Incidental Lewy Body Disease and Preclinical Parkinson Disease

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Background: The significance of Lewy bodies detected at autopsy in the brains of clinically normal individuals is uncertain but may represent preclinical Parkinson disease (PD).

Objective: To determine whether diminished striatal dopaminergic innervation and nigral cell loss are present in incidental Lewy body disease (iLBD), as one might expect if it is a forerunner of PD.

Design: Case-control study.

Setting: Medical records and archival brain tissue were obtained from a tertiary medical center for further study.

Participants: Brains from clinically healthy individuals older than 60 years with α-synuclein–immunoreactive Lewy bodies (iLBD; n=12) were compared with those from clinically healthy individuals with no α-synuclein pathological findings (n=31) and patients with PD (n=25).

Main Outcome Measures: Striatal dopaminergic integrity assessed in sections of putamen by immunofluorescence for tyrosine hydroxylase (TH) and vesicular monoamine transporter 2 (VMAT2), neuronal loss score in the substantia nigra, and distribution of Lewy bodies according to PD stage.

Results: Among the participants with iLBD, decreased striatal dopaminergic immunoreactivity was documented for both TH (33%) and VMAT2 (42%), compared with the pathologically normal subjects; as expected, the reductions were even greater in PD (73% decrease for TH and 96% decrease for VMAT2). Substantia nigra neuronal loss inversely correlated with both striatal TH (r=−0.84) and VMAT2 (r=−0.77). In addition, PD stage inversely correlated with both striatal VMAT2 (r=−0.85) and TH (r=−0.85).

Conclusions: The results indicate that iLBD has nigrostriatal pathological features that are intermediate between those in pathologically normal persons and those with PD. The findings suggest that iLBD probably represents presymptomatic PD, rather than nonspecific, age-related α-synuclein pathological changes.

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The Lewy body is the neuropathological hallmark of both Parkinson disease (PD) and dementia with Lewy bodies (DLB), which are among the most common neurodegenerative disorders of aging. The prevalence of PD is approximately 1% among those older than 65 years.1 Prevalence of clinical DLB is of the same order of magnitude, although with more varied estimates.2 Pathological findings in Lewy bodies, however, are severalfold more common on routine postmortem examination. They are found in 10% to 12% of clinically healthy people older than 60 years3,4; this finding has been termed incidental Lewy body disease (iLBD). Incidental Lewy body disease may represent preclinical PD, or perhaps DLB, suggesting that the propensity to develop these disorders may be much more common than the actual cases diagnosed later in life. If so, this has important epidemiologic and pathogenetic implications.

Limited evidence supports the proposal that iLBD represents preclinical PD or DLB. Certainly, the distribution of Lewy bodies in vulnerable brainstem nuclei in iLBD is similar to that observed in sporadic PD.5,6 Moreover, iLBD has been associated with modest reductions in substantia nigra (SN) neuronal counts.6,7 On the other hand, iLBD might reflect nonspecific pathological changes related to aging, similar to the incremental accumulation of neurofibrillary tangles with age.8,9

The motor abnormalities of PD, as well as DLB, are primarily due to loss of dopaminergic neurons in the SN and their pro-
Intracellular packaging of dopamine by vesicular monoamine transporter 2 (VMAT2), which translocates monoamines into synaptic vesicles by a proton gradient, has also been considered a reliable marker of dopaminergic neurons.12,13 In the present immunofluorescence microscopy study of the striatum, we used these 2 dopaminergic markers and image analysis to determine whether iLBD is associated with findings consistent with preclinical PD. If iLBD represents an early stage of PD, striatal dopaminergic immunoreactivity should be less than control values, but not as much reduced as in PD.

METHODS

POSTMORTEM HUMAN BRAINS

Three groups were compared in this study: persons with iLBD (n=12), persons with PD (n=25), and neurologically normal control subjects (n=31). The iLBD cases were obtained from a larger autopsy cohort (n=106) with tissue stored in the Tissue Registry of the Mayo Clinic, Rochester, Minnesota.1 The medical records of all cases in this cohort were screened without knowledge of neuropathological findings to exclude neurologic and psychiatric disorders in life. The clinical inclusion criteria were specified a priori: (1) age at least 60 years, (2) at least 2 years of Mayo Clinic records at the end of life, (3) clinical examination within the last year of life, and (4) availability of brain tissue with postmortem intervals of less than 24 hours. Cases were excluded if the clinical records disclosed the presence of (1) parkinsonism, tremor, dementia, or other chronic central nervous system disorders; (2) any major psychiatric disorder with psychosis, hallucinations, delusions, or paranoia, or requiring neuroleptic treatment; (3) a primary intracerebral event as the cause of death; (4) brain tumor (except incidental meningiomas); (5) systemic disorders likely to cause chronic brain damage; or (6) mental retardation. All iLBD cases were found to have Lewy bodies in brainstem as well as other regions of the central nervous system by means of α-synuclein immunohistochemistry. From this same cohort, 19 neurologically normal control subjects without Lewy bodies were also included for further studies. The patients in the PD group had clinically probable PD in life and pathologically confirmed PD at autopsy.17 The brains were obtained through the Neuropathology Core of the Udall Center for Excellence in Parkinson Disease Research and were from patients who had been monitored by movement disorder specialists in Jacksonville, Florida (n=14), or Rochester, Minnesota (n=11). The control group included cases from the above-described cohort (n=19) as well as healthy controls from the Mayo Clinic Jacksonville brain bank (n=12). The latter included some individuals who were participants in prospective longitudinal studies of aging and dementia (Einstein Aging Study) as described previously, as well as neurologically normal patients who were monitored by Mayo Clinic clinicians from the hospital autopsy service. All control subjects were free of significant abnormalities on neuropathological studies. For image analysis studies, formalin-fixed and paraffin-embedded tissue samples were taken at the level of the anterior commissure, including the putamen. A section of midbrain was taken at the level of the third nerve. Tissue was sectioned at 5-µm thickness for immunohistochemistry and immunofluorescence microscopy. After the paraffin was removed with xylene, glass-mounted sections were hydrated through graded ethanol series and rinsed in 0.1M phosphate-buffered saline, pH 7.1.

IMMUNOFLUORESCENCE FOR TH AND VMAT2

Immunofluorescence staining for TH and VMAT2 was performed after pretreatment of sections for 6 minutes in a solution of proteinase K (S3020; DAKO, Glostrup, Denmark; 0.05 mol/L), followed by several washes in 0.1M phosphate-buffered saline, pH 7.1. Next, sections were blocked in all-purpose blocking serum (DAKO) for 90 minutes at room temperature. Sections were then incubated overnight at 4°C using the primary antibody to TH (1:600; rabbit polyclonal, OPA04050; Affinity Bioreagents, Golden, Colorado) and VMAT2 (1:250; rabbit polyclonal, A11767; Chemicon, Temecula, California). The secondary fluorochrome, anti-rabbit (Alex Fluor 568 A11036; Invitrogen Corp, Carlsbad, California), was used for both antibodies with a working dilution of 1:300 and incubated for 90 minutes at room temperature. Following 3 washes in phosphate-buffered saline, the sections were immersed in a solution of 1% Sudan black B (1 g per 100 mL of 70% ethanol) for 2 minutes to block autofluorescence. The sections were then coverslipped in mounting medium for fluorescence (Vector Shield H-1000; Vector Laboratories, Burlingame, California). No primary negative control was used for immunohistochemistry.

IMAGE ANALYSIS

Fluorescent images were analyzed with a microscope (Olympus BX50; Olympus Corp, Melville, New York) with a digital camera (Olympus DP70). For each case and for each stain, 5 nonoverlapping images were captured from the putamen (×400 magnification for TH and ×600 for VMAT2). Each field was confined to the putamen and was taken so as not to contain large blood vessels. The images were converted to monochrome gray scale. Images were analyzed without knowledge of clinical and pathological information by means of software (MetaMorph; Molecular Devices, Sunnyvale, California) that measures pixel intensity after individual threshold adjustments are made to maximize the signal to noise ratio. The immunolabeled pixels were measured, and the TH and VMAT2 burden was defined as the ratio of the immunoreactive pixels to the total pixels of the entire imaged field. The final value for each case was the average of the 5 fields for each stain.

SEMIQUANTITATIVE ASSESSMENT OF SN NEURONAL LOSS

Dopaminergic cell loss in the SN was scored semiquantitatively on sections stained with hematoxylin-eosin. Neuronal loss in the ventrolateral tier of the SN was specifically assessed because this is the population of neurons that projects to the putamen. The sections were scored at ×100 magnification, with the evaluator (H.F.) blinded to clinical and pathological information, by means of the following 4-point scale: 0, no loss of melanized neurons; 1, focal neuronal loss; 2, moderate neuronal loss; and 3, almost complete neuronal loss.

STATISTICAL METHODS

Differences between the control, iLBD, and PD groups were assessed by Kruskal-Wallis 1-way analysis of variance on ranks, followed by pairwise comparisons using the Dunn test. Correlations were performed with the Spearman rank order test. All data were analyzed with Sigma Stat for Microsoft Windows (version 3.01; Systat Software Inc, San Jose, California). The significance level was set at P<.05.
RESULTS

SUMMARY OF CLINICAL AND PATHOLOGICAL FINDINGS

Clinical and pathological features are summarized in Table 1. The 3 groups (control, iLBD, and PD) did not differ in age at death or sex distribution. Tissue storage time was significantly longer in the iLBD group than in the control and PD groups, but storage time did not correlate with any clinical or pathological variable, including TH and VMAT2 immunoreactivity in the striatum.

Table 1. Clinical and Pathological Features of Cases and Controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n=31)</th>
<th>ILBD (n=12)</th>
<th>PD (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at death, mean (SD), y</td>
<td>75 (11)</td>
<td>75 (10)</td>
<td>78 (8)</td>
</tr>
<tr>
<td>Sex, M:F ratio</td>
<td>18:13</td>
<td>6:6</td>
<td>17:8</td>
</tr>
<tr>
<td>Tissue storage time, ya</td>
<td>8 (4-11)</td>
<td>12 (11-13)</td>
<td>6 (6-8)</td>
</tr>
<tr>
<td>SN neuronal loss scorea</td>
<td>0 (0-0)c</td>
<td>1 (1-2)</td>
<td>2 (1-3)</td>
</tr>
<tr>
<td>PD stagea</td>
<td>0 (0-0)c</td>
<td>3 (2-4)</td>
<td>4 (3-5)</td>
</tr>
<tr>
<td>Striatal TH, mean (SEM), %</td>
<td>11.2 (0.2)c</td>
<td>7.5 (0.3)d</td>
<td>3.0 (0.4)</td>
</tr>
<tr>
<td>Striatal VMAT2, mean (SEM), %</td>
<td>8.4 (0.4)c</td>
<td>4.4 (0.3)d</td>
<td>0.5 (0.07)</td>
</tr>
</tbody>
</table>

Abbreviations: iLBD, incidental Lewy body disease; PD, Parkinson disease; SN, substantia nigra; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2.

a Expressed as median (25th-75th percentile).
b P<.05 compared with control and PD groups.
c P<.05 compared with ILBD and PD groups.
d P<.05 compared with PD group.

The Braak PD stage and the substantia nigra neuronal loss score were greater in the iLBD and PD groups than in the control group, but the difference between the iLBD and PD groups was not statistically significant.

STRIATAL TH AND VMAT2 IMMUNOREACTIVITY

Immunofluorescence for both TH and VMAT2 disclosed fine varicose processes in the neuropil of the putamen (Figure 1). There was no immunoreactivity within neuronal cell bodies. The PD cases had markedly decreased immunoreactivity for both markers, while there was more variability in the iLBD cases. Immunofluorescent image analysis was performed on the putamen with the investigator (A.D.) blinded to clinical and pathological information. The results of this quantitative analysis confirmed the subjective observations, with both TH and VMAT2 being decreased in the iLBD and PD groups compared with the control group and decreased in the PD group compared with the iLBD group (Table 1). In the PD group there was a 73% reduction in TH and a 96% reduction in VMAT2 compared with controls (Figure 2). In the iLBD group there was a 33% reduction in TH and a 42% reduction in VMAT2 immunoreactivity (Figure 2).

Figure 1. Tyrosine hydroxylase (TH) and vesicular monoamine transporter 2 (VMAT2) immunoreactivity in controls, patients with incidental Lewy body disease (iLBD), and patients with Parkinson disease (PD). Representative fields from the putamen show decrease in density of fine neuritic profiles in PD compared with controls, with intermediate density for iLBD. Bars in lower right corner indicate 20 µm.

Figure 2. Striatal tyrosine hydroxylase (TH) (A) and vesicular monoamine transporter 2 (VMAT2) (B) immunoreactivity in controls, patients with incidental Lewy body disease (iLBD), and patients with Parkinson disease (PD). Box plots show median and 25th and 75th percentiles, with whiskers showing 10th and 90th percentiles. Individual dots show outliers. For both TH and VMAT2, P<.001 for control vs iLBD, control vs PD, and iLBD vs PD.
RELATIONSHIP OF STRIATAL TH AND VMAT2 IMMUNOREACTIVITY TO BRAAK PD STAGE

The relationship of TH and VMAT2 immunoreactivity to Braak PD stage was also evaluated, considering all cases together. The PD stage highly correlated with both striatal TH and VMAT2 immunoreactivities (TH, \( r = -0.85 \); VMAT2, \( r = -0.85 \)) (Table 2). Given the small number of cases in some of the stages, we chose to combine them as follows: stage 0 (\( n = 31 \)); stages 1 to 3 (\( n = 15 \)); stage 4 (\( n = 14 \)); and stages 5 and 6 (\( n = 8 \)). Post hoc analysis demonstrated that mean (SEM) TH immunoreactivity decreased as the PD increased from stage 0 (11 [0.2]) to stages 1 to 3 (5 [0.8]), to stage 4 (5 [0.4]), and then to stages 5 and 6 (3 [1.0]). The VMAT2 values were similarly distributed (stage 0, 8 [0.3]; stages 1 to 3, 1 [0.6]; stage 4, 1 [0.6]; and stages 5 and 6, 0.2 [0.03]). There were significant differences between stage 0 and stages 1 to 3; stages 0 and 4; and stage 0 and stages 5 and 6; but not between stage 4 and stages 5 and 6 for TH. There were significant differences between stages 0 and 1 to 3, but not between stages 1 to 3 and 4 or between stage 4 and stages 5 and 6 for VMAT2. The results suggest that there is a gradual decrease in TH with increasing PD stage, whereas VMAT2 has a more rapid decline and then plateaus (Figure 3).

RELATIONSHIP OF STRIATAL TH AND VMAT2 IMMUNOREACTIVITY TO SN NEURONAL LOSS

Substantia nigra neuronal loss in the ventrolateral tier of the pars compacta was estimated on the basis of a 4-point scale: no neuronal loss (\( n = 28 \)), mild neuronal loss (\( n = 15 \)), moderate neuronal loss (\( n = 12 \)), and severe neuronal loss (\( n = 8 \)). Midbrain sections were inadequate for scoring in 5 cases.) There were significant correlations between SN neuronal loss and striatal TH and VMAT2 immunoreactivity (Table 2). Post hoc analysis demonstrated that immunoreactivity for both TH and VMAT2 decreased as SN neurons decreased. The mean (SEM) TH immunoreactivity for cases with no neuronal loss was 11 (0.3), declining to 7 (0.6) in cases with mild neuronal loss, 4 (0.6) in those with moderate loss, and 2 (0.6) in those with severe neuronal loss in the ventrolateral SN. This was also true for VMAT2: no neuronal loss, 8 (0.5); mild loss, 3 (0.7); moderate loss, 1 (0.5); and severe loss, 0.5 (0.1). Similar to the findings with striatal dopaminergic markers as related to PD stage, these findings suggest that there is a gradual decrease in TH with further SN neuronal loss, whereas VMAT2 has a more rapid decline and then plateaus as SN neurons are lost.

CORRELATIONS BETWEEN CLINICAL AND PATHOLOGICAL VARIABLES

Striatal TH and VMAT2 immunoreactivity were highly correlated, and each was also very highly correlated with SN neuronal loss as well as Braak PD stage (Table 2). Braak PD stage was also highly correlated with SN neuronal loss. There were weak correlations between age and SN neuronal loss, but not for striatal TH or VMAT2 immunoreactivity or for Braak PD stage. None of the variables correlated with sex.

Abbreviations: PD, Parkinson disease; SN, substantia nigra; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2.
Pathological staging of PD as proposed by Braak and coworkers7,8 was a major advance in our conceptualization of the PD neurodegenerative process. It crystallized evidence for PD beginning in nondopaminergic lower brainstem and olfactory neurons, as well as providing a neuropathological continuum for advanced PD stages with dementia. It generated a paradigm shift from earlier concepts of PD as simply synonymous with nigrostriatal neurodegeneration.19,20 It is, however, fundamentally predicated on iLBD cases, which are the basis for stages 1 and 2 plus early stage 3. Thus, the assumption that iLBD reflects preclinical PD is crucial to the whole Braak staging scheme.

The rationale for the early stages of Braak staging was solely the presence of α-synuclein–immunoreactive Lewy–related pathological findings, without reference to neuronal loss or other evidence of degenerative change.7,8,21 By itself, this is compelling, but there is no complete assurance that α-synuclein pathology may still be nonspecific and related to aging, similar to neurofibrillar tangles and beta-amyloid plaques.10 Previously reported associations of iLBD with reduced nigral neuronal counts have been based on small sample sizes9 or showed nonsignificant trends.6

The present data complement these earlier studies and provide further evidence that iLBD represents preclinical PD, rather than a nonspecific aging phenomenon. This replicates the previous evidence linking iLBD to reduced nigral neuronal counts and extends this by documenting nigrostrial dopaminergic deficits intermediate between control and PD values. A recent study using enzyme-linked immunosassays to detect TH immunoreactivity came to a similar conclusion, namely, that patients with iLBD have decreases that are intermediate between control and PD values.22 Thus, iLBD appears to represent cases in which PD would eventually develop with further longevity, or where the PD neurodegenerative process was aborted.

The tendency of iLBD to have early Braak stages also fits with the emerging recognition that certain nonmotor symptoms predate clinical PD by many years, including olfactory dysfunction, constipation, and rapid eye movement sleep behavior disorder.23–25 Thus, the validity of iLBD as preclinical PD has implications for early diagnosis. This may be crucial if therapeutic strategies directed at the inciting process are subsequently developed; early treatment might then abort clinical PD.

We have discussed our findings in the context of preclinical PD, which is the focus of Braak staging; however, this ignores the other major Lewy body disorder, DLB. Most patients with DLB also have nigrostriatal degenerative changes and many have parkinsonism, albeit usually without tremor, and recent publications emphasize the striking overlap with late-stage PD with dementia.26–27 Conceivably, some of the iLBD cases may represent preclinical DLB, rather than PD. Obviously, DLB must start at some point, but preclinical DLB has received little attention. Interestingly, when we previously reported more detailed neuropathological findings for our iLBD cases, we noted that 4 of these cases had Lewy bodies in temporal and limbic cortex and 1 also had frontal neocortex Lewy bodies.3 Whether these reflected preclinical DLB, rather than PD, is open to speculation. Prospective clinical studies using sensitive test instruments to detect subtle cognitive and behavioral deficits are needed to address this issue. It is possible that such subtle deficits might have been missed in the present retrospective study. In addition, given the syndromic nature of PD, it may be that the pathogenesis of iLBD similarly has a heterogeneous basis.

The reductions in striatal dopaminergic markers in our iLBD cases were less than the usually cited 50% threshold values for the motor signs of PD.28,29 This may explain the absence of such clinical evidence of parkinsonism ante mortem. All of the patients with iLBD had been seen by Mayo Clinic clinicians in the last year of their life and had no documented evidence of parkinsonism, tremor, or dementia. An antemortem neurologic consultation was not an inclusion criterion because referral to a neurologist would obviously have been ordered for a neurologic disorder and we sought to exclude patients with neurologic disease. Admittedly, however, subtle clinical findings could have been overlooked. On the other hand, our patients with PD exceeded the 50% threshold (73% decrease for TH and 96% decrease for VMAT2), as might be expected in patients with clinically overt parkinsonism.

The findings in this study suggest that dopaminergic deficiency occurs relatively early in the symptomatic disease process at a time when there is not yet marked neuronal loss in the SN. This might indicate that initial changes in the nigrostriatal dopaminergic system are in the distal termini and make a case for a dying-back-type disease process. There is evidence to suggest that this is the case in several experimental models of PD. For example, systemically applied rotenone, a selective mitochondrial complex I inhibitor that causes oxidative stress and degeneration of SN neurons with α-synuclein abnormality,30 is associated with initial disease in the striatum followed by SN neuronal loss. In a viral gene transfer model, although the SN neuronal cell bodies were the site of viral infection with α-synuclein or tau constructs, deficits in TH immunoreactivity were detected in the striatum before there was neuronal loss in the SN.31 A dying-back pathological process can be related to toxic effects in the cell body or loss of trophic support from the target domain. While therapies aimed at replacing trophic factors, such as glial-derived neurotrophic factor, are currently popular,32 evidence of primary deficits in these factors in PD are less well established.33

Dopaminergic nigrostriatal integrity was assessed with 2 measures with results that were highly correlated but not equal. The reductions in VMAT2 immunoreactivity were consistently lower than those in TH immunoreactivity. Perhaps contributing to this difference is the fact that TH protein levels may be regulated differently than VMAT2, and also that TH enzyme activity is related to posttranslational modifications such as phosphorylation.34,35 Different methods (eg, the same microscopic fields were not sampled with both antibodies) and issues related to variability of the stability of antigens in tissue might also have contributed to the differences in immunoreactivity for TH and VMAT2.
A potential weakness of this study is that the 3 groups were obtained from archival tissue collections with varying storage time. Storage times were significantly longer in the iLBD group than in the PD and control groups, but this is unlikely to be a significant problem because storage time did not correlate with TH or VMAT2 immunoreactivities (TH, \( r = 0.04 \); VMAT2, \( r = 0.15 \)).

The method used to assess neuronal loss in the substantia nigra was not ideal but was necessary because of the retrospective nature of the study. Unbiased stereologic methods would have allowed more accurate estimates of neuronal loss, but this method is not possible with archival tissue samples. If performed under optimal conditions (consistent angle of sectioning and consistent section thickness), neuron counts on single sections of the SN have been shown to accurately reflect values obtained with stereologic methods.\(^3\) For a subset of the brains in the control and PD groups that were processed in the Jacksonville brain bank, a standardized section of midbrain was taken at the level of the third cranial nerve, which gives a consistent sampling of the vulnerable neuronal group in the ventrolateral tier of the pars compacta. For the other controls and PD cases and all of the iLBD cases obtained from the Mayo Clinic Tissue Registry, only a more rostral or caudal section of midbrain was available; consequently, 2 iLBD cases, 2 PD cases, and 1 control were not included in analyses of correlations between neuronal loss scores and striatal dopaminergic markers.

A random sampling scheme was used to assess TH and VMAT2 immunoreactivity in the putamen. Although unbiased stereologic methods might have been preferable, we were unable to use this technique because of lack of anatomically precise boundaries. A single standardized section of the putamen at the level of the anterior commissure was examined, whereas stereologic methods would have required sampling throughout the entirety of the putamen with serial sectioning.\(^3\) No frozen tissue was available for the iLBD group and some of the controls, so it was impossible to measure neurotransmitter levels in tissue, which would be important confirmation that the enzyme and transporter immunoreactivities reflect actual dopaminergic deficiencies.\(^2\)

These results give further credence to the presumption that iLBD reflects an integral component of PD (and possibly DLB) neurodegeneration. Until recently, iLBD cases have been overlooked in the epidemiologic search for potential pathogenic factors. Because iLBD cases are severalfold more prevalent than either PD or DLB, this suggests that pathogenic clues may be found in broader arenas and that epidemiologic studies extending to these iLBD cases may be insightful.

CONCLUSIONS

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