disorders were staged according to Braak [16] as well as the Dementia with Lewy Bodies Consortium [67]. Apolipoprotein E genotyping was done on postmortem brain tissue using a standard technique [45].

Histologic methods

Diagnostic histologic methods were performed on standard blocks of tissue that were fixed in 4% buffered formaldehyde and then either dehydrated and embedded in paraffin or cryoprotected and cut on a freezing, sliding microtome. Paraffin sections from the olfactory bulb and tract, anterior medulla (two levels anterior to the obex), anterior and mid-pons, mid-amygdala with adjacent transentorhinal area, anterior cingulate gyrus (1–3 cm posterior to the coronal slice containing the genu of the corpus callosum), middle temporal gyrus (at the level of the lateral geniculate nucleus), middle frontal gyrus (4–5 cm posterior to the frontal pole), and inferior parietal lobule were stained immunohistochemically for α-synuclein using a polyclonal antibody raised against an α-synuclein peptide fragment phosphorylated at serine 129, after epitope exposure with proteinase K. The process leading to the choice of immunohistochemical method, as well as details of the method, have been described in a previous publication [6]. The density of α-synuclein-immunoreactive Lewy bodies and neurites in each of the aforementioned brain regions was scored, for more than 90% of slides, by a single observer (TGB), without knowledge of diagnosis, as none, sparse, moderate, frequent and very frequent, using the templates provided by the Dementia with Lewy Bodies Consortium [67]. The remaining slides were scored by trainees under the instruction of the primary observer. For the substantia nigra (SN), LTS was estimated using the same scoring method but on thioflavine-S-stained thick (40 μm) sections due to the standard laboratory practice of sectioning the SN in this manner for unbiased morphometric analysis.

Assessment of nigrostriatal system degeneration

The degree of nigrostriatal degeneration was assessed histologically and biochemically. Histologic evaluation of SN pigmented neuron loss was graded as none, mild, moderate or severe on H & E-stained microscopic sections taken at or close to the level of egress of the oculomotor nerve. The descriptive terms were converted to numerical scores from zero to three for statistical purposes.

Biochemical evaluation consisted of an enzyme-linked immunoassay (ELISA) for tyrosine hydroxylase (TH), the rate-limiting enzyme for the production of dopamine. The dissected sample was taken from frozen putamen at the level of the globus pallidus pars interna. This site has been reported to show the earliest loss of dopaminergic markers in PD [8]. The ELISA method has been described in a prior publication [4].

Statistical analysis

For statistical purposes, semi-quantitative microscopic lesion density estimates were converted to numerical scores from zero to three for CERAD neuritic plaque density, zero to four for LTS (DLB Consortium III templates) and zero to six for Braak neurofibrillary stage. For correlational analyses, the α-synuclein-immunoreactive density scores of the ten evaluated brain regions were summed to give a single global score for each subject.

Statistical analyses consisted, for comparing group means, analysis of variance (ANOVA), or, for non-parametric data, Kruskall–Wallis ANOVA. Proportional measures were compared using chi-square tests. Correlations were performed using linear regression for continuous variables and Spearman's rank correlation for discontinuous variables.

Results

Basic characteristics of the study subjects

Four-hundred and seventeen subjects had a full set of brain paraffin blocks (all ten regions specified in the methods section) as well as neuropathological diagnoses within the targeted groups. Descriptive measures of these groups are given in Tables 1, 2, 3. The subjects were all of advanced age (Table 1) and the group means differed significantly ($P < 0.0001$), with the youngest group (PD) having a mean age of 79.2 while the oldest group (ILBD) mean was 86.6. The diagnostic groups differed significantly ($P = 0.003$) in terms of disease duration (from first onset of symptoms until death), with DLB subjects having the shortest duration, at 6.9 years, while PD subjects had the longest duration, at 10.6 years. Subjects with AD were in between, with ADNLB subjects having a shorter duration than ADLB subjects. Subjects with PD, DLB, and ILBD were more likely to be males (61–69% male) while normal subjects and subjects with AD (ADLB, ADNLB) were almost gender-neutral (46–51% male). Subjects with ADLB, ADNLB, and DLB were much more likely to have one or more apoE-ε4 alleles (53–55%), in comparison with normal subjects, subjects with PD and subjects with ILBD (26–30%). The median postmortem intervals were uniformly short, ranging from 2.66 to 3.08 h and the group means were not significantly different.

Basic cognitive (MMSE) and neuromotor (Part 3 of the UPDRS) performance characteristics of the diagnostic groups are given in Table 2. The diagnostic groups differed

Table 1 General characteristics of the study subjects, by neuropathologic diagnosis, age, gender, apoE genotype and postmortem interval (PMI)

Diagnosis (*N*)	Age (SD)[a]	Disease duration, years (SD)[b]	Gender (%M)[c]	ApoE-ε4 (%)[d]	PMI, hours, median, mean (SD)[e]
Normal (87)	84.8 (6.9)	N/A	51.7	26.4	2.7, 3.3 (2.8)
ILBD (26)	86.6 (5.5)	N/A	61.5	30.0	2.9, 3.1 (1.6)
PD (66)	79.1 (6.9)	10.6 (8.7)	69.7	30.3	2.7, 3.8 (3.3)
DLB (40)	80.7 (6.6)	6.9 (4.7)	65.0	55.0	3.1, 4.5 (4.7)
ADLB (85)	83.4 (8.1)	9.0 (4.7)	50.6	55.3	3.0, 6.0 (8.9)
ADNLB (113)	82.8 (9.5)	7.3 (4.7)	46.0	53.9	3.0, 6.3 (11.4)

Means and standard deviations (SD) are given. Thirty-four of the DLB cases and 27 of the PD cases also met neuropathologic diagnostic criteria for AD

ILBD incidental Lewy body disease, *PD* Parkinson's disease, *DLB* dementia with Lewy bodies, *ADLB* Alzheimer's disease with Lewy bodies, *ADNLB* Alzheimer's disease with no Lewy bodies

[a] Group means were significantly different ($P < 0.0001$)

[b] Group means were significantly different ($P = 0.003$)

[c] Subjects with ADNLB differed in gender distribution from those with PD and DLB ($P < 0.01$ for the comparison with PD, $P < 0.05$ for the comparison with DLB); subjects with ADLB differed from those with PD ($P < 0.05$)

[d] Normal subjects and ILBD subjects differed from those with DLB, ADLB, and ADNLB in the proportion that were apoE-ε4 positive ($P < 0.01$ for comparisons with normal, $P < 0.05$ for comparisons with ILBD)

[e] Group means were not significantly different

Table 2 Cognitive and neuromotor screening test results for the study subjects, in terms of Mini Mental State Examination (MMSE) and motor scores (part 3) from the Unified Parkinson's Disease Rating Scale (UPDRS)

Diagnosis	MMSE (SD) N[a]	MMSE—months before death (SD)[b]	UPDRS (SD)[c]	UPDRS—months before death (SD)[d]
Normal	28.6 (1.5) 54	21.2 (16.7)	9.8 (9.7) 55	14.3 (11.3)
ILBD	27.8 (2.0) 15	16.7 (13.2)	7.8 (4.2) 18	15.8 (12.3)
PD	20.3 (8.7) 39	16.8 (13.8)	41.0 (22.5) 27	15.1 (17.1)
DLB	12.9 (8.9) 26	13.4 (14.7)	52.1 (26.0) 9	14.2 (9.2)
ADLB	9.9 (7.9) 54	15.0 (12.7)	31.5 (28.6) 19	22.3 (26.0)
ADNLB	11.1 (8.0) 68	15.1 (13.5)	28.0 (23.4) 25	24.4 (20.8)

The time elapsed since the last clinical testing dates are given. Note that only a subset of the subjects listed in Table 1 had data available. Means and standard deviations are given where noted

Abbreviations as given in Table 1

[a] Group means were significantly different ($P < 0.0001$)

[b] Group means were not significantly different

[c] Group means were significantly different ($P < 0.0001$)

[d] Group means were not significantly different

significantly in their mean MMSE scores ($P < 0.0001$). Normal subjects and subjects with ILBD had higher mean MMSE scores (28.6 and 27.8, respectively) than all other subjects while subjects with ADLB, ADNLB, and DLB had lower scores (9.9–12.9) than all other subjects. The diagnostic groups also differed significantly in their mean UPDRS scores ($P < 0.0001$). Normal subjects and subjects with ILBD had lower mean Part 3 UPDRS scores (9.8 and 7.8, respectively) than all other subjects while subjects with DLB had higher scores than all other subjects (mean score 52.1). Subjects with PD had higher UPDRS scores than normal subjects or subjects with ILBD. Subjects with AD had increased UPDRS scores compared with normal and ILBD subjects, but ADLB subjects (mean score 31.5) did not differ appreciably from ADNLB subjects (mean score 28.9).

The elapsed times, for each diagnostic group, between the final clinical testing dates and death were compared (Table 2). For MMSE, the interval ranged, in the diagnostic groups, from 13 to 21 months, but the group means were not significantly different. Similarly, for UPDRS, the interval between the final testing dates and death ranged from 14 to 24 months, but the group means were not significantly different.

Table 3 Neuropathological and neurochemical characteristics of the study subjects, by neuropathological diagnosis, CERAD neuritic plaque density, Braak neurofibrillary stage, substantia nigra (SN) pigmented neuron loss score and striatal tyrosine hydroxylase concentration (TH; ng/mg protein)

Diagnosis (*N*)	Neuritic plaque density (SD)[a]	Braak neurofibrillary stage (SD)[a]	SN pigmented neuron loss score (SD)[a]	Striatal TH (SD) N[a]
Normal (87)	1.26 (1.04)	2.77 (0.95)	0.39 (0.58)	90.35 (92.75) 31
ILBD (26)	1.01 (0.93)	2.85 (0.78)	0.72 (1.02)	45.86 (105.42) 13
PD (66)	1.29 (1.03)	2.86 (0.97)	2.71 (0.70)	10.55 (14.41) 17
DLB (40)	2.20 (0.97)	3.72 (0.85)	2.15 (0.81)	–
ADLB (85)	2.76 (0.47)	5.22 (0.85)	1.41 (0.96)	99.7 (85.3) 14
ADNLB (113)	2.70 (0.48)	4.71 (1.08)	1.04 (0.94)	75.53 (64.23) 13

Note that the lattermost was only available for a subset of the cases. For striatal TH concentrations, N for normal is 31, N for ILBD is 13, N for PD is 17, N for DLB is 2; N for ADLB is 14, N for ADNLB is 13. Abbreviations as given in Table 1

[a] Group means were significantly different ($P < 0.0001$)

Relevant neuropathological characteristics of the study subjects are given in Table 3. The group means for all measures differed significantly ($P < 0.0001$). In terms of AD-related lesions, normal subjects and subjects with ILBD and PD had lower neuritic plaque scores and lower Braak neurofibrillary stages than subjects with DLB, ADLB, and ADNLB. SN pigmented neuron loss was moderate to severe in PD and DLB. This was greater for PD subjects than for any other group. Subjects with ADLB had mild to moderate pigmented neuron loss, while subjects with ADNLB had only mild neuron loss, similar to that seen in normal and ILBD subjects. Subjects with PD had markedly decreased striatal TH concentrations as compared with normal subjects. For subjects with ILBD, TH was 50% depleted compared to normal subjects. Striatal TH correlated inversely with SN pigmented neuron loss score ($R = -0.44$, $P = 0.002$).

Regional analysis of Lewy-type LTS

Figure 1 shows photomicrographs of the immunohistochemical staining for α-synuclein in the brain regions examined. Generally, sections with positive staining contained fibers, dot-like structures and neuronal perikaryal staining (a, b, c, g, k, l, m, n, o, p, q, s), but occasionally sections or regions contained just fibers or dots (d, e, f, h, r, t) or just perikaryal staining. The perikaryal staining was either diffusely distributed in the cytoplasm (i, j) or condensed into defined inclusions (a, b, c, g, k, l, m, n, o, p, q, s), a subset of which resembled classical Lewy bodies (c, k). The amygdala (n, o, p) was often the most densely stained region, especially those nuclei that lie superficially along the ambient and semilunar gyri. The cerebral cortex sections, other than the cingulate gyrus (r, s, t), were the least likely regions to be heavily stained but occasionally could display relatively dense arrays of immunoreactive structures (r).

Figure 2 shows graphically the presence and density of Lewy bodies and related neurites in the ten selected brain areas and the four affected diagnostic groups (ILBD, PD, DLB, ADLB). The sub-regional distribution of LTS was not systematically examined but generally was consistent with that described previously in detail by others (10, 48, 59, 80, 85]. It can be immediately appreciated from the frequency graphs in Fig. 2a, c, e, g that subjects with ILBD and ADLB have one pattern of involvement while those with PD and DLB have a second pattern. In ILBD and ADLB, there are marked regional differences in the frequency of involvement (between 10 and 90%) while in PD and DLB all regions are frequently affected (70–90%). In both ILBD and ADLB, the regions most frequently affected are the olfactory bulb, medulla, pons, and amygdala. Closer examination shows three major differences between ILBD and ADLB. The olfactory bulb is a most-affected region in both conditions but is the single most affected region in ADLB. While the medulla is frequently affected in both groups, it is much less frequently affected in ADLB than in ILBD. In ILBD, the brainstem regions, as a whole, are more frequently affected than the limbic regions, while in ADLB it is the reverse.

The graphs depicting regional density scores (Fig. 2b, d, f, h), only for those regions with a score of 1 or greater, are generally similar to the frequency graphs in that scores are uniformly higher in the PD and DLB groups and lower in the ILBD and ADLB groups. Again, the olfactory bulb is more heavily involved in ADLB than in ILBD, and again, in ADLB the limbic areas are, as a whole, more heavily affected than the brainstem while in ILBD there is a rough equivalency.

In all regions and diagnostic groups, the neocortical regions other than cingulate gyrus have the lowest frequencies of involvement and the lowest density scores when affected. Generally, the temporal lobe section was

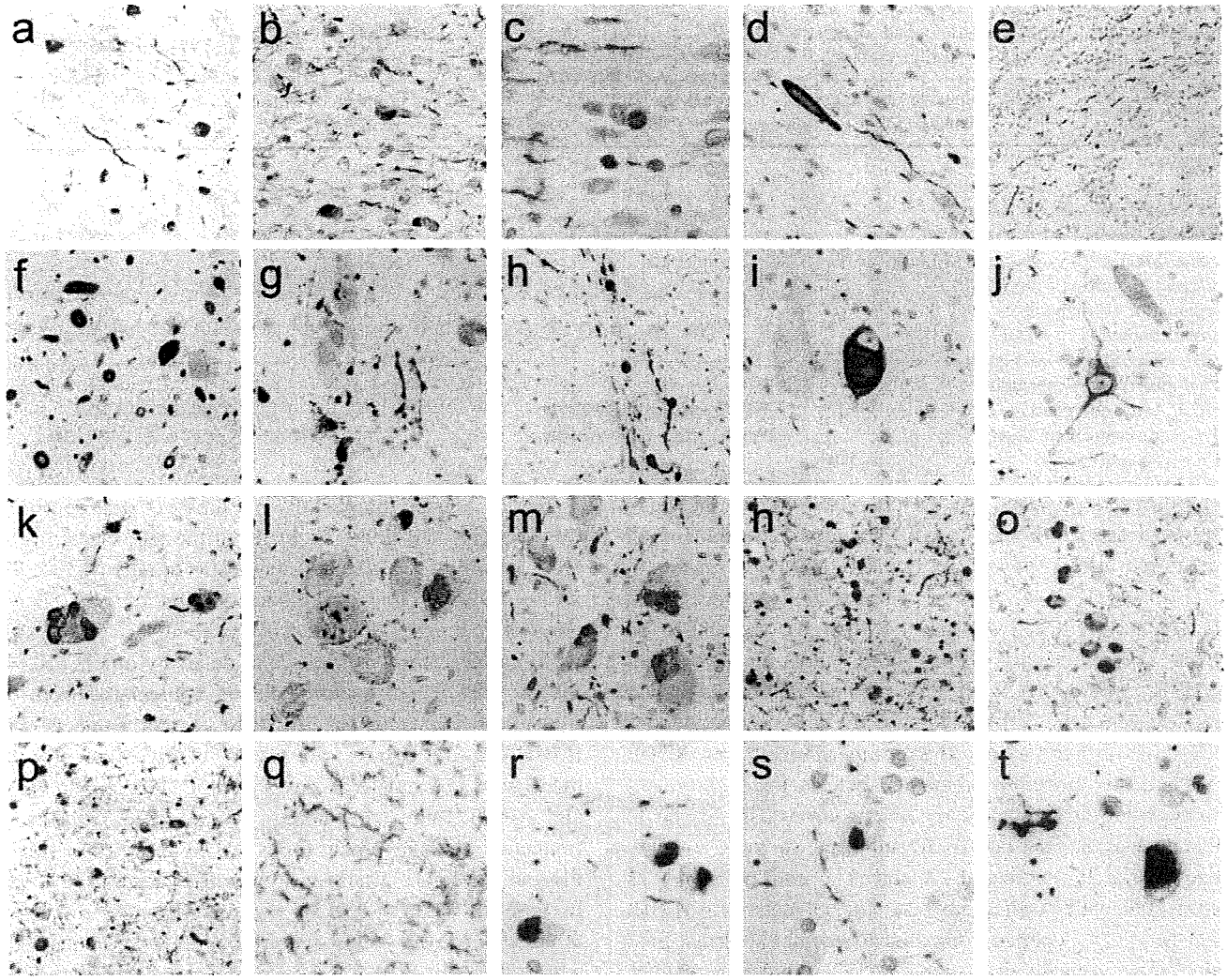

Fig. 1 Photomicrographs depicting immunohistochemical staining for α-synuclein in the brain regions investigated. Positive immunostaining is black; the counterstain is Neutral Red. (**a–e**) The olfactory bulb and tract. The anterior olfactory nucleus of the olfactory bulb is shown in (**a–c**); both neuronal perikaryal inclusions as well as fibers are present. An enlarged, abnormal neurite within the olfactory tract is shown in (**d**). Positive fibers coursing in parallel array through the internal plexiform layer are shown in (**e**). (**f–j**) The anterior medulla. The dorsal motor nucleus of the vagus nerve is shown in (**f**), the raphe nucleus in (**g**) and (**i**), the internal tract of the IXth nerve in h and the lateral reticular nucleus in (**j**). (**k–m**) The locus ceruleus in the pons. (**n–p**) The amgydala. (**q**) The cingulate gyrus. (**r**) The middle temporal gyrus. (**s**) The middle frontal gyrus. (**t**) The inferior parietal lobule

affected more frequently and had higher density scores than the frontal or parietal sections.

In an attempt to establish the brain region most likely to be initially involved by LTS, cases affected in only a single area were analyzed. The results are presented in Fig. 3. There were no cases of PD or DLB that involved only a single brain region; for the latter condition, the diagnostic criteria preclude this possibility. For both ILBD and ADLB, the olfactory bulb was the brain region most frequently involved when only a single brain region was affected. For ILBD subjects, the medulla and pons lagged only slightly behind the olfactory bulb while for ADLB subjects, it was rare for another brain region to be the sole focus.

Correlations of LTS density scores with nigrostriatal degeneration, UPDRS and MMSE

For these analyses, again only the subjects with LTS were included (ILBD, PD, DLB, ADLB). The global, sum-of-all-areas LTS density scores correlated significantly with both measures of nigrostriatal degeneration (correlations with striatal TH concentration shown in Fig. 4, correlations with SN pigmented neuron loss score in Fig. 5). The correlations were relatively strong and highly significant when all cases were considered together, regardless of diagnostic grouping (Figs. 4a, 5a) as well as when all cases were subdivided by the presence (Figs. 4f, 5g) or absence (Figs. 4e, 5f) of diagnostic levels of AD histopathology

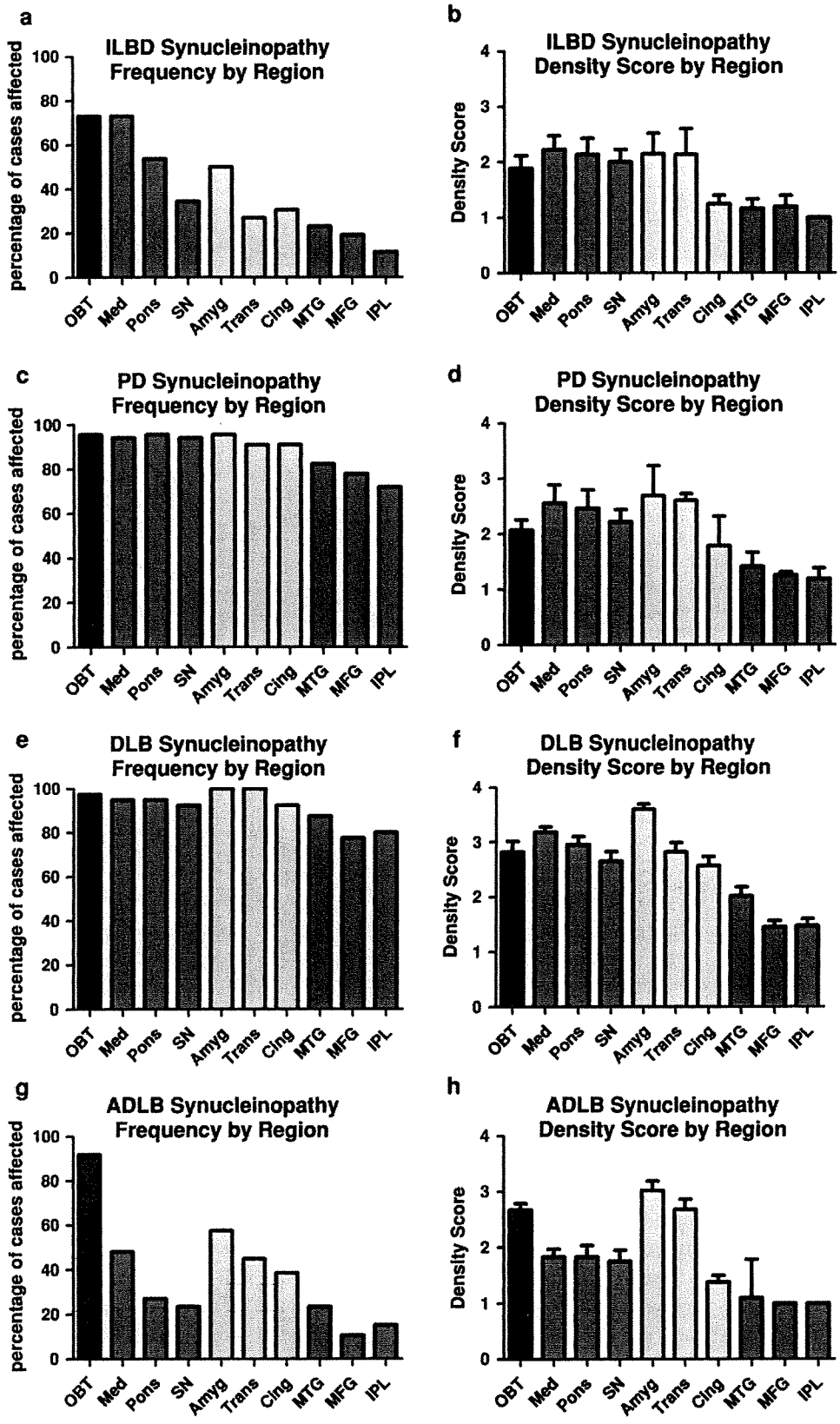

Fig. 2 Graphic depiction of Lewy-type synucleinopathy frequency (**a**, **c**, **e**, **g**) and density (**b**, **d**, **f**, **h**) by brain region. *Purple bars* are for olfactory bulb, *red* are for brainstem regions, *yellow* are for limbic regions and *green* are for neocortical regions. *Error bars* are standard error of the mean. Mean density scores differed significantly between regions for all diagnostic groups shown ($P < 0.001$). *OBT* olfactory bulb and tract, *Med* medulla, *SN* substantia nigra, *Amyg* amygdala, *Trans* transentorhinal cortex; *Cing* cingulate gyrus, *MTG* middle temporal gyrus, *MFG* middle frontal gyrus, *IPL* inferior parietal lobule

(NIA-Reagan intermediate or high probability). For the ILDB group, there was a trend for LTS density scores to inversely correlate ($R = -0.53$; $P = 0.06$) with striatal TH (Fig. 4b) while the correlation with SN pigmented neuron loss score showed the same trend (Fig. 5b; $P = 0.13$). For PD subjects, there was no significant correlation with

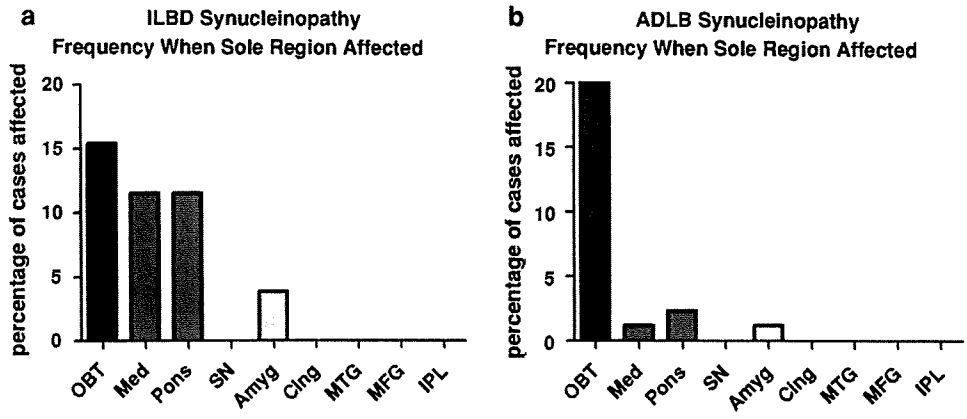

Fig. 3 Graphic depiction of the frequency with which brain regions were the sole region affected by Lewy-type synucleinopathy. (**a**) For ILBD subjects. (**b**) For ADLB subjects

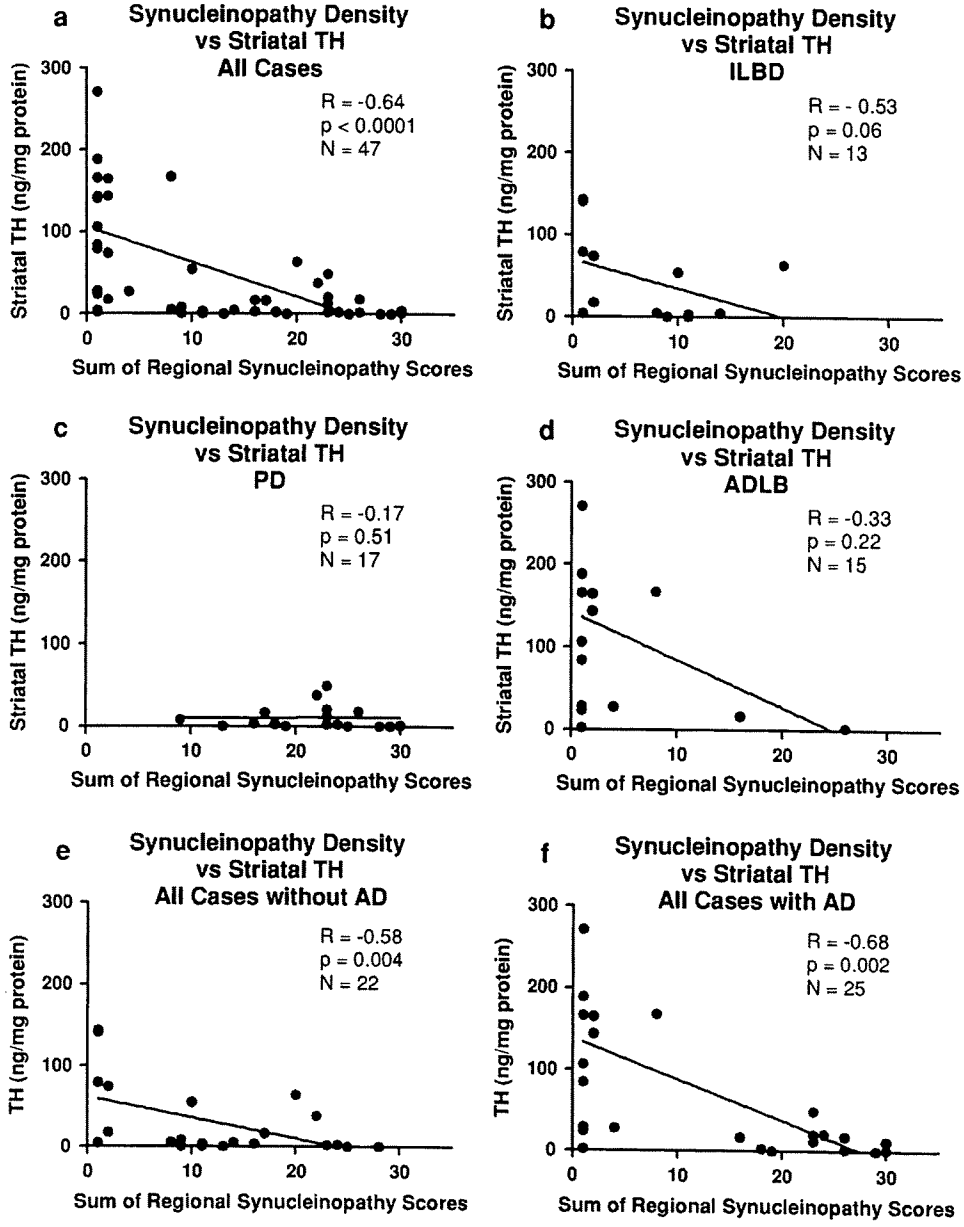

Fig. 4 Graphs depicting correlations between the sum of brain regional synucleinopathy density scores and measures of striatal tyrosine hydroxylase (TH), in subjects divided or not divided by diagnostic group and presence or absence of AD. As there were only two DLB subjects with TH measurements available, there is no graph for this category, but the two subjects are included in graphs (**a**) and (**f**). The graph titled "All Cases" included all subjects with Lewy body pathology and TH measurements available, regardless of diagnosis. The graph titled "with AD" included data from all subjects who also met NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. The graph titled "without AD" included data from all subjects who did not meet NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. *R* Spearman Rho, *N* number of subjects included in the analysis; *p* probability. Abbreviations otherwise as for Fig. 2

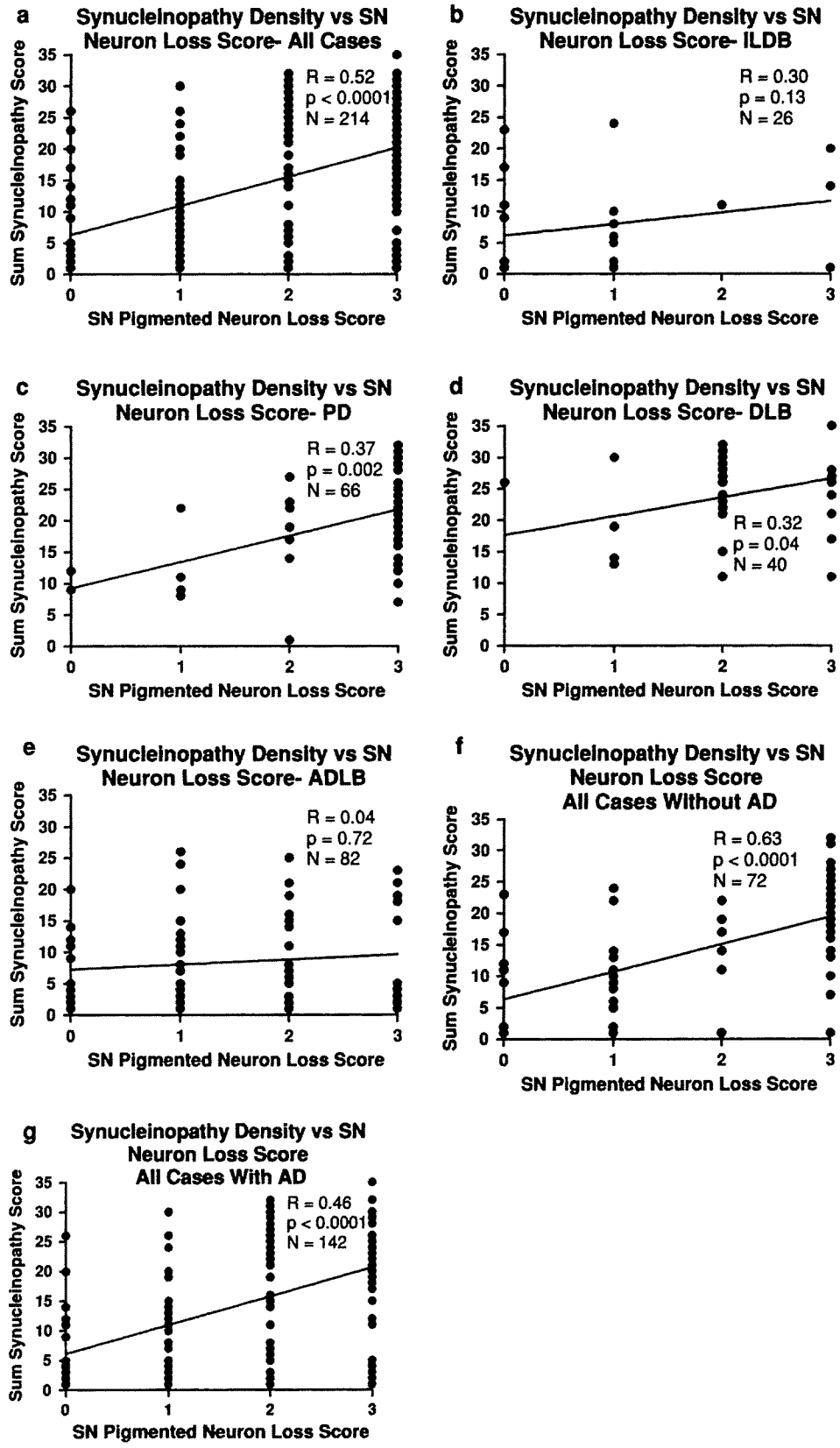

Fig. 5 Graphs depicting correlations between the sum of brain regional synucleinopathy density scores and substantia nigra (SN) pigmented neuron loss score, in subjects divided or not divided by diagnostic group and presence or absence of AD. The graph titled "All Cases" included all subjects with Lewy body pathology regardless of diagnosis. The graph titled "with AD" included data from all subjects who also met NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. The graph titled "without AD" included data from all subjects who did not meet NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. *R* Spearman Rho, *N* number of subjects included in the analysis, *p* probability. Abbreviations otherwise as for Fig. 2

striatal TH due to uniformly low TH values (Fig. 4c), while the correlation with SN pigmented neuron loss score was significant (Fig. 5c; $P = 0.002$). The DLB group had too few striatal TH measurements available for correlation analysis, but the correlation with SN pigmented neuron loss score was significant (Fig. 5d; $P = 0.04$). For the ADLB group there were no significant correlations (Figs. 4d, 5e).

There is a relatively strong ($R = 0.45$) and significant ($P < 0.0001$) correlation between LST and UPDRS but not between LTS and MMSE when all subjects are considered together (Figs. 6a, 7a). In general, it appeared that significant correlations were obscured when subjects with diagnostic levels of AD were included. This is most dramatically illustrated for the correlation with MMSE (Fig. 7a, e, g), where there are no significant correlations when subjects with AD are included but a highly significant correlation when subjects with AD are excluded (Fig. 7f). Similarly, but less dramatically, for UPDRS motor scores, when only subjects with AD are considered there are no significant correlations (Fig. 6d, f). For subjects with PD and DLB, both of which have large fractions of subjects meeting diagnostic criteria for AD, the correlations for both UPDRS and MMSE are significant or near-significant for PD subjects, with a smaller fraction of

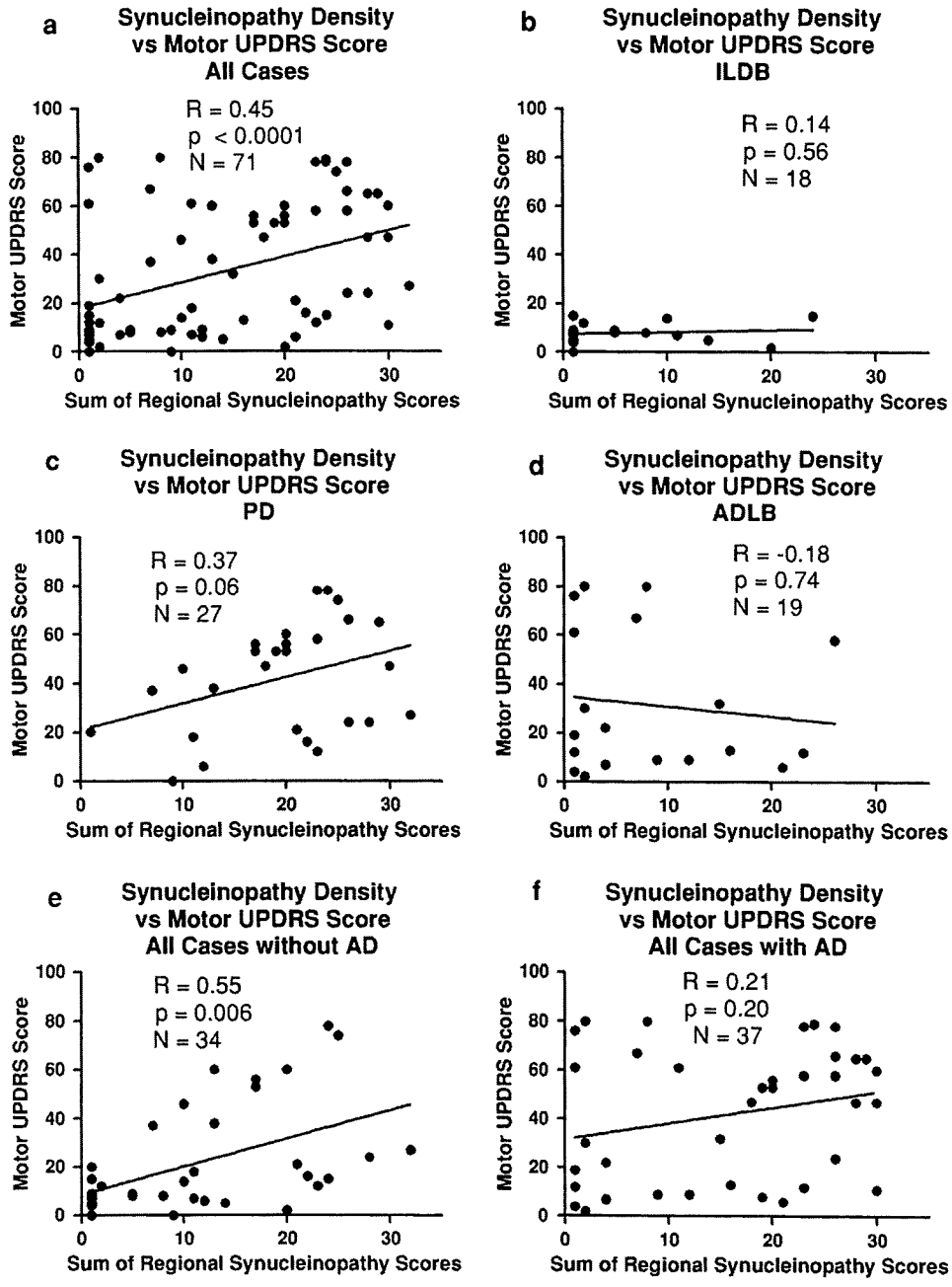

Fig. 6 Graphs depicting correlations between the sum of brain regional synucleinopathy density scores and motor scores on the Unified Parkinson's disease rating scale (UPDRS), in subjects divided or not divided by diagnostic group and presence or absence of AD. As there were too few DLB subjects with UPDRS scores available, there is no graph for this category, but these subjects are included in graphs **a**, **e** and **f**. The graph titled "All Cases" included all subjects with Lewy body pathology regardless of diagnosis. The graph titled "with AD" included data from all subjects who also met NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. The graph titled "without AD" included data from all subjects who did not meet NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. *R* Spearman Rho, *N* number of subjects included in the analysis, *p* probability. Abbreviations otherwise as for Fig. 2

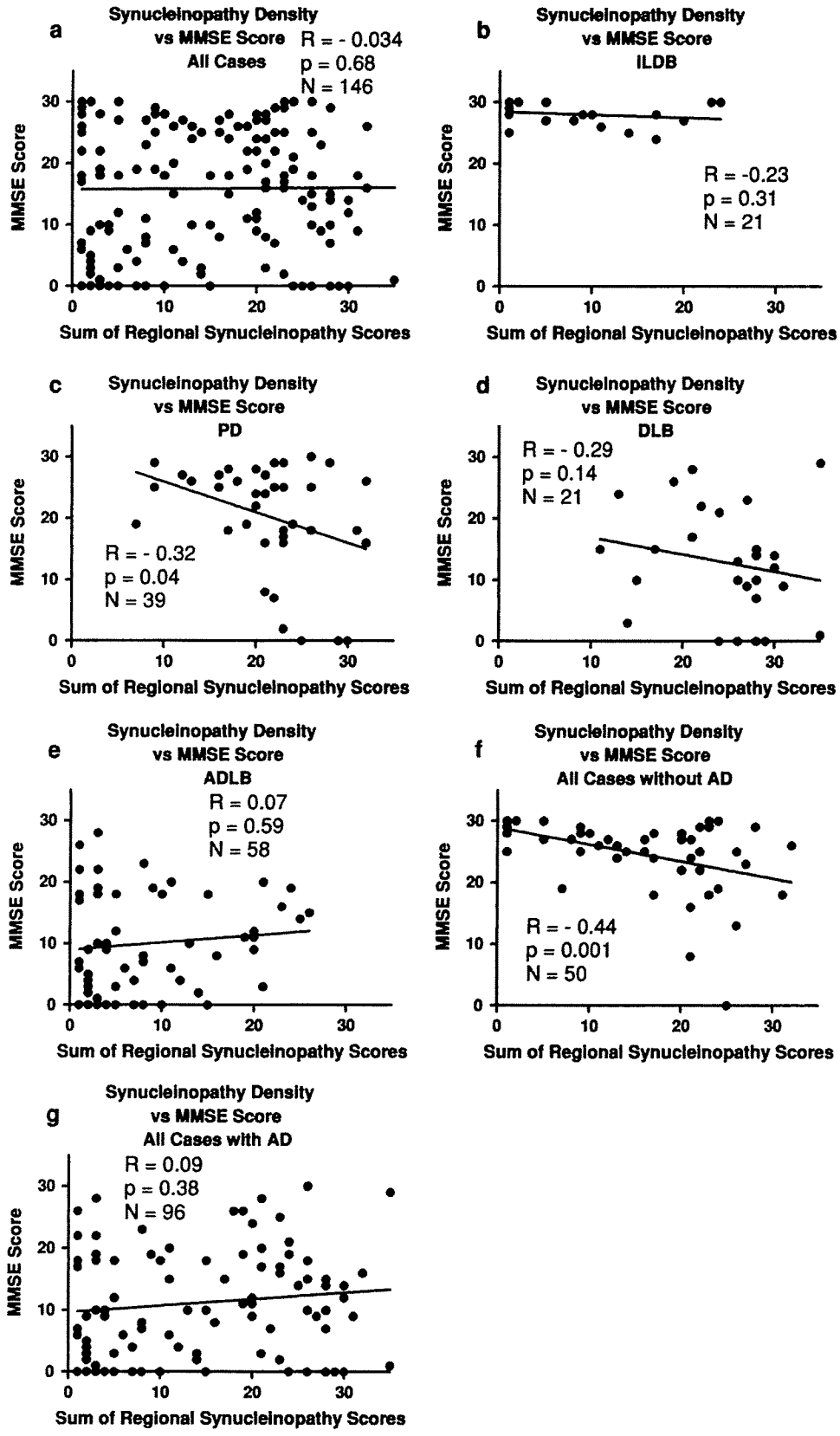

Fig. 7 Graphs depicting correlations between the sum of brain regional synucleinopathy density scores and scores on the Mini Mental State Examination (MMSE), in subjects divided or not divided by diagnostic group and presence or absence of AD. The graph titled "All Cases" included all subjects with Lewy body pathology regardless of diagnosis. The graph titled "with AD" included data from all subjects who also met NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. The graph titled "without AD" included data from all subjects who did not meet NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. *R* Spearman Rho, *N* number of subjects included in the analysis, *p* probability. Abbreviations otherwise as for Fig. 2

co-existing AD (Figs. 6c, 7c) but not significant for DLB subjects, with a larger fraction of co-existing AD (only the correlation with MMSE is shown, due to too few DLB subjects with a motor UPDRS score). For ILBD subjects there are no significant correlations with either UPDRS or MMSE, consistent with the asymptomatic clinical status of this group.

Correlations of LTS density scores with disease duration and age

For the correlations with disease duration (Fig. 8), all subjects with positive LTS density scores and a symptomatic disease condition (PD, DLB, ADLB) were included, while for the correlations with age (Fig. 9), all subjects with a positive LTS score were included, regardless of clinical status (ILDB, PD, DLB, ADLB). The influence of co-existing AD was again explored by analyzing all subjects with and without AD.

The only significant correlation between LTS regional density scores and disease duration was within the ADLB group (Fig. 8d; $P = 0.02$), where the sum of the regional LTS density scores increased mildly ($R = 0.25$) with increasing age. In contrast, there were significant correlations between age and LTS regional density scores when all subjects were considered together (Fig. 9a; $P < 0.0001$)

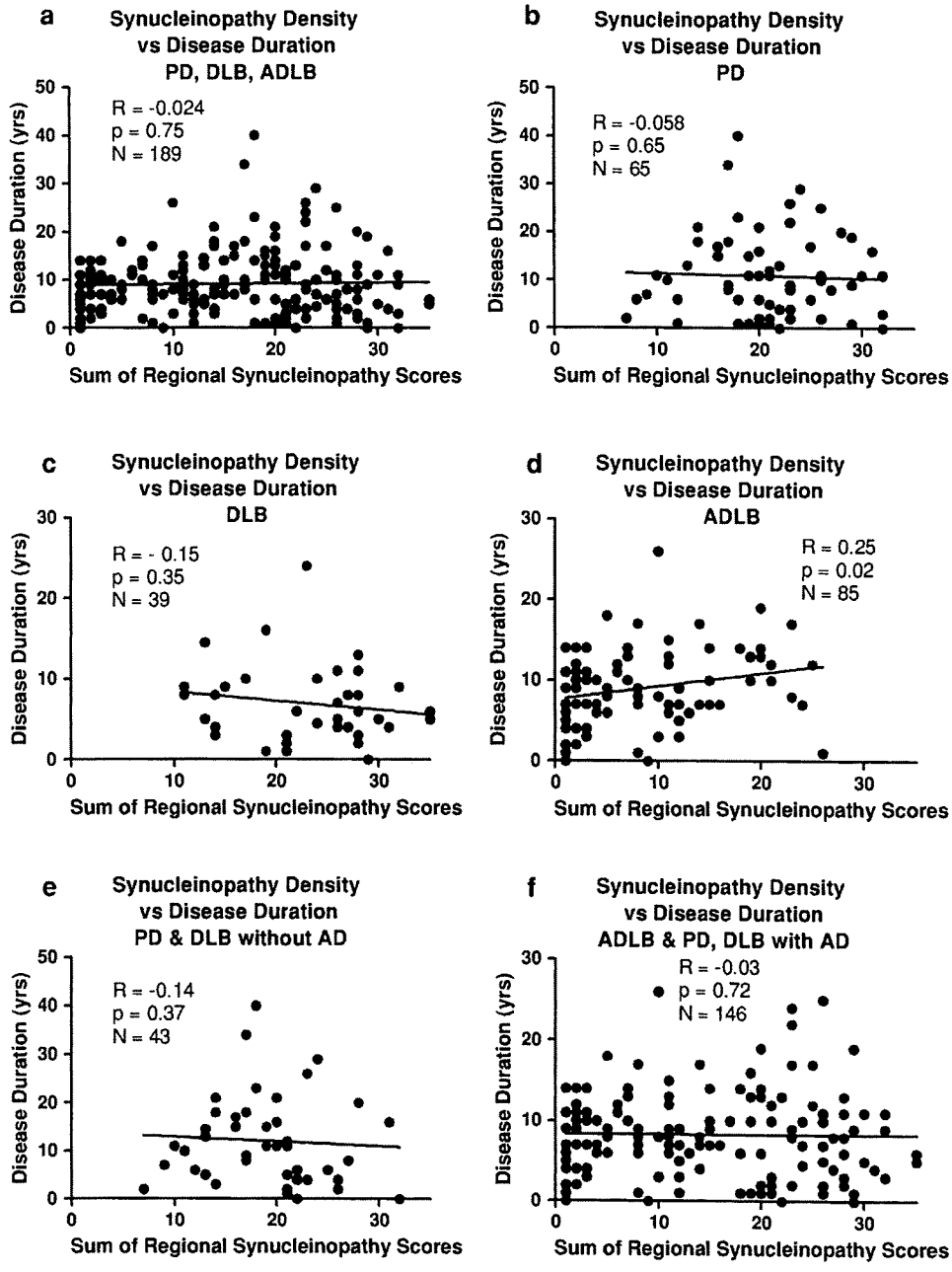

Fig. 8 Graphs depicting correlations between the sum of brain regional synucleinopathy density scores and disease duration, in subjects divided or not divided by diagnostic group and presence or absence of AD. The graph titled "with AD" included data from all subjects who also met NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. The graph titled "without AD" included data from all subjects who did not meet NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. *R* Spearman Rho, *N* number of subjects included in the analysis, *p* probability. Abbreviations otherwise as for Fig. 2

Table 6 Classification rules for the proposed unified staging system for Lewy body disorders

Regional score (DLB III)	I. Olfactory bulb-only	IIa. Brainstem predominant	IIb. Limbic predominant	III. Brainstem/limbic	IV. Neocortical
Olfactory bulb	Score 1–4	Score 0–4	Score 0–4	Score 0–4	Score 0–4
Brainstem	Scores all 0	Either a or b	Either a or b	Either a or b	Scores 0–4
		a. Scores 1–2	a. Scores 0	a. Scores 1–2	
		b. Scores 3–4	b. Scores 1–2	b. Scores 3–4	
Limbic	Scores all 0	Match a & b with above	Match a & b with above	Match a & b with above	Scores 0–4
		a. Scores 0	a. Scores 1–2	a. Scores 1–2	
		b. Scores 1–2	b. Scores 3–4	b. Scores 3–4	
Neocortical	Scores all 0	Scores 0–1	Scores 0–1	Scores 0–1	Scores 2–4

Density scores are derived from the Third Dementia with Lewy Bodies Consortium conference. Scores refer to range of scores for all regions within a subdivision. The criteria for each stage are outlined in the columns and are dependent on the LTS density scores within the regions, i.e., olfactory bulb, brainstem, limbic, and neocortical. Scores given in the table are for any individual area within that region. For example, a subject would be scored as brainstem predominant if the highest brainstem area score range (1–2 or 3–4) was higher than the highest limbic score range (0–1 or 1–2, respectively). Additionally, to qualify as any stage less than neocortical, the highest neocortical area score would have to be 0 or 1. For olfactory bulb-only stage, all other regional scores must be 0. Otherwise, the olfactory bulb score does not affect scoring for any other stage

multiple neocortical LTS density scores of 1 do not affect staging.

Figure 11a–d show the distribution of subjects within the proposed staging system, organized by disease entity. Most ILBD cases are classified as Stage IIa, Brainstem Predominant, most PD and DLB subjects are in Stages III, Brainstem and Limbic or IV, Neocortical, and most ADLB subjects are in stage I, Olfactory Bulb-Only or IIb Limbic Predominant. Tables 7 and 8 show the proportions of PD subjects classified into the various stages after subdivision according to presence or absence of dementia, the latter further divided by the presence of absence of neuropathologically confirmed Alzheimer's disease. Non-demented subjects with PD had a much higher percentage of Stage IIa Brainstem Predominant cases (30%) than the subjects with PD and dementia, with or without AD (3–12%), and many fewer subjects with Neocortical Stage IV (13% for non-demented PD vs 31–55% for PD with dementia), but still a large percentage (47%) with Stage III (Brainstem and Limbic). Surprisingly, PD/AD subjects had a much higher percentage of cases in the Neocortical Stage IV than the PDD subjects without AD (55 vs. 31%).

Correlation of proposed Unified Lewy body disorder staging system with MMSE, UPDRS and measures of nigrostriatal degeneration

Figure 11e–h graphically depicts how the proposed unified Lewy body staging system relates to measures of cognitive impairment (MMSE; Fig. 11g), motor dysfunction (UPDRS Part 3; Fig. 11h) and nigrostriatal system degeneration (striatal TH; Fig. 11e; SN pigmented neuron loss score; Fig. 11f). As preliminary analyses indicated that inclusion of subjects with ADLB obscured relevant correlations with the other groups, ADLB subjects were excluded when this occurred. The results indicate that cognitive impairment and motor dysfunction generally progressively worsen with increasing stage. Similarly, measures of nigrostriatal degeneration become progressively greater passing from stage 0 to stage IV, with the exception of stage IIb (Limbic Predominant), which, predictably, has less nigrostriatal degeneration than stage IIa (Brainstem Predominant). The stepwise progression is most clearly seen with UPDRS scores (Fig. 11h).

Discussion

The usefulness of any disease staging system depends on the degree of its applicability to the intended population and on its ability to divide affected subjects into biologically significant stages. The two current major staging systems in use for PD and DLB, that of Braak [16] and the DLB Consortium [67], do not meet the first of these two criteria very well. Reports by several groups as well as the current study have indicated that both systems fail to classify up to 50% of subjects [34, 51, 52, 59, 62, 76]. Both systems have proposed stages based on a topographical progression that begins in the brainstem and then spreads progressively to the limbic lobe and the cerebral neocortex. Neither system allows for large numbers of subjects who have LTS confined to the olfactory bulb or who pass through a limbic-predominant pathway that at least initially bypasses the brainstem. A recent publication has acknowledged this difficulty, proposing a modification allowing a limbic-only stage [60]. This study did not include the olfactory bulb, however, and did not, therefore, note the additional difficulties with classifying olfactory

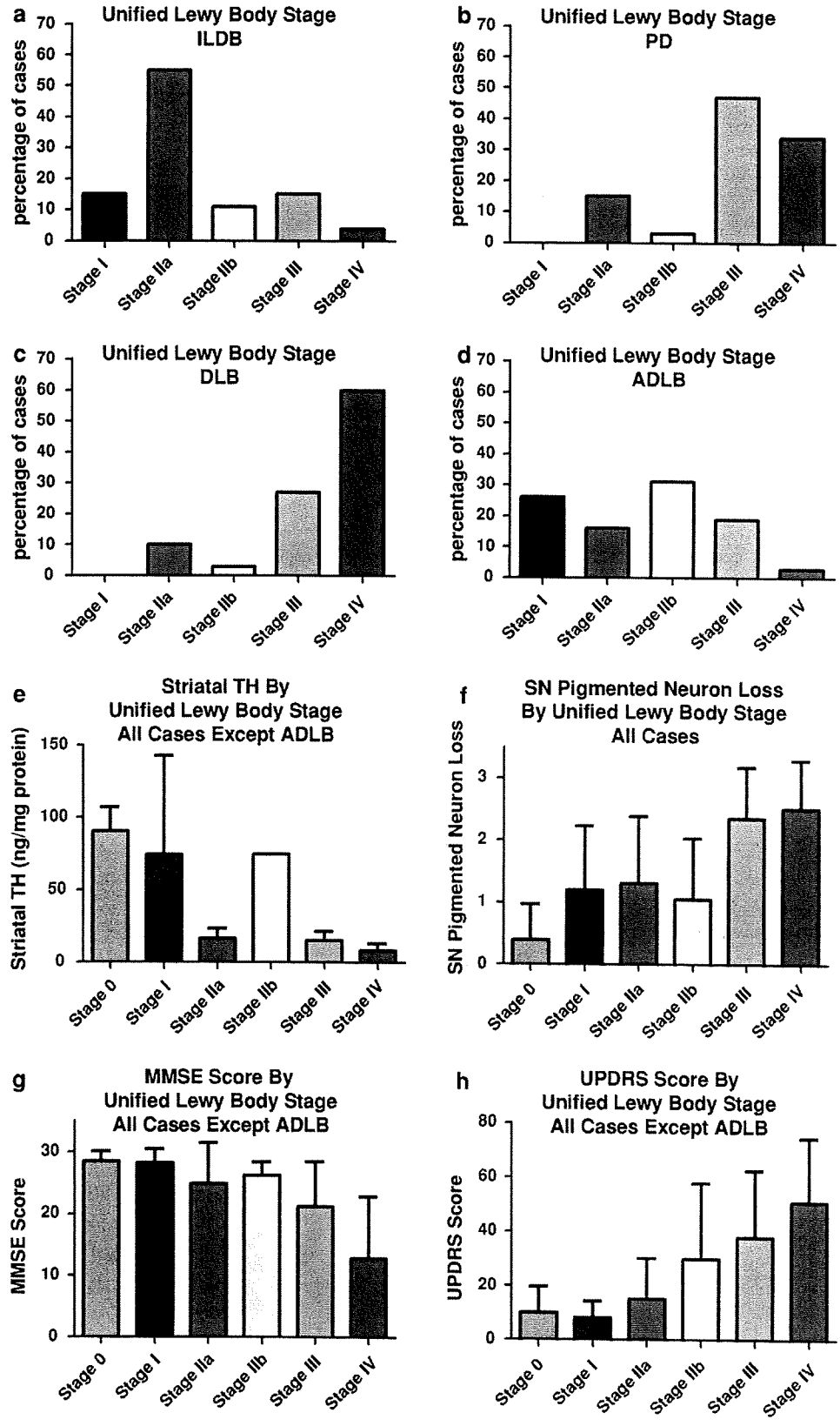

Fig. 11 Graphic depiction of differential characteristics of the subjects classified by the proposed unified Lewy body staging system. For all graphs, the 0 stage data is from the normal control group. (**a**) Incidental Lewy body disease (ILBD) subjects classified by Unified Lewy body stage. (**b**) Parkinson's disease (PD) subjects classified by Unified Lewy body stage. (**c**) Dementia with Lewy bodies (DLB) subjects classified by Unified Lewy body stage. (**d**) Alzheimer's disease with Lewy bodies (ADLB) subjects classified by Unified Lewy body stage. (**e**) Unified Lewy body stage by striatal TH concentration. Subjects with ADLB were excluded from the analysis. *Error bars* show standard error of the mean. The means of the stages differed significantly ($P < 0.001$). (**f**) Unified Lewy body stage by substantia nigra pigmented neuron loss score. All subjects with Lewy body pathology were included. *Error bars* indicate standard deviation. The means of the stages differed significantly ($P < 0.001$). Unified Lewy body stage by MMSE (**g**) and UPDRS (**h**) scores. *Error bars* indicate standard deviation in both graphs. The means of the stages differed significantly ($P < 0.0001$)

bulb-only cases. The results of the current study suggest a new, unified staging system that allows for the classification of all subjects with Lewy body disorders. The biological significance of these stages, as with the original staging systems, largely remains to be determined, but the generally stagewise worsening of cognition, motor function

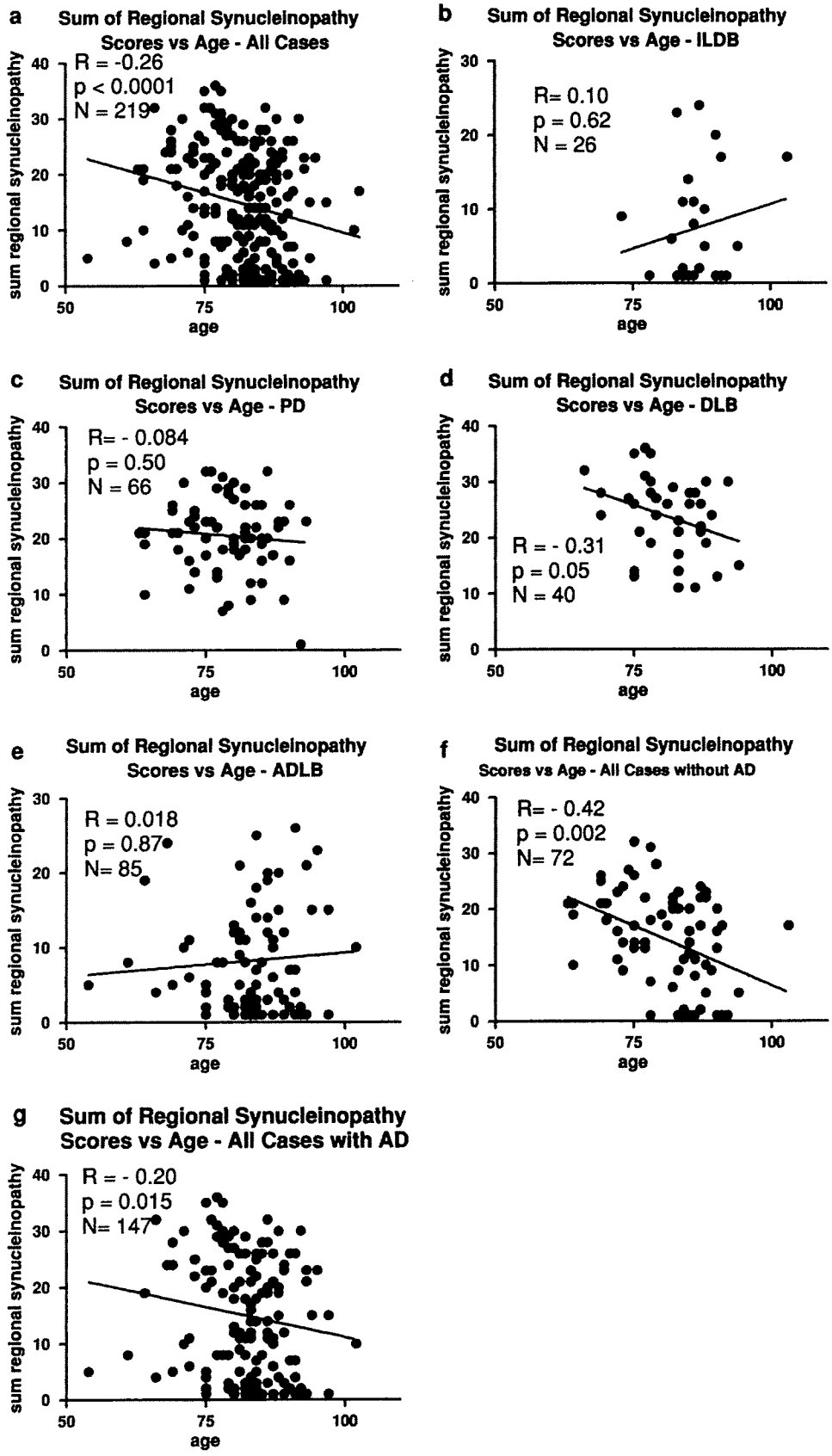

Fig. 9 Graphic depiction of the relationship between age and the sum of regional synucleinopathy densities, in subjects divided or not divided by diagnostic group and presence or absence of AD. The graph titled "with AD" included data from all subjects who also met NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. The graph titled "without AD" included data from all subjects who did not meet NIA-Reagan "intermediate" or "high" criteria for AD, regardless of their other diagnoses. *R* Spearman Rho, *N* number of subjects included in the analysis, *p* probability. Abbreviations otherwise as for Fig. 2

and also when all subjects with (Fig. 9g; $P = 0.015$) or without a co-existing diagnosis of AD were considered (Fig. 9f; $P = 0.002$). Additionally, there was an inverse correlation of age with LTS density within the DLB group (Fig. 9d; $P = 0.05$). In contrast, the ILDB (Fig. 9b) and ADLB (Fig. 9e) groups showed a trend, although non-significant, for a positive correlation with age.

LTS-based staging of study subjects

When study subjects were classified, in terms of their LTS regional involvement, by either the Braak system [16] or the DLB Consortium system [67], there were a high proportion of subjects that could not be assigned a stage (Tables 4, 5). For the Braak system, 42% of subjects were not classifiable while for the DLB Consortium system, 50% of subjects were unclassifiable. For both systems, approximately one-third of the unclassifiable subjects were due to olfactory bulb-only cases, while approximately two-thirds were due to limbic system involvement in the absence of brainstem pathology. Neither system provides for the latter pattern of involvement while the Braak system has been unclear as to the status of olfactory bulb-only cases.

A new staging system was, therefore, devised so that all subjects could be assigned to a defined stage. The results of the regional frequency and density analyses (Figs. 2a–h, 3a–b) were used as the basis for constructing an hypothetical regional progression pathway and unified staging system for Lewy body disorders (Fig. 10 and Table 6). As the olfactory bulb is the most frequently involved brain region, and the most frequent region to be solely involved, Stage I is defined as cases with pathology only in the olfactory bulb. However, as occasionally the olfactory bulb may not be involved, Stage I is not required and subjects may be classified as any higher stage without regard to olfactory bulb score. From the olfactory bulb, the pathway diverges into one that has brainstem-predominant involvement (Stage IIa) and one that has involvement predominantly of the limbic system (Stage IIb). The basis for this distinction is made by comparing LTS density scores in brainstem and limbic regions, as detailed in Table 6; the distinction is made when scores in all areas of one region are clearly higher than the scores in all areas of the other region. Stage III (Brainstem and Limbic) subjects are those for whom LTS density scores in brainstem and limbic regions are roughly equivalent. The neocortical stage (Stage IV) is reached when any single neocortical region has an LTS density score of 2 or higher, signifying unequivocal involvement of the neocortex. Single or

Table 4 Classification of study subjects (all diagnoses together) by the Braak Lewy body staging system

	I	II	III–IV	V	VI	Unclassifiable
N (%)	12 (5.5)	1 (0.5)	3 (3)	11 (5)	96 (44)	90 (42)

Twenty-six of the unclassifiable cases were due to involvement of the olfactory bulb only while 66 were due to non-sequential involvement of regions

Table 5 Classification of study subjects by Dementia with Lewy Body Consortium (III) system

	Brainstem Predominant	Limbic (Transitional)	Neocortical	Unclassifiable
N (%)	5 (2.3)	76 (35)	29 (13)	108 (50)

Twenty-six of the unclassifiable cases were due to involvement of the olfactory bulb only while 83 were due to non-sequential involvement of regions

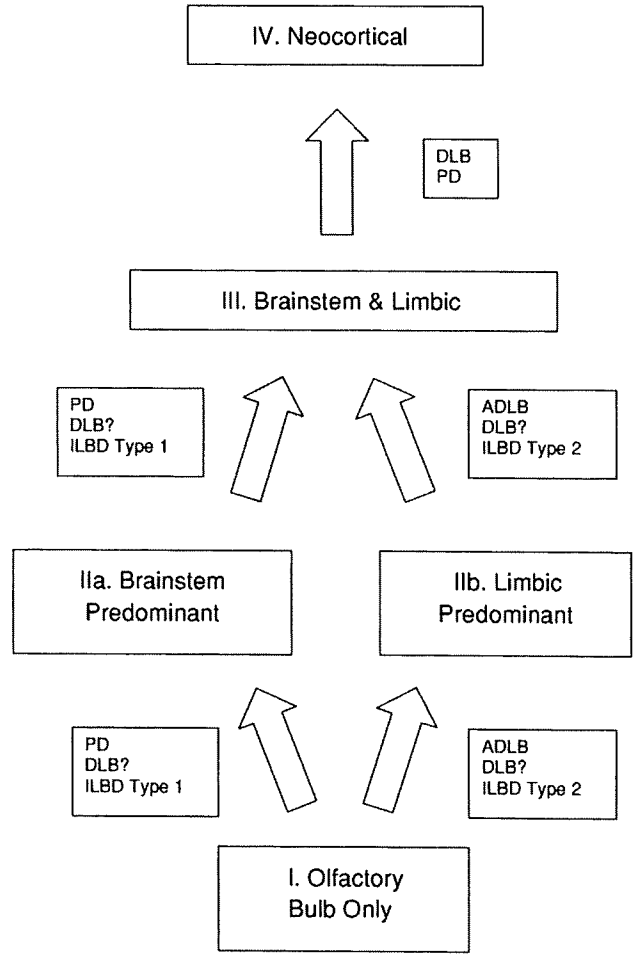

Fig. 10 Schematic depiction of hypothetical progression pathways and stages for Lewy body disorders. The pathway for PD seems likely to proceed through Stage IIa (brainstem predominant) while that for ADLB passes through Stage IIb. The pathway followed by DLB is uncertain as the current definition of DLB largely excludes all but the neocortical stage. There may be two types of ILBD, one that leads to PD and another that leads to ADLB and possibly DLB. Only PD and DLB progress to the neocortical stage

Table 7 Basic characteristics of subjects with PD, subdivided by presence or absence of dementia and neuropathological Alzheimer's disease

Diagnosis (N)	Age (SD)[a]	Gender (%M)[b]	ApoE-ε4 +ve (%)[c]	PMI hours[d], median, mean (SD)
PDND (23)	80.5 (7.6)	65.2	21.7	2.7, 3.8 (3.3)
PDD/AD (27)	80.8 (5.6)	70.4	37.0	2.5, 3.6 (3.0)
PDD/NAD (16)	74.1 (6.2)	70.6	31.2	3.0, 3.9 (4.0)

PDND Parkinson's disease, non-demented, *PDD/AD* Parkinson's disease, demented, Alzheimer's disease, *PDD/NAD* Parkinson's disease, demented, no Alzheimer's disease

[a] PDD/NAD subjects are significantly younger than other subjects ($P < 0.05$)

[b] Gender distribution differences were not significant

[c] PDD/AD subjects have significantly higher proportions that are positive for the ε4 allele, as compared with PDND subjects ($P < 0.05$)

[d] Differences in PMI were not significant

Table 8 Classification of PD subjects into the proposed Lewy body staging system, after subdivision by presence or absence of dementia and neuropathological Alzheimer's disease

	I. Olfactory Bulb-Only	IIa. Brainstem Predominant	IIb. Limbic Predominant	III. Brainstem/ Limbic	IV. Neocortical
PD/ND ($N = 23$)	0	7 (30.4)	2 (8.7)	11 (47.8)	3 (13.0)
PDD/AD ($N = 27$)	0	1 (3.7)	0 (0)	11 (40.7)	15 (55.5)
PDD/NAD ($N = 16$)	0	2 (12.5)	0 (0)	9 (56.2)	5 (31.2)

Figures shown are number of subjects and percentage of category

and nigrostriatal degeneration provides initial support for its clinical and biological relevance. These correlations are generally not evident within the ADLB group, perhaps due to the masking of a restricted and low-intensity LTS by a much more widespread and higher-intensity AD histopathology. In general, accompanying diagnostic-level AD histopathology tended to obscure LTS-related associations within all of the groups under consideration but within the DLB and PD groups correlations with LTS are generally still evident even with co-existing AD. It may be pertinent that some authors [35, 71] have suggested that LTS and tauopathy, at least in some situations, may be generated by the same stimulus, with the outcome possibly having an inverse relationship dependent on genetic background or possibly environmental factors.

The current study suggests a new schematic for the temporal progression and topographical distribution of LTS (Fig. 10), although the temporal aspect must be admittedly regarded as speculative. However, it seems likely that the earliest stages are most clearly seen in clinically normal elderly subjects, up to 25% of which harbor LTS in one or more brain regions and have been termed incidental Lewy body disease (ILBD). Early stages may also be exemplified by subjects with AD and Lewy body pathology insufficient to meet DLB Consortium criteria (ADLB). In both of these groups, the olfactory bulb is often the only brain region affected and for this reason such cases are assigned Stage I. From the olfactory bulb, subjects with ILBD and ADLB may diverge. Most subjects with ILBD pass into the Brainstem Predominant Stage IIa (ILBD Type 1) while most subjects with ADLB go directly to the limbic system, especially the amgydala, as the Limbic Predominant Stage IIb. A sizeable subset of ILBD cases are also classified as Stage IIb (ILBD Type 2). Following the two parts of Stage II, the pathways converge again into a single Brainstem and Limbic Stage III. The Neocortical Stage IV is reached virtually exclusively by subjects meeting diagnostic criteria for PD and DLB.

As subjects with clinically evident PD and DLB frequently have involvement of all of the regions under consideration, the pathways that these subjects initially followed become more of a matter of speculation. However, in both of these groups, more subjects were classified as Stage IIa (brainstem predominant) than stage IIb (limbic predominant), suggesting that most of these subjects pass through the brainstem prior to the limbic system, and are, therefore, unlike ADLB cases, most of which proceed to the limbic Stage IIb before significantly involving the brainstem. It seems unlikely that the differing patterns and densities of LTS are a result only of differing disease durations, as even though the greatest densities and distributions of LTS occur in PD and DLB, these have the longest and shortest disease durations, respectively, and disease duration did not correlate significantly with LTS in any diagnostic group except ADLB.

As ADLB has been defined almost accidentally by the most recent revision of the DLB Consortium, it seems at first consideration unlikely to be a condition completely separate from both PD and DLB. With respect to its accompanying AD pathology and high apoE-ε4 prevalence (Table 1], ADLB seems very similar to DLB, but the greater male gender distribution and greater nigrostriatal degeneration in DLB ally that condition more closely with PD. Although both ADLB and DLB have high neuritic plaque density scores (Table 3), the fact that DLB subjects have generally lower Braak neurofibrillary stages than ADLB cases, and that disease duration is much shorter in DLB than in ADLB, argue against the hypothesis that ADLB might represent early DLB. In some ways, ADLB resembles ILDB most closely, as with both there is a topographically restricted LTS that increases gradually with age while both DLB and PD have widespread LTS that has an inverse correlation with age. At the present time, it appears that ADLB, PD, and DLB are sufficiently different from one another as to justify their separate existence while the LTS common to all is suggestive of at least some shared pathogenic pathways [27, 64, 85].

An important disclaimer is that the brain region frequency analysis has shown that it is possible that many cases may not follow the hypothetical temporal progression pathway outlined in Fig. 10. Some cases have brainstem and/or limbic involvement prior to the olfactory bulb and rare cases have isolated and/or sparse neocortical involvement before the preceding two stages are reached. Other cases, perhaps including PD and DLB, may begin with simultaneous and widespread but light involvement of many regions. This does not preclude these from classification, however, as the rules established here allow for non-sequential progression. The persistence of a subset of cases with an apparently random direction of pathological spread suggests, however, that progression through a specified topographical sequence is not an obligate form of disease advancement, but rather is indicative that the observed patterns of involvement are due to probabilistic rather than deterministic factors.

Prior published work has increasingly questioned the relevance of LTS for clinically relevant phenomena such as cognitive or motor dysfunction [18, 51, 76]. The results presented here show that there are clear and significant correlations of LTS density scores with those for MMSE, UPDRS, SN pigmented neuron loss and striatal TH concentration. Also apparent, however, is the ability of coexistent AD histopathology to obscure these relationships, especially within the ADLB group. Inclusion of such cases in previous studies, which are probably relatively numerous in most populations, may have similarly prevented the appreciation of relevant correlations with LTS in these investigations. As ADLB is a condition with an LTS distribution and density that is much reduced compared to PD and DLB, and in fact resembling that seen in ILBD, it should not be surprising that this relatively light level of pathology has correspondingly little effect on clinical, biochemical, or structural brain measures.

The results of this study are supportive of prior published evidence that subjects with ILBD have increased nigrostriatal degeneration relative to normal elderly subjects without LTS. Both in terms of SN pigmented neuron numbers [23, 33, 79] and striatal dopaminergic markers [23, 25], subjects with ILBD have significant degeneration that places them intermediate between PD and age-similar normal elderly subjects. This important finding makes it very likely that ILBD is not a benign disorder and may represent the earliest stage of other Lewy body disorders. As the current study and other recent work [50, 69, 81] have shown that between 16 and 30% of the asymptomatic elderly have ILBD, this greatly expands the estimated prevalence of Lewy body disorders in the human population. Indeed, the clinically manifest Lewy body disorders may be regarded as truly the tip of the iceberg in terms of the numbers of people affected as it is now apparent that the most common Lewy body disorder by far is ILBD. Further study of this crucial group may identify clinical or laboratory biomarkers that would allow for presymptomatic detection and preventative therapy.

The present work indicates that the olfactory bulb has a special vulnerability for LTS. It has long been observed that subjects with Parkinson's disease (PD) have decreased olfactory sensation [1, 26, 42, 56]. Attempts to use tests of olfaction for clinical diagnosis have had mixed results, however, due to loss of olfactory ability in other conditions as well [53, 68]. Lewy bodies and LTS have been reported to be present in the olfactory bulbs of subjects with PD, DLB, ILBD, and ADLB [12, 21, 35, 48, 77, 83], suggesting that olfactory bulb LTS is common to all Lewy body disorders and occurs at an early stage of disease. We have recently published results showing that the presence of LTS in the olfactory bulb is an excellent biomarker for the presence of a more widely distributed Lewy body disorder, with sensitivity and specificity of close to or greater than 90% for subjects with PD, DLB, or ADLB [7]. It is now clear, from the large number of cases identified in the present study as having LTS exclusively in the olfactory bulb, that for many subjects this is the first area affected. Other authors have offered reasons for this special vulnerability, citing thinly myelinated and lengthy axons [15], an unusually high degree of neuronal plasticity [28, 48] or the spatial proximity to the external environment, with its associated exposure to toxins and infectious agents [14, 43, 58]. These remain speculative, but it seems likely that further investigations of the olfactory bulb may yield new insights into Lewy body disorders. The virtually universal

involvement of the olfactory bulb is a unifying feature of the Lewy body disorders, providing some support for the concept that they are all part of a continuum, with the variability coming from the varying genetic and environmental background of affected individuals.

The correlation analyses of LTS versus disease duration and LTS versus age showed a mild progression of LTS with age in ILBD and ADLB but an inverse correlation with age in DLB and PD. This is similar to what has been widely known about AD histopathology for many years, i.e., younger cases have more severe pathology. While AD and Lewy body disorders are clearly age-associated, and their respective histopathologies are both more prevalent in the population with increasing age, it appears that individuals with more susceptibility to these conditions are affected earlier and more severely while those with less susceptibility are affected later and less severely. Individuals with ILDB and ADLB may contain disproportionate numbers of less-susceptible individuals.

It may be maintained that because the Lewy body disorders may differ biologically, they each should have their own unique staging systems. The failure, within ADLB subjects, to achieve a significant correlation between LTS density, MMSE scores and UPDRS scores, might suggest that these subjects are biologically different from the others. However, it is also possible that ADLB subjects, like ILDB subjects, have LTS that is too limited, in both distribution and density, to affect clinical measures. It is suggested here that there are insufficient data to answer the question of biological similarity. Supporting biological similarity is the frequent presence in subjects in all of the categories, i,e., ILDB, PD, DLB, and ADLB, of plaques, tangles and Lewy bodies. Another unifying feature is that, as discussed earlier, virtually all cases have olfactory bulb involvement. The overall similarities within the pairing of ILBD and ADLB, as well as within the pairing of PD and DLB, with respect to the topographical patterns of their LTS density and distribution frequencies, are supportive of two separate groups with two disorders in each, but these two groups may simply represent earlier and later stages of the same disorder. Justifiably, some researchers still argue that all of these conditions are part of a continuum.

Although the Braak and DLB Consortium systems work relatively well within their targeted diagnostic groups, it is difficult to decide which system to use for ILBD and ADLB, neither one of which is well served by either. The proposed unified staging system would be a simple way to quickly and efficiently describe the topographical distribution of Lewy bodies in any single subject, in any diagnostic category. Importantly, this would also allow researchers to easily compare Lewy body stages across diagnostic categories, something that has not been easily possible with the usage of multiple staging systems.

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