), or are likely to be, involved in the effects of dopamine, acting through D_1 dopamine receptors on striatal neurons. Hence they represent excellent probes for assessing the integrity of the striatal neurons, and, in particular, the integrity of their signal transduction machinery. The specificity of possible changes in the levels of these proteins was addressed by the simultaneous measurement of several other markers. Tyrosine hydroxylase was used as an index of the disappearance of dopaminergic fibers. Synapsin I and synaptophysin (also called P38) were used as markers of the nerve terminals (13). Finally, glial fibrillary acidic protein and myelin basic protein were measured as markers of astrocytes and oligodendrocytes, respectively.

METHODS

Postmortem Samples. Brains were obtained at autopsy from 23 patients with idiopathic Parkinson disease, 13 patients with progressive supranuclear palsy, and 24 controls without neurological or psychiatric disease (Table 1). The criteria used for the diagnosis of Parkinson disease included the clinical record and at least one of the following types of evidence: (*i*) histopathological evidence of neuronal loss in the substantia nigra with presence of Lewy bodies, (*ii*) low dopamine levels in the striatum, or (*iii*) decreased levels of [^{3}H]dihydrotetrabenazine (CEA, Saclay, France), an index of dopaminergic fibers (15). The criteria used for the diagnosis of progressive supranuclear palsy were the clinical record and the existence of typical lesions upon neuropathological examination (5). The premortem severity index (14)

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Abbreviations: ARPP-16, -19, -21, c*A*MP-*r*egulated *p*hos*p*hoproteins of M_r 16,000, 19,000, and 21,000, respectively; DARPP-32, *d*opamine- and c*A*MP-*r*egulated *p*hospho*p*roteins.

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Table 1. Clinical parameters

	Control	Parkinson disease	Progressive supranuclear palsy
Men, no.	8	16	7
Women, no.	16	7	6
Age, yr	79 ± 9	75 ± 8	70 ± 6
Interval between cessation of L-dopa and death, day		3.4 ± 4.4	3.5 ± 5.1
Premortem severity index	2.6 ± 1.7	2.8 ± 1.2	2.0 ± 1.7
Duration of disease, yr		15.0 ± 11.2	6.4 ± 2.0

Data represent means ± SD. The premortem severity index is an estimation of the severity of hypoxia and hypovolemia before death (14), graded from 0 to 4 in this study.

was a clinical index taking into account the degree of hypoxia and hypovolemia just before death. Postmortem delay was defined as the time interval between death and freezing of the brain. Brains were stored at −70°C until dissection (5).

Measurement of Proteins of Interest. Brain samples (12–45 mg) were homogenized in boiling 2% (wt/vol) $NaDodSO_4$ by sonication and further maintained in a boiling-water bath for 10 min. Protein concentrations were determined with BCA reagent (Pierce; uses bicinchoninic acid) using bovine serum albumin as standard. Equal amounts of protein were added to "stop solution" and boiled; 100-μg aliquots were then subjected to polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (concentration of polyacrylamide, 13%) (16) and transferred to nitrocellulose (pore size, 0.2 μm; Schleicher & Schuell) (17). The transferred proteins were fixed in isopropanol/acetic acid/water (1:1:8) and stained with amido black or Ponceau red for identification of molecular weight standards and possible transfer artifacts. In preliminary experiments the migration of the proteins of interest was determined, and in most subsequent experiments nitrocellulose filters were cut according to the position of molecular weight markers to incubate only the relevant part of the filter with the corresponding antibody. This approach allowed the study of several proteins from the same gel, a valuable procedure when starting material was available in limited amounts. Dyes were removed before immunoblotting by use of either 8 M urea and bovine serum albumin (200 mg/ml) at 60°C for 1 hr for amido black or 0.2 mM NaOH at room temperature for 5 min for Ponceau red.

Immunoblotting was performed at room temperature as described (18), using nonfat dry milk as blocking agent (19). The monoclonal antibodies utilized were anti-tyrosine hydroxylase (Boehringer Mannheim) at 0.8 mg/liter, anti-glial fibrillary acidic protein (Boehringer Mannheim) at 0.2 mg/liter, anti-myelin basic protein (Boehringer Mannheim) at 2 mg/liter, anti-bovine DARPP-32 (mixture of two monoclonal antibodies, a gift of H. C. Hemmings, Jr.; final dilution 1:5000). Filters were incubated for 2 hr in the presence of monoclonal antibodies and, after washing, they were incubated for an additional 2 hr in the presence of affinity-purified rabbit IgG anti-mouse IgG (Cappell Laboratories, diluted 1:500). The filters were washed again and incubated with ^{125}I-labeled protein A (Dupont/NEN, 85 μg/liter, specific activity, 80 Ci/g; 1 Ci = 37 GBq) for 4–10 hr and used for autoradiography as described (18). The other antibodies used were rabbit antisera against bovine ARPP-16 and ARPP-19 (G153, a gift of A. Horiuchi; diluted 1:500), against rabbit skeletal muscle protein phosphatase inhibitor I [G48 and G187, a gift of A. C. Nairn and H. C. Hemmings, Jr.; diluted 1:500], against rat synaptophysin [G95, a gift of R. Jahn and M. Bahler; diluted 1:500), and against bovine synapsin I (affinity-purified G116, a gift of A. J. Czernick; diluted 1:1000). After 5-hr incubation with the appropriate rabbit antiserum, filters were washed and incubated with ^{125}I-labeled protein A as described above.

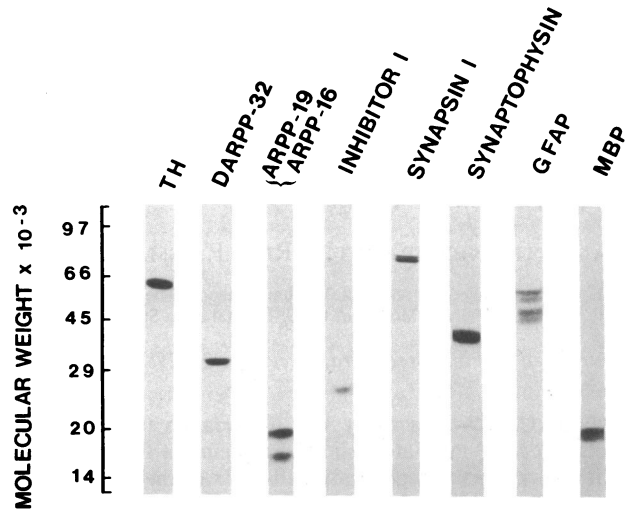

FIG. 1. Autoradiograms of immunoblots of proteins studied in human striatal samples obtained postmortem. Frozen samples were homogenized in 2% boiling $NaDodSO_4$. Equal amounts of protein were separated by electrophoresis on 13% polyacrylamide gels and transferred to nitrocellulose filters. Filters were sequentially incubated with the appropriate antibodies and ^{125}I-labeled protein A as described in text. TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein. ARPP-16 and ARPP-19 have a high degree of primary structure homology (21), and the serum used in the present study reacted equally well with both. In most samples GFAP immunoreactivity appeared to be composed of several bands, possibly as a consequence of partial proteolysis.

Quantification of the proteins of interest was achieved by cutting the nitrocellulose pieces that corresponded to the immunoreactive bands, using autoradiograms as guides, and counting the radioactivity with substraction of background radioactivity (estimated from pieces of nitrocellulose from the same filters of equal area which contained no immunoreactivity). Purified protein standards including bovine DARPP-32, bovine ARPP-16 and ARPP-19 (a gift of A. Horiuchi), bovine synapsin I (a gift of A. J. Czernick), and rat synaptophysin (a gift of F. Benfenati) were used on all gels for standardization. When no purified protein was available, standardization was achieved by loading aliquots of the same

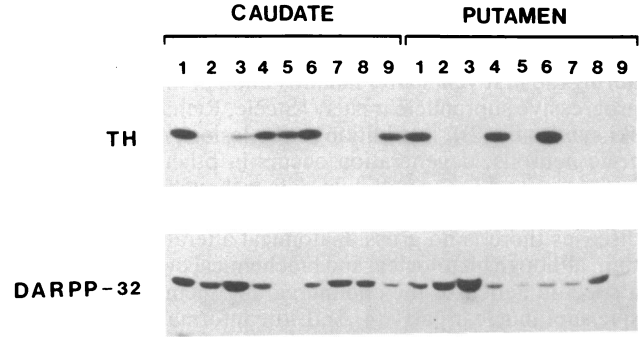

FIG. 2. Autoradiograms of immunoblots comparing the amounts of tyrosine hydroxylase (TH) and DARPP-32 in samples of caudate nucleus and putamen obtained from the brains of three control subjects (lanes 1, 4, and 6), three patients with Parkinson disease (lanes 2, 5, and 8), and three patients with progressive supranuclear palsy (lanes 3, 7, and 9). Experimental procedures were as described in the legend for Fig. 1. In many cases the levels of TH were more markedly decreased in the putamen than in the caudate nucleus (e.g., lanes 5 and 9).

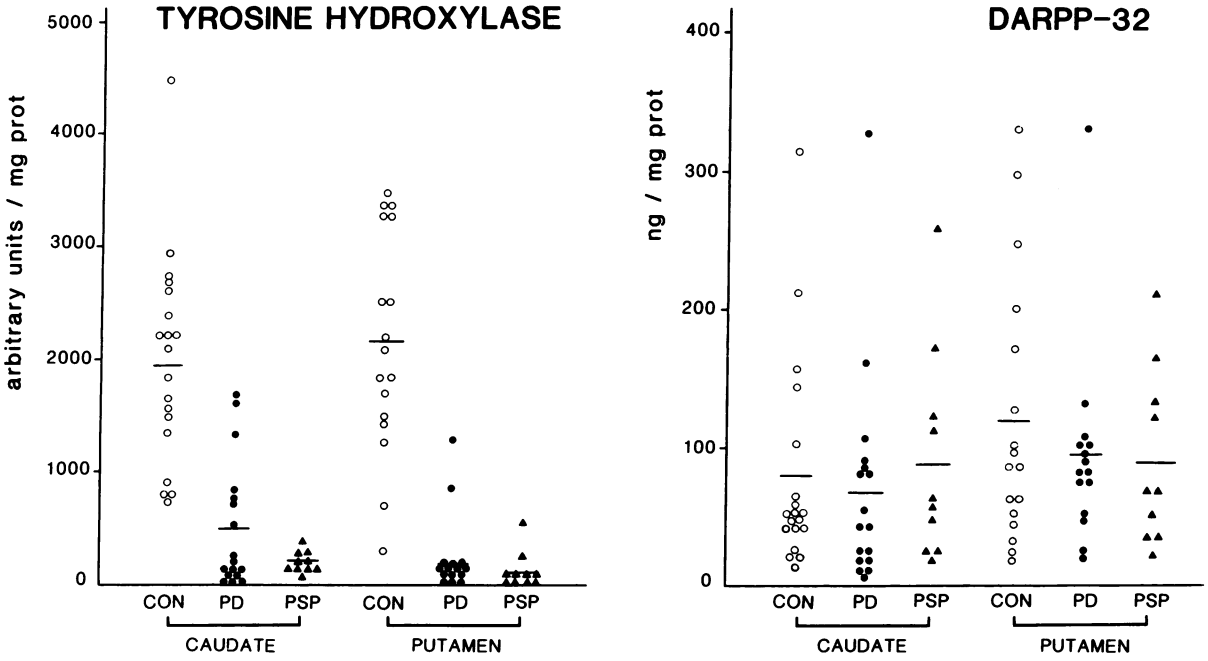

FIG. 3. Levels of tyrosine hydroxylase and DARPP-32 in caudate nucleus and putamen of controls (CON), patients with Parkinson disease (PD), and patients with progressive supranuclear palsy (PSP), determined by immunoblotting as described in text. Horizontal bars indicate the means. Statistical analysis was done with one-way analysis of variance followed, when significant, by Tukey's test. Means ± SD for tyrosine hydroxylase were as follows: caudate nucleus—CON 1960 ± 950, PD 480 ± 585, PSP 220 ± 88 ($F = 27.7$, $P < 0.001$; differences between controls and the two other groups $P < 0.001$); putamen—CON 2115 ± 994, PD 213 ± 362, PSP 121 ± 165 ($F = 41.3$, $P < 0.001$; differences between controls and the two other groups $P < 0.001$). The means ± SD for DARPP-32 were as follows: caudate nucleus—CON 81 ± 77, PD 70 ± 79, PSP 90 ± 78 ($F = 0.22$, not significant); putamen—CON 113 ± 93, PD 96 ± 72, PSP 92 ± 65 ($F = 0.28$, not significant).

brain extracts on every gel. The radioactivity measured increased linearly with the amount of protein (either purified standards or tissue homogenates) in the range studied (data not shown).

Another phosphoprotein enriched in the striatonigral neurons, ARPP-21, could not be assayed by immunoblot, because of its poor yield of transfer to nitrocellulose. Therefore ARPP-21 was measured by *in vitro* phosphorylation with the catalytic subunit of cAMP-dependent protein kinase (a gift of A. C. Nairn) and [γ-^{32}P]ATP followed by immunoprecipitation with a specific antiserum (G154) as described (20). Comparison of data obtained from immunoblots and phosphorylation/immunoprecipitation for ARPP-16, ARPP-19, and DARPP-32 showed that the two methods gave similar results (data not shown).

RESULTS

The proteins from human brain appeared to have properties (immunoreactivity, M_r values) qualitatively similar to those previously described for the corresponding proteins from rodent or bovine brain (Fig. 1). Moreover two-dimensional proteolytic mapping of ARPP-16, ARPP-19, ARPP-21, and DARPP-32 from human brains, phosphorylated with cAMP-dependent protein kinase, yielded phosphopeptides similar to those obtained from purified bovine proteins, further demonstrating their close structural similarities (data not shown). However, variations in the levels of these proteins were observed from one human brain to another, especially for DARPP-32 (Figs. 2 and 3), ARPP-16, ARPP-19, and inhibitor I, whereas these proteins are found at very constant levels in

Table 2. Correlation between level of striatal proteins and postmortem delay

	Control		Parkinson disease		Progressive supranuclear palsy	
	Caudate (n = 24)	Putamen (n = 17)	Caudate (n = 22)	Putamen (n = 15)	Caudate (n = 10)	Putamen (n = 12)
TH	0.068	0.130	−0.211	0.040	0.385	0.243
DARPP-32	−0.462*	−0.648*	−0.563*	−0.652*	−0.362	−0.623*
ARPP-16	−0.363	−0.494*	−0.624*	−0.653*	−0.384	−0.426
ARPP-19	−0.333	−0.025	−0.613*	−0.322	−0.728*	−0.442
ARPP-21	−0.249		−0.391		−0.237	
Inhibitor I	−0.585*	−0.619*	−0.506*	−0.410*	−0.268	−0.427
Synapsin I	−0.284	−0.414	−0.047	0.025	0.336	−0.169
Synaptophysin	−0.404	0.336	−0.218	0.237	−0.323	0.249
GFAP	−0.170	0.155	0.180	−0.112	0.677	−0.060
MBP	0.237	0.542	0.004	−0.009	0.261	0.040

The number of patients studied is indicated in parentheses in each column (with the exception of ARPP-21, for which only 16 controls, 15 patients with Parkinson disease, and 10 patients with supranuclear palsy were studied). TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein.

*Statistically significant ($P < 0.05$) inverse correlations between postmortem delays and concentrations of the proteins of interest.

the brains of experimental animals. This variability could not be accounted for by heterogeneity in microdissection because in several cases two different samples from the same caudate nucleus were measured and gave comparable results (data not shown). Furthermore, in control brains, levels of these proteins in the putamen and in the caudate nucleus from the same individuals correlated (DARPP-32, $r = 0.894$, $P < 0.001$; ARPP-16, $r = 0.692$, $P < 0.005$; ARPP-19, $r = 0.452$, $P < 0.10$; inhibitor I, $r = 0.711$, $P < 0.002$). Correlations between the levels of all proteins studied and several clinical parameters (sex, age of the patient, duration of the disease, interval between the cessation of L-dopa therapy and death, premortem severity index, and postmortem delay) were computed for each group of patients. The only significant correlations observed were between the levels of the striatal neuron-enriched phosphoproteins and postmortem delays (Table 2).

Given this inverse correlation, only brains with postmortem delays <24 hr were used for further study (Figs. 2 and 3, Table 3). These brains had comparable average postmortem delays (Table 3). The levels of tyrosine hydroxylase were decreased in the caudate nucleus and putamen of both Parkinson disease and progressive supranuclear palsy (Figs. 2 and 3). In contrast, the levels of DARPP-32, ARPP-16, ARPP-19, ARPP-21, and inhibitor I were not significantly altered in patients with either disease (Figs. 2 and 3 and Table 3). There was no significant correlation between the levels of tyrosine hydroxylase and those of any of the phosphoproteins studied (data not shown). Furthermore, synaptic vesicle-associated phosphoproteins synapsin I and synaptophysin and glial markers were also unchanged (Table 3).

DISCUSSION

In the present work with postmortem brain samples, it was important to consider the stability of the biochemical markers studied. In fact, the levels of striatal-enriched phosphoproteins decreased with postmortem time, indicating that these proteins may be very sensitive to proteolysis after death.

The amounts of tyrosine hydroxylase detected by immunoblot were markedly decreased in both Parkinson disease and progressive supranuclear palsy. This decrease reflects the degeneration of dopaminergic terminals in these two diseases and is in agreement with the decrease in tyrosine hydroxylase activity (3, 22). In contrast, no significant change occurred in the levels of DARPP-32 or of any other proteins studied in the brains of patients with Parkinson disease or progressive supranuclear palsy. The normal levels of nerve terminal-associated proteins, despite the disappearance of the dopaminergic fibers, is not surprising because these fibers probably account for only a small fraction of the total number of terminals in the striatum (23).

The present results are at variance with previously reported observations in which DARPP-32 levels, estimated by endogenous phosphorylation with cAMP-dependent protein kinase present in the homogenates, were found to be decreased (24). There are several possible explanations for this discrepancy. The results observed in the previous study may have been affected by various endogenous inhibitors or phosphatases present in the assay, by variations in the levels of endogenous cAMP-dependent protein kinase, and most probably by differences in postmortem delays (which were significantly longer for parkinsonian patients than for controls).

Table 3. Level of specific proteins in the caudate nucleus and putamen of patients with parkinsonian syndromes

	Control	Parkinson disease	Progressive supranuclear palsy	F
Postmortem delay, hr				
Caudate	8.4 ± 4.9	9.9 ± 6.9	9.3 ± 7.5	0.28
Putamen	7.8 ± 4.6	9.4 ± 6.3	10.0 ± 7.4	0.50
ARPP-16, ng/mg				
Caudate	17.5 ± 8.7	17.6 ± 6.2	16.3 ± 6.3	0.12
Putamen	17.1 ± 11.8	14.9 ± 7.8	13.8 ± 8.6	0.41
ARPP-19, ng/mg				
Caudate	13.2 ± 5.4	12.3 ± 5.2	11.9 ± 3.5	0.29
Putamen	12.4 ± 4.6	13.2 ± 5.9	10.2 ± 4.9	1.01
ARPP-21, au/mg				
Caudate	1050 ± 368	981 ± 396	1082 ± 421	0.17
Inhibitor I, au/mg				
Caudate	1100 ± 835	1066 ± 892	933 ± 659	0.14
Putamen	851 ± 675	706 ± 533	785 ± 547	0.23
Synaptophysin, ng/mg				
Caudate	2664 ± 662	2707 ± 557	2097 ± 670	3.45
Putamen	2499 ± 465	2353 ± 355	2514 ± 534	0.55
Synapsin I, ng/mg				
Caudate	3486 ± 1813	2899 ± 878	3433 ± 2113	0.67
Putamen	3268 ± 962	3176 ± 743	3392 ± 627	0.21
GFAP, au/mg				
Caudate	3122 ± 1164	2636 ± 997	3106 ± 1112	1.03
Putamen	2505 ± 708	2087 ± 405	2368 ± 513	2.15
MBP, au/mg				
Caudate	5731 ± 6270	6493 ± 4332	7757 ± 6792	0.41
Putamen	5949 ± 2689	7636 ± 3524	5905 ± 2722	1.50

Data represent means ± SD and is controlled for postmortem delay. The number of brains studied was as follows: controls, 19 caudates and 17 putamens; Parkinson disease, 17 caudates and 15 putamens; progressive supranuclear palsy, 10 caudates and 10 putamens. For ARPP-21 only 10 brains were studied in each group. Statistical analysis was done with one-way analysis of variance for which the F values are indicated in the last column. au, Arbitrary units; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein.

The lack of change in DARPP-32, despite degeneration of dopaminergic neurons, seen in Parkinson disease and progressive supranuclear palsy agrees with observations in rat, where acute 6-hydroxydopamine lesions of the substantia nigra resulted in no change of DARPP-32 (25) or of its mRNA (M. S. Schalling, M. Ehrlich, T. Hokfelt, and P. G., unpublished observations). Similarly, chronic treatment of rats with SCH 23390, a specific D_1-type dopamine receptor antagonist, did not induce any significant change in the levels of DARPP-32, ARPP-16, ARPP-19, or synapsin I (J. A. Grebb, J.-A.G., and P.G., unpublished work). Our results are also consistent with reports of the absence of change in the levels of D_1-type dopamine receptors (15, 24), glutamic acid decarboxylase, γ-aminobutryic acid, and several neuropeptides (22) in the striatum of patients with Parkinson disease or progressive supranuclear palsy. However, in the latter disease restricted lesions of large cholinergic interneurons may exist (4, 5) and account, at least in part, for the decrease in striatal D_2-type dopamine receptors (15). All these observations indicate that the medium-sized spiny neurons, which represent the output neurons of the striatum, do not bear any detectable lesion in Parkinson disease and in progressive supranuclear palsy, even in advanced stages. In particular, several phosphoproteins, which are likely to mediate some of the effects of dopamine acting through D_1-type dopamine receptors, are present at normal levels in both diseases.

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