

# Differential loss of striatal projection neurons in Huntington disease

(enkephalin/substance P/globus pallidus/substantia nigra/chorea)

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**ABSTRACT** Huntington disease (HD) is characterized by the loss of striatal projection neurons, which constitute the vast majority of striatal neurons. To determine whether there is differential loss among different populations of striatal projection neurons, the integrity of the axon terminal plexuses arising from the different populations of substance P-containing and enkephalin-containing striatal projection neurons was studied in striatal target areas by immunohistochemistry. Analysis of 17 HD specimens indicated that in early and middle stages of HD, enkephalin-containing neurons projecting to the external segment of the globus pallidus were much more affected than substance P-containing neurons projecting to the internal pallidal segment. Furthermore, substance P-containing neurons projecting to the substantia nigra pars reticulata were more affected than those projecting to the substantia nigra pars compacta. At the most advanced stages of the disease, projections to all striatal target areas were depleted, with the exception of some apparent sparing of the striatal projection to the substantia nigra pars compacta. These findings may explain some of the clinical manifestations and pharmacology of HD. They also may aid in identifying the neural defect underlying HD and provide additional data with which to evaluate current models of HD pathogenesis.

Huntington disease (HD) is an autosomal dominant neurodegenerative disorder characterized by choreiform movements, cognitive decline, and personality disturbance (1). The underlying genetic defect in HD is unknown, but the gene has been localized to the short arm of chromosome 4 (2). The histopathology of HD reveals cell loss and astrogliosis in several brain areas, with the most prominent alterations occurring in the striatum (3–5). Although the pathogenetic mechanism of this process is unknown, endogenous “excitotoxins” have been proposed as the mechanism of cell death (6–8). Recent findings indicate that striatal neurons are not uniformly affected in HD and that somatostatin–neuropeptide Y-containing interneurons and cholinergic interneurons are relatively spared (8, 9). Striatal interneurons, however, constitute only a small fraction of the total number of striatal neurons, and it has not been possible to correlate the preservation of interneuron populations with the clinical features of HD.

The great majority of striatal neurons are projection neurons, which are heterogeneous in terms of their projection targets and in terms of the neuropeptides they contain (10, 11). These neurons show the earliest evidence of abnormality and are progressively depleted in HD (4, 12). Previous studies, however, have not resolved whether all populations of striatal projection neurons are equally affected in HD. The

identification of the putative populations of striatal projection neurons that are earliest and most severely affected in HD, however, could provide valuable clues regarding the basis of striatal cell death in HD. To determine whether some populations of striatal projection neurons are selectively lost while others are preserved during the course of HD, we undertook an immunohistochemical study of peptidergic striatopallidal and striatonigral projection fibers in autopsy material from individuals at different stages of the disease, with a particular focus on the populations of substance P (SP)-containing neurons that project to the medial segment of the globus pallidus (GP), to the substantia nigra pars reticulata (SNr), and to the substantia nigra pars compacta (SNc), respectively, and the population of enkephalin (Enk)-containing neurons that project to the lateral segment of GP. The findings provide evidence that all striatal projection neurons are not uniformly affected in HD.

## MATERIALS AND METHODS

**Tissue.** Tissue blocks of the GP and SN were obtained from 17 pathologically verified cases of HD. Nine of these were obtained from the Pathology Department at the University of Michigan Medical Center, six were from the Brain Tissue Resource Center (Belmont, MA), and two were from the National Neurological Resource Bank (Los Angeles). Control specimens were obtained from individuals whose autopsies were performed within approximately the same range of dates as the HD cases. Eight control specimens were neurologically normal. Additional controls with Parkinson disease ( $n = 1$ ), Alzheimer disease ( $n = 1$ ), and multiple sclerosis ( $n = 3$ ) were also examined. Brains were obtained at autopsy and immersion-fixed in formalin. Those obtained at the University of Michigan were fixed in unbuffered 15% formalin. Those obtained from the other two sources were fixed in buffered 10% formalin. After 2–3 weeks, all brains were blocked and stored in 10–15% formalin/0.1 M sodium phosphate buffer (PB) (pH 7.4). The average postmortem delay from death to fixation for HD brains was 13 hr, with a range of 4–27 hr. For control cases, the average postmortem delay was 12 hr, with a range of 5–26 hr. The average age of HD cases was 56 years, with a range of 11–81 years. For controls, the average age was 48 years, with a range of 12–83 years. Differences in age and postmortem delay were not statistically significant, and HD and control cases were well

Abbreviations: Enk, enkephalin; GABA,  $\gamma$ -aminobutyric acid; GP, globus pallidus; GPi and GPe, internal and external GP segments; HD, Huntington disease; SN, substantia nigra; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SP, substance P; STN, subthalamic nucleus.

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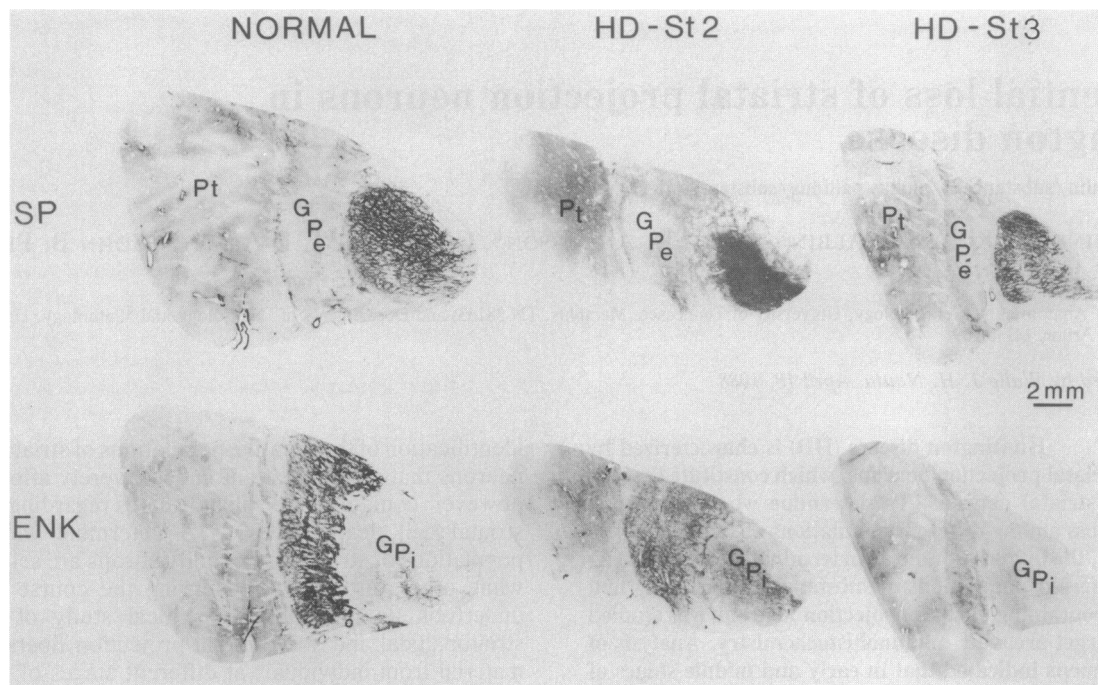


FIG. 1. Immunohistochemically labeled sections showing GPI and GPe of normal control, stage 2 HD, and stage 3 HD cases stained for SP (*Upper*) and Enk (*Lower*). In the control, there is abundant SP<sup>+</sup> staining in the GPI and abundant Enk<sup>+</sup> staining in the GPe. In the stage 2 HD specimen, Enk depletion in GPe is evident with clear preservation of SP in GPI. The contrast is even more evident in the stage 3 specimen, where Enk is markedly depleted in the atrophied GPe, while SP in GPI is still quite prominent. In stage 4 specimens, both SP<sup>+</sup> fibers in the GPI and Enk<sup>+</sup> fibers in the GPe are largely absent.

matched for agonal states. Staged<sup>¶</sup> according to the neuropathological criteria of Vonsattel *et al.* (5), the HD material consisted of four stage 2, eight stage 3, and five stage 4 specimens (earliest, stage 0; most advanced, stage 4). All stage 4 specimens were from juvenile-onset patients.

**Immunohistochemical Methods.** Tissue blocks were recovered from formalin and immersed overnight in 20% (vol/vol) sucrose/10% (vol/vol) glycerol/PB (pH 7.4). Frozen sections were cut at 40  $\mu$ m on a sliding microtome and washed in PB. To reduce nonspecific background staining, sections were serially immersed for 30 min in each of the following (with intervening PB rinses): 1% sodium borohydride in PB, 0.1 M DL-lysine in PB, and 0.2% nonfat dry milk in PB. Sections were then incubated for 48–72 hr at 4°C in primary antiserum diluted with PB/0.3% Triton X-100. The primary antisera and the dilutions used for these antisera were: anti-SP (Accurate Chemicals, Westbury, NY), 1:2000; anti-[Leu]Enk (Immuno Nuclear, Stillwater, MN), 1:1000; and anti-[Met]Enk-Arg<sup>6</sup>-Gly<sup>7</sup>-Leu<sup>8</sup> (kindly donated by G. J. Dockray, University of Liverpool, Liverpool, England), 1:250. The latter two antisera both label enkephalinergic fibers (13, 14), and the anti-[Met]Enk-Arg<sup>6</sup>-Gly<sup>7</sup>-Leu<sup>8</sup> antiserum was found to be superior to the anti-[Leu]Enk antiserum for staining tissue that had been stored in formalin for over a year. The two Enk antisera yielded identical staining patterns. The tissue was otherwise labeled according to the peroxidase-antiperoxidase procedure as described (13, 15).

## RESULTS

We chose to determine the degree of sparing of SP-containing and Enk-containing striatofugal neurons by examining their

<sup>¶</sup>Although the criteria described by Vonsattel *et al.* (5), strictly speaking, grade the degree of neuropathology, in using their criteria to classify our HD cases, we use the term stage (which refers to the disease or physical disability itself) rather than the term grade (which refers to the degree of neuropathology). We believe this is valid because the paper of Vonsattel *et al.* and the clinical data available to us on our HD cases clearly indicate that the degree of physical disability suffered by HD victims is highly correlated with the degree of neuropathology.

immunoreactive axon plexuses in the GP and SN rather than by counting their immunoreactive cell bodies in the striatum. The reason for this choice was that quantitatively reliable staining of peptidergic perikarya is difficult to achieve. In animal studies, it is necessary to pretreat with intraventricular colchicine to optimize striatal perikaryal staining. This is obviously not possible in humans.

The neurologically normal control specimens had dense plexuses of SP-positive (SP<sup>+</sup>) fibers in the internal GP segment (GPI) and in both the pars reticulata (SNr) and pars compacta (SNc) of the SN (Figs. 1 and 2). Few SP<sup>+</sup> fibers were seen in the external GP segment (GPe). Enk-positive (Enk<sup>+</sup>) fibers were abundant in the GPe and sparse in GPI. At high power, both the SP<sup>+</sup> and Enk<sup>+</sup> fiber plexuses had the configuration that Haber and Nauta have termed the "woolly fiber" pattern (Fig. 3) (16). These woolly fibers consist of the unstained dendrites of GP and SN neurons densely coated with the labeled terminals of striatal projection neurons. GP and SN staining patterns identical to those seen in the Parkinson disease, Alzheimer disease, and multiple sclerosis specimens.

Marked deviations from the normal pattern were found in the HD specimens. Enk<sup>+</sup> fibers were dramatically reduced in the GPe at all stages (Figs. 1 and 3), with evidence of progressively greater depletion of Enk<sup>+</sup> fibers from early- to later-stage cases (Figs. 1 and 3). The number of woolly fibers was greatly decreased in the early-stage cases (Figs. 1 and 3) and, in the later-stage cases, these fibers were only rarely observed. Residual staining in the later-stage cases took the form of isolated labeled terminals (Fig. 3). The SP<sup>+</sup> fibers in the SNr were markedly decreased at all stages, with some evidence of progressive depletion (Fig. 2). Again, the normal fiber configuration was absent in later stages. In the SNc, the SP<sup>+</sup> fibers were relatively abundant throughout, although at later stages loss of terminals was apparent. In contrast to the early and rapid loss of Enk<sup>+</sup> fibers in the GPe, the SP<sup>+</sup> fiber labeling pattern in the GPI of stage 2 and many stage 3 cases

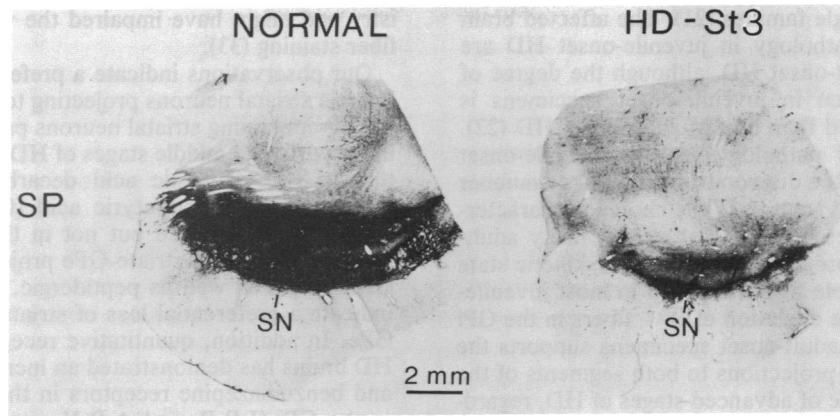


FIG. 2. Sections of normal and stage 3 HD midbrain stained for SP. SP<sup>+</sup> staining is present throughout the SN of the control specimen, while in the HD case staining is restricted to an area of neuropil surrounding the neuromelanin-containing neurons. This was also typical of our other HD cases. In primates, precise differentiation of the SNr and SNc is difficult because of the interdigitation of the dopaminergic neurons of the SNc with nondopaminergic neurons of the SNr. We used the presence of neuromelanin-containing neurons to define the location of the SNc and identified the SNr as the ventral portion of the SN without neuromelanin-containing neurons.

was very similar to that seen in controls. When examined at higher power, some reduction in the density of the SP<sup>+</sup> terminal plexus was, however, noted, especially in the stage 3 cases (Fig. 3). Nonetheless, in all stage 2 and many stage 3 specimens, there was an abundance of SP<sup>+</sup> fibers in GPi and a marked depletion of Enk<sup>+</sup> fibers in the GPe. In contrast, in all stage 4 specimens and the other stage 3 cases, marked depletion of both SP<sup>+</sup> and Enk<sup>+</sup> terminals was noted. There was considerable sparing of SP<sup>+</sup> fibers in the SNc as compared to the SNr at all stages.

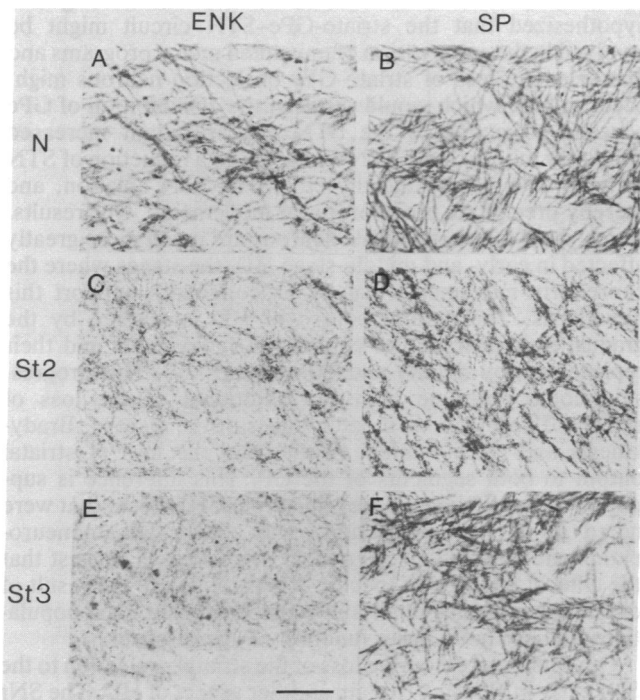


FIG. 3. Photomicrographs of SP<sup>+</sup> fibers in GPi (Right) and Enk<sup>+</sup> fibers in GPe (Left). In the control specimen (n), abundant, typical "woolly fibers" can be seen. In the stage 2 HD specimen, loss of Enk<sup>+</sup> fibers is apparent with the majority of the staining in the form of isolated terminals (although some Enk<sup>+</sup> woolly fibers are still present), while the SP<sup>+</sup> fibers in GPi are indistinguishable from that of the control. In the stage 3 specimen, Enk<sup>+</sup> woolly fibers are completely absent, while the SP<sup>+</sup> fibers in GPi are remarkably preserved, although some decrease in terminal density is apparent. By stage 4, both SP<sup>+</sup> fibers in GPi and Enk<sup>+</sup> fibers in GPe are largely absent. (Bar = 50 μm.)

Two cases merit special mention because of their atypical clinical histories. One of these was a stage 3 juvenile-onset case with chorea predominating throughout the clinical course. Examination of this case revealed Enk<sup>+</sup> fiber depletion in the GPe with substantial preservation of SP<sup>+</sup> fibers in the GPi. The other case was a stage 3 adult-onset case with a clinical presentation and course dominated by rigidity and bradykinesia. Examination of this specimen revealed depletion of both Enk<sup>+</sup> fibers in the GPe and SP<sup>+</sup> fibers in the GPi.

## DISCUSSION

The SP-containing and Enk-containing fibers of the GP and SN are known to be the terminals of striatal projection neurons (10) and a high percentage of striatofugal fibers to these targets contain one or the other of these peptides (10, 17, 18). Several studies in nonhuman primates and cats have shown that striatal projections to the GPe, GPi, SNc, and SNr derive from largely separate pools of striatal neurons; only a few neurons were found to project to more than one target (10, 19, 20). Our findings indicate that in early and middle-stage HD the enkephalinergic striato-GPe projection and the SP-containing projection to the SNr are more severely affected than the SP-containing projections to the GPi and SNc. The most likely interpretation of these results is that SP-containing striato-SNr neurons and Enk-containing striato-GPe neurons are lost at a greater rate than either SP-containing striato-SNc or SP-containing striato-GPi neurons. The differences we have found, however, are dependent on the stage of HD, as obvious changes in GPi and less obvious changes in the SP<sup>+</sup> fibers in SNc were noted in the more advanced cases of HD. Thus, by the later stages of HD, all classes of striatal projection neurons have been affected, either because of the primary pathogenetic process of HD, or as a result of transynaptic degeneration. Of all classes of fibers we examined, those projecting to the SNc appear to be the most resistant to the pathogenetic process of HD.

All our stage 4 specimens, however, were from juvenile-onset cases. Individuals with juvenile-onset HD constitute only a small fraction of all HD victims and this variant of HD is clinically distinctive: onset occurs before age 20 years, progression is relatively rapid, and a high proportion of these individuals have bradykinesia and rigidity as their initial manifestation (21). Nonetheless, several lines of evidence argue that late-stage specimens of this variant can be legitimately compared with late-stage adult-onset specimens. Juvenile-onset HD is produced by the same gene that causes adult-onset HD, a fact demonstrated by the occurrence of

both variants within single families (21). The affected brain regions and the histopathology in juvenile-onset HD are identical to that in adult-onset HD, although the degree of atrophy and gliosis seen in juvenile-onset specimens is usually more pronounced than that in adult-onset HD (22). The greater severity of pathology seen in juvenile-onset specimens accounts for the disproportionately large number of juvenile-onset cases at stage 4. While chorea is characteristic of adult-onset cases in the initial stages, many adult-onset cases eventually progress to a rigid bradykinetic state clinically indistinguishable from that seen in most juvenile-onset cases (23, 24). The depletion of SP<sup>+</sup> fibers in the GPI of some of our stage 3 adult-onset specimens supports the idea that loss of striatal projections to both segments of the pallidum is characteristic of advanced stages of HD, regardless of age of onset or initial clinical features. Furthermore, one of our stage 4 juvenile-onset specimens had prominent chorea initially, and the immunoneuropathologic findings in this case were indistinguishable from the other stage 4 specimens.

The greater preservation of striato-GPI than of striato-GPe fibers noted in this study correlates with the well-known histopathologic changes in striatal target regions in HD. Atrophy of the GPe is common in HD, while atrophy of the GPI is less frequently observed (22). It is thought that GPe atrophy is due in part to the loss of afferent striatopallidal fibers (22). Likewise, atrophy of the SNr but not the SNc has been noted frequently (25). The present findings are also consistent with observations on Golgi-impregnated HD material by Graveland *et al.* (12), who reported that while most striatal projection neurons exhibit degenerative changes in HD, some appear normal.

Although biochemical studies of HD brains have shown depletion of SP (26–29) and Enk (29) in the striatum and its projection targets, such studies have not reported the more rapid loss of Enk than SP from the globus pallidus that is indicated in the present results. Regarding this differential rate of depletion, it must be stressed that the content of neurotransmitters and neuropeptides in HD brains may be affected by a variety of factors, including differences in postmortem delay, age, or agonal status between HD patients and controls, the stage of HD, and regional differences in tissue shrinkage. In our study, control and HD specimens were closely matched in terms of postmortem delay, age, and agonal status, and specimens were stratified according to stage. Furthermore, since immunohistochemistry allows direct assessment of the morphological integrity of specific neural pathways, differential shrinkage in HD brains did not affect our interpretations. It should be added that, in the case of SN, the results of two biochemical studies (28, 29) accord well with the present observation of greater SP depletion in SNr than SNc.

The present findings are in apparent conflict with those of prior immunohistochemical studies (30–32), which concluded that both SP<sup>+</sup> and Enk<sup>+</sup> fibers are markedly and equally reduced in the GP and SN in HD. In one of these studies (30), however, the text refers to residual SP<sup>+</sup> fiber staining in the GPI and SNc and some of the figures clearly show that the Enk<sup>+</sup> fiber reduction in the GPe was greater than the SP<sup>+</sup> fiber reduction in the GPI. Since this study predated the introduction of the Vonsattel *et al.* (5) staging system for HD, it is likely that these investigators combined their findings on early- and late-stage specimens, the appearance of the latter obscuring the significance of the former. The same reservation can be raised regarding the other two studies (31, 32). Furthermore, both of these latter groups of investigators examined only a few HD specimens and used paraffin-embedded sections for immunocytochemistry, which commonly reduce the sensitivity of immunocytochem-

istry and might have impaired the visualization of residual fiber staining (33).

Our observations indicate a preferential loss of enkephalinergic striatal neurons projecting to GPe as compared with the SP-containing striatal neurons projecting to GPI, at least in the early and middle stages of HD. Spokes (34) has shown that in HD, glutamic acid decarboxylase, the synthetic enzyme for  $\gamma$ -aminobutyric acid (GABA), is significantly decreased in the GPe but not in the GPI. Since both the striato-GPI and the striato-GPe projections are known to be GABAergic as well as peptidergic, these results, likewise, indicate a preferential loss of striatal neurons projecting to GPe. In addition, quantitative receptor autoradiography of HD brains has demonstrated an increased density of GABA and benzodiazepine receptors in the GPe and SNr but not in the GPI (J.B.P. and A.B.Y., unpublished observations; ref. 35), consistent with receptor "up-regulation" due to deafferentation, which also implies a greater loss of GABAergic input to the GPe and SNr than to the GPI.

The preferential loss of striato-GPe projections may help explain the choreiform movements observed in HD. In a recent theoretical treatment of basal ganglia motor function, Penney and Young (36) noted that a general loss of striatal projection neurons could not underlie the choreiform movements observed in HD because neither experimental, traumatic, nor ischemic lesions of the striatum (which nonselectively destroy striatal neurons) in humans and nonhuman primates produce chorea. In contrast, it was noted that ablation of the subthalamic nucleus (STN), which receives a prominent inhibitory GABAergic input from GPe, or injection of the GABA antagonist bicuculline into the GPe (which mimics the loss of inhibitory striato-GPe neurons) do produce chorea (37, 38). On this basis, Penney and Young (36) hypothesized that the striato-GPe–STN circuit might be involved in the suppression of unwanted motor programs and that selective loss of striato-GPe projection neurons might occur in HD, which would result in the disinhibition of GPe neurons projecting to the STN. The resultant increased inhibitory output to the STN would cause a reduction of STN activity, thus mimicking the effects of STN ablation, and thereby precipitate the choreiform movements. Our results, which show that striato-GPe neurons are much more greatly affected in early- and middle-stage HD (the stages where the choreiform movements usually predominate), support this hypothesis. The natural course of HD is marked by the gradual disappearance of choreiform movements and their replacement by rigidity and akinesia (23, 24). This progression could correlate with the additional severe loss of striato-GPI projections in later stages of the disease. Bradykinesia and rigidity might result from the loss of striatal output to both segments of the GP. This inference is supported by the findings in the two atypical HD cases that were noted in the results section. The clinico-immunoneuropathologic correlations seen in these two cases suggest that the clinical features of specific HD patients are the result of differential changes in striatal projection neuron subpopulations and are not a strict function of age of onset.

Our finding of extensive loss of the striatal projection to the SNr early in HD may explain another aspect of HD. The SNr is known to play a major role in the control of saccadic eye movements (39). Abnormalities of saccades are an early and prominent manifestation of HD (23, 24, 40) and the early loss of striatal input to the SNr could account for this clinical manifestation.

The early and relatively selective loss of enkephalinergic and GABAergic striato-GPe projections may also account for the efficacy of dopamine antagonists in ameliorating the choreiform movements of HD and subthalamic lesions. Dopamine receptor antagonists are known to increase the synthesis of Enks by striatal neurons (41, 42). SP production

is either unchanged or diminished (42, 43). Similarly, studies in rats with unilateral 6-hydroxydopamine-induced loss of the nigrostriatal dopamine fibers indicate that dopamine suppresses the release of GABA from striatal neurons projecting to the GPe (44). Increases in the production of Enk and GABA by surviving striato-GPe neurons in HD patients could partially overcome the reduction in the number of Enk<sup>+</sup> and GABAergic neurons projecting to GPe. By the same token, in the case of STN lesions, increased Enk and GABA release in the GPe could compensate for the diminished STN function by decreasing pallidal inhibition of the remaining STN neurons.

Further study of the Enk-containing striato-GPe and SP-containing striato-SNr neurons may reveal common features that predispose them to early cell death and, thereby, aid in the identification of the biological defect underlying HD. Comparison with the relatively resistant SP-containing striato-SNc neurons may be especially fruitful. Our specimens did not include any presymptomatic, stage 0, or stage 1 cases of HD. Studies of such early cases of HD would reveal which striatal projection neurons are most affected at the onset. Our material also did not include any adult-onset stage 4 specimens. Examination of such cases will be necessary to confirm that depletion of SP<sup>+</sup> fibers in GPe is characteristic of stage 4 in general and not only of stage 4 juvenile-onset cases. Examination of early- to middle-stage juvenile-onset specimens would allow confirmation of the proposed association of the rigid bradykinetic clinical picture with depletion of projections to both segments of the pallidum. Finally, the present findings may provide additional criteria by which to evaluate experimental models or theories that attempt to explain the pathogenesis of HD (6–8).

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