

Lewy pathology is not the first sign of degeneration in vulnerable neurons in Parkinson disease



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ABSTRACT

Objective: To determine whether evidence of neuronal dysfunction or demise preceded deposition of Lewy pathology in vulnerable neurons in Parkinson disease (PD).

Methods: We examined the extent of nigral dysfunction and degeneration among 63 normal, incidental Lewy body disease (ILBD), and PD cases based on tyrosine hydroxylase (TH) immunoreactivity and neuron densities, respectively. The relationship between these markers and Lewy pathology (LP) burden in the substantia nigra (SN) and Braak PD stage was assessed.

Results: Compared with normal subjects, ILBD cases displayed a significantly higher percentage of TH-negative cells and lower neuronal densities in the SN as early as Braak PD stages 1 and 2, before LP deposition in the nigrostriatal system. ILBD nigral neuron densities were intermediate between normal subjects and PD cases, and TH-negative percentages were higher in ILBD than either normal or PD cases. Furthermore, neuron density and neuronal dysfunction levels remained relatively constant across Braak PD stages in ILBD.

Conclusions: These results suggest that significant neurodegeneration and cellular dysfunction precede LP in the SN, challenging the pathogenic role of LP in PD and the assumption that ILBD always represents preclinical PD. *Neurology*® 2012;79:2307-2314

GLOSSARY

ABC = avidin-biotin-peroxidase; **aSyn** = α -synuclein; **DL** = dorsolateral; **DM** = dorsomedial; **HAAS** = Honolulu-Asia Aging Study; **ILBD** = incidental Lewy body disease; **LB** = Lewy bodies; **LN** = Lewy neurites; **LP** = Lewy pathology; **PD** = Parkinson disease; **SN** = substantia nigra; **TH** = tyrosine hydroxylase; **THN** = tyrosine hydroxylase negative; **THP** = tyrosine hydroxylase positive; **VL** = ventrolateral; **VM** = ventromedial.

Parkinson disease (PD) is a neurodegenerative disorder characterized by motor impairment including tremor, bradykinesia or rigidity, and cell loss in the substantia nigra (SN) pars compacta, most severely in the ventrolateral tier.^{1,2} α -Synuclein (aSyn) aggregates comprising Lewy bodies (LB) and Lewy neurites (LN), collectively referred to as Lewy pathology (LP), are required for the postmortem diagnosis of definite PD³ and are considered a precursor for neuronal degeneration.⁴ However, some authors have suggested that LP may be protective or an epiphenomenon rather than deleterious to neurons,⁵ although there is little evidence to date for cell dysfunction or loss unrelated to LP in PD.

The SN is considered particularly vulnerable to LP-induced neurodegeneration,⁶ and Braak proposed a staging system whereby LP deposition follows a nonrandom pattern of progression based on selective vulnerability and connectivity, involving the SN in Braak PD stage 3.^{7,8} LP is also found in the brains of 10% to 30% of aged subjects without parkinsonism in a condition known as incidental Lewy body disease (ILBD).^{9,10} Because incidental pathology affects

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Supplemental Data



CME



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approximately the same selectively vulnerable neuronal populations as PD pathology and nigrostriatal degeneration in these subjects is intermediate between that of controls and PD,^{11–14} it has been proposed that ILBD represents a premotor stage of PD. However, a clear understanding of the relationship among LP, neuronal dysfunction, and cell loss has yet to be elucidated in ILBD. If ILBD is a precursor to PD, some ILBD nigral neurons might display signs of dysfunction, such as diminished production of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis and a validated marker for dopaminergic neuronal integrity.^{11,13,15,16}

Examination of cases of ILBD with early pre-nigral (Braak LB stages 1 and 2) pathology should identify whether any pathologic features occur within the nigrostriatal system before LP deposition. Herein, we examine the extent of dopaminergic cell dysfunction and loss respectively based on TH immunoreactivity and neuron densities in the SN of normal, ILBD, and PD cases. These measures were analyzed in the context of Braak PD stage and nigral aSyn burden to explore the relationship between LP and SN neuronal dysfunction and loss.

METHODS Subjects and materials. The Honolulu-Asia Aging Study (HAAS)¹⁷ is a longitudinal prospective study of risk factors for the development of PD and dementia in a large cohort of Japanese-American men born between 1900 and 1919. The study is approved by the Kuakini Medical Center Institutional Review Board and participants signed informed consents at all examinations. All study participants were screened for parkinsonism during a structured interview. Those with a history or signs of parkinsonism were referred to a study neurologist who administered standardized questions about symptoms and the onset of parkinsonism, previous diagnoses, and medication use, followed by a comprehensive and standardized neurologic examination. A final diagnosis of PD was by consensus of 2 neurologists according to published criteria.¹⁸ Further description of the diagnosis of PD is described elsewhere.^{19,20} The HAAS provided 10- μ m-thick formalin-fixed, paraffin-embedded sections from 325 subjects who had sections available from sufficient anatomical regions to allow Braak LB staging. These sections were immunohistochemically stained for aSyn using a protocol described previously²¹ and cases with ILBD were identified. Of these cases, a convenience sample of 63 cases with sufficient nigral sections to allow analyses was chosen including all available cases of PD.

Briefly, slides were deparaffinized, rehydrated in graded ethanols, and endogenous peroxidase activity was quenched in a 30% H₂O₂/methanol bath for 30 minutes. Slides were then transferred to an antigen retriever (Antigen Retriever 2100, catalog #62700; EMS, Hatfield, PA) with commercial buffer (R-Buffer U, catalog #62706; EMS) overnight. The next day, the slides were washed, immersed in 88% formic acid for 30 minutes, and blocked with

2% diluted goat serum. The sections were immunostained with monoclonal antibodies against either oxidized aSyn (Syn 303, 1:16,000; generously donated by Virginia Lee et al.),²² or TH (1:1,000; Pel-Freez, Rogers, AR) overnight at 4°C. The next day, a species-specific secondary antibody (Vector, Burlington, CA) was applied. After an hour incubation, the sections were processed using the avidin-biotin-peroxidase (ABC) method with a Vectastain ABC Kit (Peroxidase Standard; Vector), and diaminobenzidine as chromogen.

Braak staging. A trained histotechnologist (J.V.N.), blinded to the diagnosis, analyzed the presence of immunostaining for aSyn in 15 brain regions, including the olfactory bulb, medulla, pons, midbrain, hippocampus, amygdala, striatum at the level of the nucleus accumbens, basal forebrain, and 7 neocortical regions (midfrontal, anterior superior and midtemporal, inferior parietal, calcarine, and primary motor/sensory gyri). Appropriate foci for Braak staging were evaluated from each brain region, including at least 2 foci for each Braak stage,⁷ and were assigned a semi-quantitative density score regardless of LP morphology, as suggested previously.²³

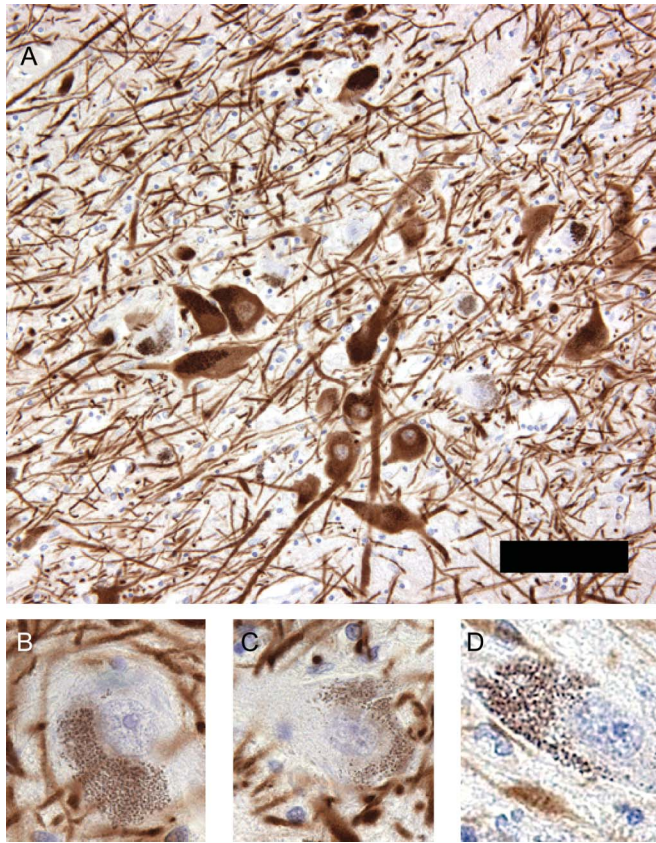
Braak stage was assigned for the highest stage that included LP in at least one of the relevant foci. For the subjects without a clinical history of PD or dementia with Lewy bodies, those with Braak LB stage 0 were considered normal controls and those with stages >0 were considered to have ILBD. In addition, a total LP burden score was assessed for each case by adding the density score for each area examined.

Neuron counting. Midbrain sections were immunostained for TH at the level of the red nucleus and the exit of the third nerve. All experiments included positive control sections to confirm uniform staining and no cases were devoid of TH-positive (THP) neurons. Using image analysis software (Stereo Investigator; MBF Biosciences, Williston, VT), the SN of each section was contoured using the cerebellar peduncles and red nucleus as ventral and dorsal boundaries, respectively. The left or right SN of each case was chosen randomly unless only one side was available or intact and exhaustively analyzed at 200 \times magnification using the Meander Scan feature of Stereo Investigator for pigmented THP neurons and TH-negative (THN) neurons (figure 1). A dopaminergic neuron was only counted if it contained black granular neuromelanin pigment and a distinct nucleus and nucleolus. Previous studies have confirmed neuromelanin's validity in identifying dopaminergic neurons.²⁴ The density of THN and THP neurons (neurons/mm²) as well as the THN percentage of total dopaminergic neuron count (THN%) were calculated.

Each SN contour was further sectioned into 4 quadrants (ventromedial, VM; ventrolateral, VL; dorsomedial, DM; and dorsolateral, DL) using image editing software (Adobe Illustrator CS4; Adobe Systems Inc., San Jose, CA). In accordance with methods used previously by Ross et al.,¹² a primary line was drawn from the maximal medial-to-lateral extent of the nucleus and curved to fit its shape to segment the ventral and dorsal halves of the SN. A perpendicular secondary line was drawn at the primary line's midpoint to segment the lateral and medial halves of the SN. Markers of THN and THP neurons were then quantified per quadrant. Quadrants with zero total dopaminergic neuron counts were excluded from THN% calculations.

Statistical analysis. Statistical calculations were performed using SPSS Statistics 18 (IBM Corp., Somers, NY) and graphed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Standard 1-way analysis of variance was conducted on group means from parametric data, whereas Kruskal-Wallis tests were conducted on

Figure 1 Tyrosine hydroxylase (TH)-positive and TH-negative neurons in the substantia nigra



(A) Low-power magnification of the substantia nigra immunostained for TH. In addition to typical TH-positive pigmented neurons, several TH-negative pigmented neurons, which are typical in incidental Lewy body disease, can be seen at low-power magnification and higher-power magnification (B-D). Scale bar = 30 μm (A), 10 μm (B-D).

group medians from nonparametric data. Pairwise comparisons between ≥ 3 groups were conducted on group medians using Mood's median tests. Two-tailed p values are adjusted for multiple comparisons when appropriate and reported with p values ≤ 0.05 considered significant.

Standard protocol approvals, registrations, and patient consents.

The study was approved by the Philadelphia Veterans Affairs Medical Center Institutional Review Board. Informed consent was obtained by the HAAS. There are no recognizable persons in the study.

RESULTS Of the 63 cases examined, 17 were normal, 33 were ILBD cases, and 13 were PD cases with a mean disease duration of 8.3 years (table e-1 on the *Neurology*[®] Web site at www.neurology.org). Interestingly, ILBD cases with Braak PD stages 5 and 6 had significantly lower total LP burden than PD cases with Braak PD stages 5 and 6 ($p = 0.004$; figure 2). There was no significant difference in the mean age at death between groups ($p > 0.887$), but PD brains had a higher mean Braak PD stage ($p < 0.001$; table e-1).

Analysis of nigral neuron density and dysfunction.

Total density and THN% assessment was reliable in a test-retest setting (intraclass correlation

coefficient = 0.902, $p < 0.001$; intraclass correlation coefficient = 0.730, $p = 0.001$) separated by approximately 1 year. Also, our neuron densities correlated well with prior estimations by Ross et al.¹² in the same cases ($\rho = 0.758$, $p < 0.001$). THN neurons were found in 12 of 17 normal subjects (71%), 10 of 13 cases with PD (77%), and 33 of 34 cases with ILBD (97%). Mean SN area was only statistically higher in ILBD compared with PD ($p = 0.013$) (tables 1 and e-2).

Age at death did not correlate with SN neuron density or THN% in any quadrant (whole SN: $p = 0.345$, $p = 0.981$; each quadrant: $p > 0.168$); however, the limited range in ages of the subjects may limit our ability to identify subtle correlations. There was a negative trend between PD duration and total neuron density, strongest in VL ($r^2 = 0.580$, $p = 0.004$). THN% showed no relationship with disease duration in PD cases ($p = 0.387$).

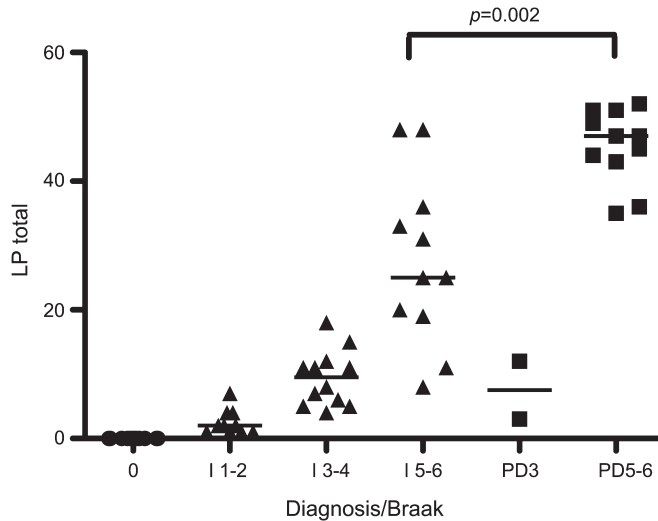
Regarding neuron density in the SN, ILBD was intermediate between normal ($p = 0.001$) and PD ($p = 0.037$) (tables 1 and e-2; figure 3A). Median neuron density in ILBD was 39.8% lower than that of normal ($p = 0.001$), whereas PD was 66.7% lower than normal ($p < 0.001$). For THN% (figure 3B), ILBD was higher than both PD ($p = 0.005$) and normal ($p < 0.001$). Additionally, PD had significantly higher THN% than normal ($p = 0.025$) as shown previously.¹⁵

SN quadrant analysis. ILBD was consistently intermediate in neuron density between normal and PD (figure e-1A) and exhibited the highest THN% in all quadrants (figure e-1B). Of the 4 quadrants, VL had the lowest neuron density in ILBD relative to normal ($p = 0.004$), and in PD relative to ILBD ($p < 0.001$) (tables 1 and e-2). Between normal and PD, VL had the largest difference ($p < 0.001$), whereas DL had the smallest difference ($p = 0.087$). Regarding THN%, VL had the highest in ILBD relative to normal ($p < 0.001$), and in PD relative to normal ($p = 0.014$). DM and DL showed no significant changes in THN% across diagnoses (table e-2).

Lower neuron densities in ILBD relative to normal were observed with similar percent differences in all SN quadrants ($p = 0.207$; figure e-1A). In contrast, VL was disproportionately affected in ILBD with a higher THN% relative to dorsal quadrants ($p < 0.001$) and VM ($p = 0.039$; figure e-1B). In PD, VL exhibited the highest THN% although there were no significant differences between quadrants ($p = 0.352$).

Analysis of Lewy pathology. Nigral neuron density was observed to decrease as aSyn burden became more severe ($\rho = 0.401$, $p = 0.007$; figure 4A). In contrast, nigral THN% was not associated with aSyn burden (figure 4B).

Figure 2 Relationship between Lewy pathology burden and disease state by Braak PD stage



Lewy pathology burden scores (LP total) were derived by summing semiquantitative scores from 16 foci throughout the brain (table e-1). Median LP total scores were compared between normal (0), incidental Lewy body disease (ILBD) with Braak Parkinson disease (PD) stages 1 and 2 (1 1-2), 3 and 4 (1 3-4), and 5 and 6 (1 5-6), as well as PD with Braak PD 3 (PD3) and 5 and 6 (PD5-6). Note that median LP total in ILBD with Braak PD stages 5 and 6 was significantly lower than that in PD with Braak PD stages 5 and 6. The circles represent normal subjects, the triangles ILBD, and the squares PD. Horizontal bars indicate median values.

When total neuron density was analyzed across Braak PD stages in ILBD, Braak PD stages 1–6 showed density levels similar to one another but significantly lower than normal and higher than

PD (figure 4C). Likewise, there was no significant difference in THN% between Braak stages in ILBD (figure 4D), although all ILBD groups were significantly higher than normal.

DISCUSSION To date, the relationship between ILBD and PD has been unclear. It has been hypothesized that ILBD represents an intermediary step between normal aging and PD, offering a unique window to observe the pathophysiology of premotor disease processes. However, the presence of asymptomatic subjects with high LP burdens and the fact that the majority of patients described to date with ILBD are older than the median age at onset of PD,²⁵ suggest that the relationship may be more complex.

The findings of this report provide some evidence that ILBD may be an intermediary between normal and PD. In accord with previous estimations,^{16,24,26,27} nigral neuronal density in PD was 67.5% less than normal, and ILBD was intermediate between normal and PD values, as has been reported previously.¹² Overall, our SN neuron densities are lower than previously reported,¹² which is almost certainly an artifact of our strict counting criteria.

However, the observation that nigral neuronal density seems to be independent of the regional distribution of LP deposition in ILBD supports the notion that at least some ILBD cases may represent a process distinct from PD. It is interesting that ILBD subjects

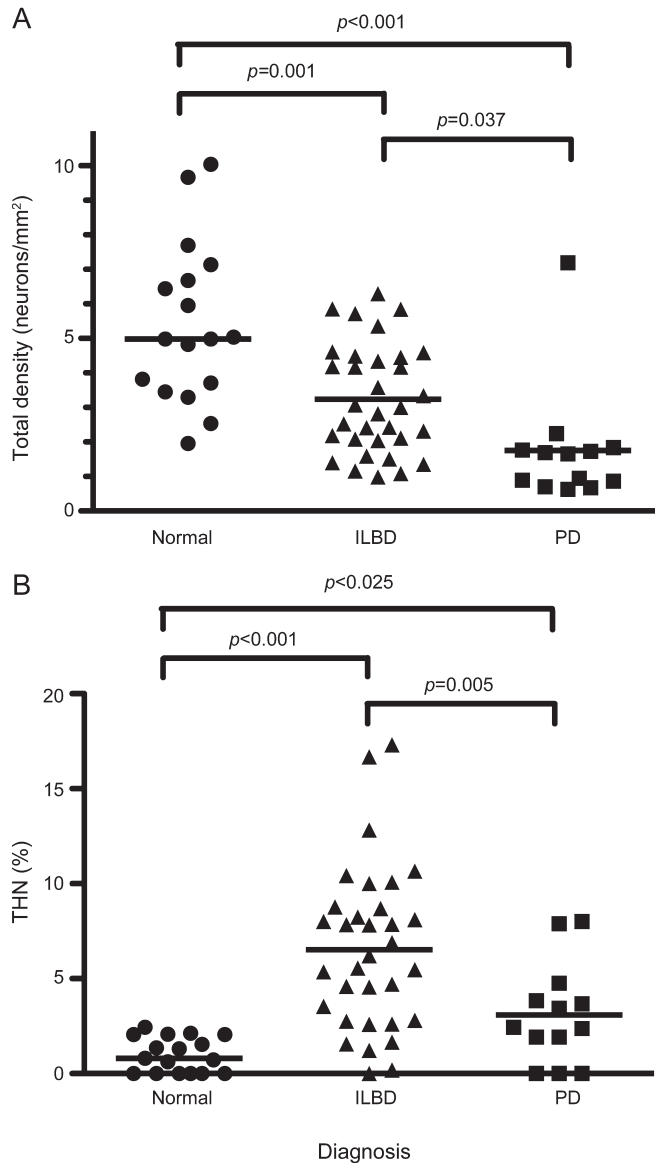
Table 1 Summary of mean pathologic results^a

		Normal (n = 17)	ILBD (n = 33)	PD (n = 13)
Total SN	SN area (mm ²)	29.70 ± 2.23	34.94 ± 1.41	26.88 ± 2.17
	Neuron count	148.53 ± 11.69	110.48 ± 9.44	41.54 ± 8.52
	Neuron density	5.42 ± 0.56	3.24 ± 0.27	1.76 ± 0.48
	THN count	1.65 ± 0.34	7.21 ± 0.86	1.31 ± 0.38
	THN%	1.01 ± 0.22	6.53 ± 0.74	3.10 ± 0.73
Ventral SN	Neuron density	5.74 ± 0.77	3.60 ± 0.35	1.48 ± 0.45
	THN%	1.46 ± 0.41	10.47 ± 1.24	6.20 ± 2.04
VM	Neuron density	5.30 ± 0.71	3.76 ± 0.46	1.77 ± 0.55
	THN%	1.12 ± 0.64	7.89 ± 1.75	5.33 ± 2.81
VL	Neuron density	6.24 ± 0.98	3.54 ± 0.41	1.07 ± 0.38
	THN%	1.61 ± 0.64	14.50 ± 2.17	9.48 ± 4.68
Dorsal SN	Neuron density	5.39 ± 0.87	2.86 ± 0.28	2.02 ± 0.54
	THN%	1.10 ± 0.47	3.47 ± 0.78	1.87 ± 0.88
DM	Neuron density	6.17 ± 0.91	3.69 ± 0.41	2.12 ± 0.43
	THN%	0.74 ± 0.39	2.86 ± 0.91	0.64 ± 0.64
DL	Neuron density	4.53 ± 0.94	1.98 ± 0.19	1.86 ± 0.79
	THN%	1.38 ± 0.64	4.87 ± 1.35	3.41 ± 2.05

Abbreviations: DL = dorsolateral; DM = dorsomedial; ILBD = incidental Lewy body disease; PD = Parkinson disease; SN = substantia nigra; THN = tyrosine hydroxylase negative; VL = ventrolateral; VM = ventromedial.

^aData are presented as means and standard errors. Neuronal density = number of neurons per area/regional area; THN% = number of THN neurons/total number of neurons per area.

Figure 3 Nigral neuron density and percentage of tyrosine hydroxylase-negative (THN) neurons between diagnostic groups



(A) Neuron density (total density) of pigmented neurons and (B) percentage of THN neurons to total neuron count (THN%) in the substantia nigra of normal, incidental Lewy body disease (ILBD), and Parkinson disease (PD) cases. Circles represent normal subjects, triangles represent ILBD cases, and squares represent PD cases. Horizontal bars indicate median values.

with Braak PD stages 5 and 6 had lower total LP burden than PD patients with similar Braak PD stages 5 and 6, as a possible explanation for why some patients with equivalent distributions of pathology do not exhibit the same clinical phenotype. Because a previous report found the opposite relationship, with ILBD cases with widespread LP distribution having higher total LP burden than respective PD cases, further investigation is needed to resolve this discrepancy.

Further evidence that ILBD may not be merely premotor PD arises from an examination of SN subregions. In PD, SN cell loss mirrored previously observed regional patterns,^{24,26} with the VL quadrant experiencing the most severe difference in cell density

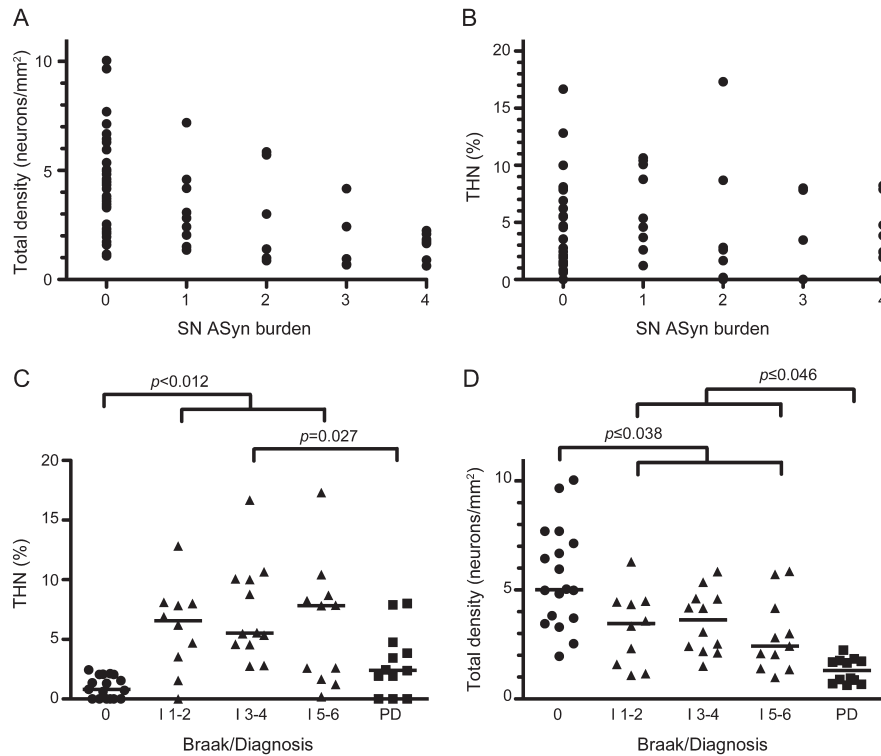
between normal and PD. However, all quadrants were equally affected by changes in cell density between normal and ILBD, suggesting that the pathogenic process subserving cell loss in ILBD does not discriminate among SN subregions. This symmetric cell loss is further evident by the profound difference in nigral neuron density between Braak PD stage 0 and 1/2 (figure 4C). In contrast to neuron density, however, disruption of TH production predominates in the neurons of the VL quadrant in ILBD as well as in PD (figure e-1B).

Interestingly, pre-nigral ILBD cases with Braak PD stages 1 and 2 already demonstrate neuronal dysfunction and loss in the nigrostriatal system (figure 4C). To our knowledge, this is the first time that neurodegeneration was demonstrated in a selectively vulnerable population of neurons before aSyn deposition in those neurons, and it raises serious questions about LP pathogenicity. It is also interesting that although SN neuronal dysfunction and demise were evident in the absence of microscopically detectable LP in some cases, neuronal densities did linearly decline as nigral aSyn burden increased. However, THN% did not associate with nigral aSyn burden, which further suggests that LP is not the only cause of nigral neuron loss.

Compared with PD cases, THN% is also increased in ILBD, suggesting that these dysfunctional neurons, perhaps struggling to survive in ILBD, have died by end-stage PD. This possibility is supported by the observation of lower nigral neuron density in PD compared with all Braak PD stages of ILBD. At this point, it is impossible to say whether the remaining THN neurons in PD are in the process of degeneration and will eventually die, but the lack of TH production suggests that they represent a component of the neurons responsible for clinical parkinsonism. However, it should be recognized that all THN neurons in ILBD and PD also represent a target for neurorestorative therapies and that these therapies should be initiated during the premotor stage to be most successful.

If cell loss precedes LP accumulation in the SN, which our data suggest, what is the cellular insult that causes cell dysfunction and death? Many in vitro studies have implicated the LB precursors, protofibrillar soluble oligomers that are undetectable at the light microscopic level, as the culprits directly responsible for cell death, and this remains plausible.^{28,29} Additionally, previous reports support the notion that aSyn aggregation begins in the axonal compartment as LN and progresses toward the cell soma in a retrograde manner.^{7,22,30,31} However, the ILBD Braak PD stage 1/2 cases reported herein were examined for the presence of LN in the striatum as described previously²² and none were detected (data not shown). A possible mechanism for this discrepancy is presynaptic LP, as reported in several recent articles,^{32,33} which can be demonstrated in areas of the brain in the absence of

Figure 4 Relationship between Lewy pathology and nigral neuron density and dysfunction



The relationship between Lewy pathology and nigral neuron dysfunction and density was assessed by examining α -synuclein (aSyn) pathology within the substantia nigra (SN) (A and B) and throughout the brain (C and D). The burden of aSyn pathology in the SN was assessed as described previously²³ with semiquantitative scores (0 = none; 1 = trace; 2 = mild; 3 = moderate; 4 = severe) in all cases. (A) Neuron density (total density) of pigmented neurons decreased in a linear manner ($p = 0.498$; $p < 0.001$) as SN aSyn burden increased. (B) There was no significant linear association between the percent of tyrosine hydroxylase-negative (THN%) neurons and SN aSyn burden in all cases. Lewy pathology was also assessed by assigning Braak Parkinson disease (PD) stages to each case (see Methods) and grouping cases into those with preclinical pathology (Braak PD stages 1 and 2), precortical pathology (Braak PD stages 3 and 4), and diffuse pathology (incidental Lewy body disease [ILBD] Braak stages 5 and 6). (C) Total nigral neuron density in the SN of normal subjects (Braak 0) was significantly increased compared with all groups of ILBD as well as PD. Similarly, all groups of ILBD had mean neuronal densities higher than PD. (D) Regarding the relationship between Braak PD stage and THN%, all groups of ILBD had significantly higher mean percents than normal subjects, and ILBD Braak PD stages 3 and 4 were significantly higher than PD. In all figures, circles represent normal subjects, triangles represent ILBD cases, and squares represent PD cases. Only significant comparisons are shown. Horizontal bars indicate median values.

LN or LB (Schulz-Schaeffer, personal communication, 2011). In addition, localized LP deposition might engender systemic activation of innate inflammatory responses in resident microglia and astrocytes that might affect vulnerable neurons elsewhere.

There are several limitations in this study that should be acknowledged. As with all human neuropathologic examinations, each individual is characterized by a “snapshot” in time and it is impossible to determine the effect of this on the associations observed. The comprehensive examinations involved in the HAAS cohort are conducted at approximately 2- to 3-year intervals, so some mild symptoms of parkinsonism could have been missed in a proportion of subjects, although PD incidence in the HAAS cohort is similar to comparable population-based prevalence data. Importantly, the significance of many of these observations is independent of whether or not some of the ILBD cases were actually

early clinical PD, because it is the absence of LP in the SN that is important, regardless of whether the parkinsonian phenotype was evident.

In addition, these studies are limited because the HAAS cohort is all male and of Japanese ancestry. In addition, although single-section counts of the SN have been shown to strongly relate to stereologic methods,³⁴ it is possible that the counting protocols used do not accurately reflect the density of neurons in SN subregions. Unfortunately, rigorous stereologic assessment was not possible in HAAS tissues.

We have attempted to understand the relationships among neuronal dysfunction, neuronal loss, and aSyn pathology in what many consider the premotor phase of PD. In summary, ILBD is characterized by dopaminergic cell dysfunction and intermediate cell loss in the SN despite the absence of clinical parkinsonism. Although these individuals may have eventually

developed PD if they had lived longer, an alternate explanation is that variation in other factors such as concomitant inflammatory responses or baseline differences in reserve make some forms of ILBD distinct from PD. These results suggest that there may be a dissociation among aSyn accumulation, cell loss, and cell dysfunction, as has been seen in other neurodegenerative disorders, including Huntington disease.³⁵ As these and other processes involved in neurodegeneration begin to unfold, novel in vivo diagnostic options and treatment targets will hopefully be introduced that will lead to earlier diagnosis and intervention in these patients.

AUTHOR CONTRIBUTIONS

Conception and design (J.M.M., J.E.D., G.W.R.), data collection (J.M.M., J.V.N., G.W.R., J.E.D.), data analysis (J.M.M., J.E.D., J.F.M.), drafting of manuscript (J.M.M., J.E.D., J.F.M.), and editing of manuscript (all authors).

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