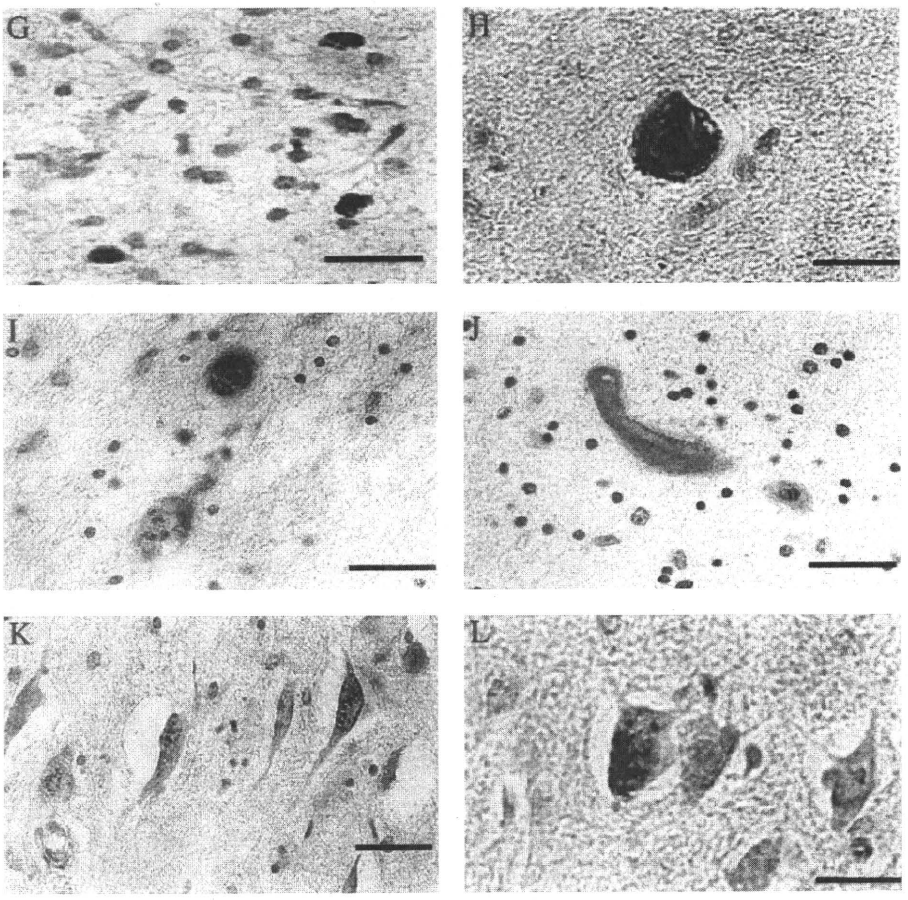


Fig. 3 (*continued*).

4.1. Alpha-Syn pathology in DNTC

The area of limbic region seemed to be the most vulnerable for aSyn pathology as well as tau pathology in DNTC (Table 1). These observations are consistent with the previous reports [21,22].

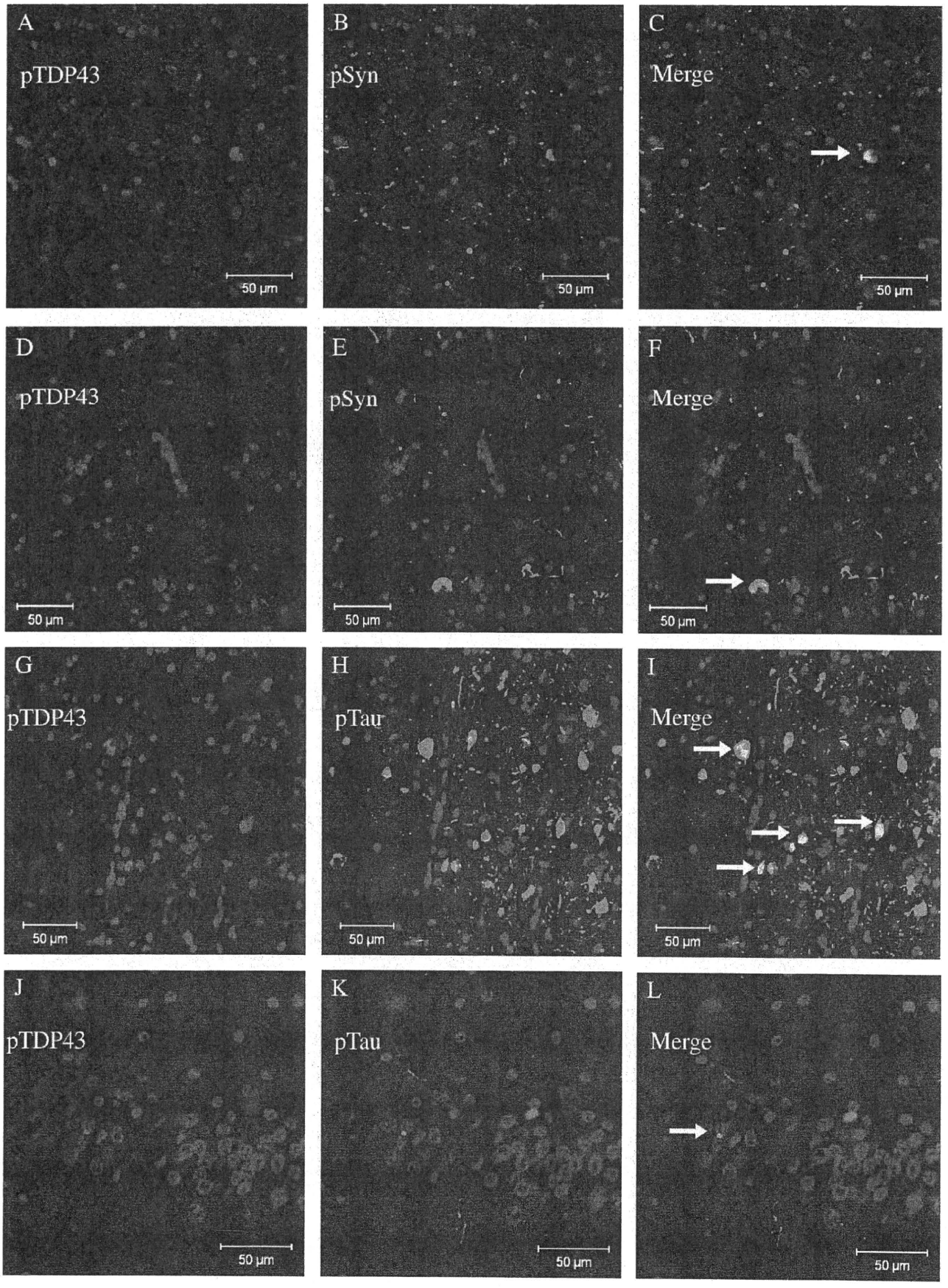
4.2. TDP-43 pathology in DNTC

We demonstrated a high frequency of TDP-43 pathology in 9 out of 10 DNTC cases (90%). Among the 9 cases having pTDP-43 pathology, 2 cases (Cases 1, 6) (22%) were identified as the 'limbic type,' while the other 7 cases (Cases 2, 3, 4, 5, 7, 8, 10) (77%) were "diffuse type," according to the classification by the study of Amador-Ortiz et al. [10]. The vulnerability of the limbic region to pTDP-43 accumulation found in our DNTC series was consistent with the previous reports of AD, DLB and AGD [10,11,13,14,23].

We mainly observed the pathology of DNTC for the purpose of comparison with FTLD. Recently, the TDP-43 pathology in FTLD has been classified into 4 subtypes according to the cortical TDP-43 pathology [24–26]. In the present study, 6 out of 7 cases (86%) with cortical TDP-43 pathology showed many NCIs/GCIs and sparse DNs, similar to the FTLD with ubiquitin-positive, tau-negative, TDP-43 positive neuronal cytoplasmic inclusions (FTLD-TDP), Type 2, using current terminology [27]. Only one case (Case 7) showed many NCI/GCIs and DNs, similarly to FTLD-TDP, Type 3. These findings of TDP-43 pathology in DNTC differ from those of AD and DLB cases, which almost all present Type 3 pathology [13,28,29].

In this point, the question arises as to whether this high frequency of TDP-43 pathology in DNTC cases represents a concurrent primary pathological process of FTLD-TDP or a secondary change occurring in susceptible neuronal populations. Although the high frequency of GCIs in DNTC is similar to the pathology of FTLD-TDP, Type 2, a simple coincidence of FTLD-TDP and DNTC seems unlikely based on our findings as follows. First, the main TDP-43 pathology in DNTC is similar to Type 2, but the frequency of TDP-43 positive NCIs in the dentate gyrus was low (one out of 9 cases; 11%) in our DNTC cases with TDP-43 immunoreactivity in comparison with those (about 80%) in FTLD-TDP, Type 2 [25,30]. Second, there are previous reports that 25–40% of FTLD-TDP cases with Type 2 pathology tend to be associated with clinical features of motor neuron disease [25,31]. In contrast, there was no case with Type 2-like pathology showing clinical features of motor neuron disease in our DNTC series.

Fig. 4. Double labeling immunofluorescence in Case 8 (A–F) demonstrates that most alpha-synuclein positive inclusions (green fluorescence in B, E) and pTDP-43 positive inclusions (red fluorescence in A, D) in the CA3 area of the hippocampus are independent (F), while there is partial colocalization of alpha-synuclein and pTDP-43 in some neuronal cytoplasmic inclusions in the deep layer of the entorhinal cortex (arrows in C). Double labeling immunofluorescence in Case 2 (G–L) demonstrates that most tau-positive neuropil threads (green fluorescence in H, K) and pTDP-43 positive inclusions (red fluorescence in G, J) in the entorhinal cortex are independent (L), while there is partial colocalization of tau and pTDP-43 in some neuronal cytoplasmic inclusions and the dentate gyrus of the hippocampus (arrows in I). Scale bar: A–L, 50 μm.


A
pTDP43
50 μm
B
pSyn
50 μm
C
Merge
50 μm
D
pTDP43
50 μm
E
pSyn
50 μm
F
Merge
50 μm
G
pTDP43
50 μm
H
pTau
50 μm
I
Merge
50 μm
J
pTDP43
50 μm
K
pTau
50 μm
L
Merge
50 μm

Therefore, it may be possible to speculate that TDP-43 pathology in DNTC has different characteristics from that of FTLD-TDP.

There arises another query concerning the possibility that the results in this study were driven by cross-reactive phospho-epitopes because the antibodies used for tau, aSyn and TDP-43 in this study were all phosphorylation-specific. Although there is some evidence of cross-reactivity with phosphorylated aSyn and phosphorylated tau [32], the anti-phosphorylated aSyn antibody used in this study did not immunostain NFT in AD brains, suggesting that it does not have cross-reactivity with phosphorylated tau.

Since the posttranslational modification is known to be a common motif in pathological protein accumulation in many neurodegenerative disorders and also this process can involve phosphorylation [33–36], the phosphorylation-dependent antibodies could mostly detect the pathological TDP-43 inclusions [13,17,37]. These mechanisms of phosphorylation might proceed in the pathologies of tau [34] or synuclein [38].

We found a scarce colocalization of pTDP-43 and tau and/or aSyn in some neuronal cytoplasmic inclusions in this study. Such a partial colocalization of tau (grain, or NFT), aSyn, and TDP-43 is consistent with that previously reported in AD, DLB, Pick's Disease, AGD, G-PDC and corticobasal degenerations to a varying degree [10–14,23]. As for the double-labeled cytoplasmic inclusions within the neuron, some reports [11,14] indicated that the TDP-43 was hardly superimposed with tau or observed only in a part of tau-positive cytoplasmic inclusions in AD or AGD based on double confocal microscopic observation. Also in this study, pTDP-43 scarcely coexisted with tau in neuronal cytoplasmic inclusions. These facts suggested that the pathway of appearance of the TDP-43 pathology might be different from that of the tau pathology but could relate closely in some way to tau proteinopathy of neurodegenerative diseases including DNTC.

It is possible that these findings were resulted from a non-specific vulnerability of the limbic region against these proteinopathies. However, there may be common factors or mechanisms that affect the conformation or modification of those proteins, leading to their intracellular accumulation. Especially, it was interesting to note that the results of the present study further supported the previous report that indicated similarity between DNTC and G-PDC [39]. Both disorders are assumed to be some type of endemic disease, and show frontotemporal atrophy and accumulation of tau, aSyn and TDP-43 without Amyloid β deposition. These findings could offer a hint toward understanding of the pathogenesis of these disorders.

4.3. Clinical features and pathology in DNTC

The phosphorylated aSyn pathology was observed in the brain stem of 5 cases of DNTC, but only one of these 5 cases had presented parkinsonism clinically. There seems to be a discrepancy between the presentation of parkinsonism and the pathological findings of phosphorylated aSyn appearance in DNTC.

There are some reports referring to an association between a specific clinical symptom and an underlying pathology in diverse degenerative disorders [40–43]. In our DNTC cases, the limbic region seemed to be the most vulnerable to abnormal accumulation of tau, aSyn and TDP-43. Thus, there is a possibility that not only accumulation of tau but also accumulation of aSyn and TDP-43 in the limbic region may be associated with early onset cognitive decline in DNTC. Indeed, in AD, concurrent TDP-43 pathology was reported to be associated with more severe cognitive decline [40].

In this study, we found a comprehensive association between pTDP-43 pathology and the frontotemporal symptoms (apathy, disinhibition, motor disturbances) in DNTC. Clinically, DNTC was diagnosed as FTLD because these three symptoms are representative and appear simultaneously to various degrees, so the integration of these three symptoms was very important for clinical assessment. Velakoulis, D. et al. recently reported that abnormalities in TDP-43 nuclear expression in the hippocampus were identified in patients with late-onset psychosis and a positive family history [44]. These findings suggest that abnormality of TDP-43 may be involved in the psychiatric symptoms of DNTC.

Meanwhile, there were some reports of DNTC without sufficient clinical or pathological assessment [4,45,46]. Cases of Fahr's syndrome with neuropsychological deficits and neuropsychiatric features have also been reported based on radiological findings [3,47,48]. More investigation will be needed to identify Fahr's syndrome as having the same background pathology of DNTC.

The association between basal ganglia calcification and psychotic symptoms has also been reported [49], but it remains unknown how the pathogenesis of calcification phenomenon concerns the psychosis. In the clinical setting, DNTC might pass unnoticed because it presents various clinical symptoms during the clinical course. Further studies on such cases from clinical, radiological and pathological standpoints might help elucidate the pathogenesis of DNTC.

5. Further assignment

We declare that there are some limitations of this study. Firstly, all the tissue samples were embedded in paraffin, so we could not perform molecular investigations including the Western Blotting test. Secondly, there were only a couple of sets of neuroimaging data of low resolution computed tomography available to this study, so we could not evaluate the association among the neuroimaging, neuropathology, and clinical symptoms. To address these issues, we are currently trying to upgrade the method for preserving tissue samples, and will acquire more neuroimaging data for a future prospective study.

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ORIGINAL PAPER

Multi-organ distribution of phosphorylated α-synuclein histopathology in subjects with Lewy body disorders

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Abstract A sensitive immunohistochemical method for phosphorylated α-synuclein was used to stain sets of sections of spinal cord and tissue from 41 different sites in the bodies of 92 subjects, including 23 normal elderly, 7 with incidental Lewy body disease (ILBD), 17 with Parkinson's disease (PD), 9 with dementia with Lewy bodies (DLB), 19 with Alzheimer's disease with Lewy bodies (ADLB) and 17 with Alzheimer's disease with no Lewy bodies (ADNLB). The relative densities and frequencies of occurrence of phosphorylated α-synuclein histopathology (PASH) were tabulated and correlated with diagnostic category. The greatest densities and frequencies of PASH occurred in the spinal cord, followed by the paraspinal sympathetic ganglia, the vagus nerve, the gastrointestinal tract and endocrine organs. The frequency of PASH within other organs and tissue types was much lower. Spinal cord and peripheral PASH was most common in subjects with PD and DLB, where it appears likely that it is universally widespread. Subjects with ILBD had lesser densities of PASH within all regions, but had frequent involvement of the spinal cord and paraspinal sympathetic ganglia, with less-frequent involvement of end-organs. Subjects with ADLB had infrequent involvement of the spinal cord and paraspinal sympathetic ganglia with rare involvement of end-organs. Within the gastrointestinal tract, there was a rostrocaudal gradient of decreasing PASH frequency and density, with the lower esophagus and submandibular gland having the greatest involvement and the colon and rectum the lowest.

Keywords Parkinson's disease · Parkinsonism · Dementia with Lewy bodies · Alzheimer's disease · Incidental Lewy bodies · α-Synuclein · Spinal cord · Sympathetic nervous system · Peripheral nervous system · Autonomic nervous system · Enteric nervous system · Submandibular gland · Esophagus · Adrenal gland · Heart · Stomach · Gastrointestinal system

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Introduction

The topographical distribution and density of Lewy bodies and their associated abnormal neurites are much greater than formerly appreciated [8, 28, 31, 33, 37, 44, 46, 61, 64, 70, 71, 77, 82]. Furthermore, it is also now more clearly apparent that Lewy body pathology frequently extends to the spinal cord and peripheral nervous system [12, 14, 17–19, 29, 40, 48, 81]. Despite these recent achievements, there has not yet been published a wide survey of the distribution of Lewy-type histopathological changes in the peripheral nervous system. A sensitive immunohistochemical method for phosphorylated α-synuclein [8, 10]

was used to stain sets of spinal cord and peripheral nervous system sections from 92 subjects that had previously received neuropathological diagnoses and Lewy body central nervous system (CNS) staging. The densities and frequencies of occurrence of phosphorylated α-synuclein histopathology (PASH) in multiple regions of spinal cord, sympathetic ganglia and tissue from the major organ systems were tabulated and correlated with diagnostic category. The results are presented below.

Materials and methods

Human subjects

Deceased human subjects were autopsied at Sun Health Research Institute (SHRI), a division of the not-for-profit health care provider Banner Health, located in the Sun Cities retirement communities of northwest metropolitan Phoenix, Arizona. All subjects had volunteered for the SHRI Brain and Body Donation Program (BBDP) [5, 9]. The majority of BBDP subjects are clinically characterized at SHRI with annual standardized test batteries that include functional, neuropsychological and neuromotor components, including the Mini Mental State Examination (MMSE) and Unified Parkinson's Disease Rating Scale (UPDRS). In addition, private medical records are requisitioned and reviewed for each subject and the postmortem Dementia Questionnaire [32] or an adaptation of the Clinical Dementia Rating Scale (CDR) are administered to subject contacts to help determine the presence or absence of dementia for those subjects lacking standardized antemortem evaluations. Subjects sign consent that has been approved by the Banner Health Institutional Review Board.

Subjects were chosen by searching the BBDP database for all cases that had received a whole-body autopsy, a completed neuropathologist's examination and a neuropathologic diagnosis of a Lewy body disorder, including Parkinson's disease (PD), dementia with Lewy bodies (DLB), incidental Lewy body disease (ILBD) and Alzheimer's disease with Lewy bodies (ADLB). Comparison groups were composed of subjects without evidence of dementia or parkinsonism (normal elderly subjects) and subjects with AD but no Lewy body pathology (ADNLB).

Subjects received standardized neuropathological examinations. Specific clinicopathologic diagnostic criteria were used for AD [1], PD [36], and DLB [58]. For both AD and DLB, cases received the diagnosis if they were classified as "intermediate" or "high" probabilities in their respective classification schemes. Cases with PASH, but not meeting these diagnostic criteria were designated as either ILBD, if they had no clinical history of parkinsonism or dementia, or ADLB if they had dementia, Alzheimer's disease and Lewy bodies in any brain region, but failed to meet clinicopathologic criteria for DLB or PD.

Gross and microscopic neuropathologic assessments were made by a single observer (TGB) without knowledge of the clinical history or clinical diagnosis; subsequently the clinical history was reviewed with the neurologists (CHA, JNC, HAS, MNS) in order to make an appropriate clinicopathologic diagnosis.

Histologic methods

Diagnostic histologic methods were performed on standard blocks of tissue that were fixed in 3.75% neutral-buffered formaldehyde and then either dehydrated and embedded in paraffin or cryoprotected and cut on a freezing, sliding microtome. Each case was first staged according to the Unified Staging System for Lewy body disorders with a standard set of brain sections stained with an immunohistochemical method for phosphorylated α-synuclein as previously described [8]. The Unified Staging System is a modification of the scheme first devised by the Dementia with Lewy Bodies Consortium [8, 58, 59].

Paraffin-embedded sections from multiple body sites (Table 1) were stained in an identical fashion as the brain sections, using a polyclonal antiserum raised against an α-synuclein peptide fragment phosphorylated at serine 129, after epitope exposure with proteinase K [34]. The process leading to the choice of immunohistochemical method, as well as details of the method, has been described in a previous publication [10]. In each body region, the density of α-synuclein-immunoreactive perikaryal neuronal cytoplasmic inclusions as well as puncta and neurites was scored, at the site of highest density, 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 [58]. The total number of body sites examined varied between subjects as an initially broad sampling scheme was progressively reduced to those regions showing a greater likelihood to have positive staining. To evaluate the relative frequency of immunoreactivity in the different body regions, paraffin sections on a single stained slide from each body site listed in Table 1 were used. Following this analysis, selected regions of interest were further evaluated with up to five additional paraffin sections and/or 80 μm thick formalin-fixed, frozen sections.

Alzheimer's disease histopathology was staged and graded on 40 μm thick sections stained with the Gallyas method for neurofibrillary tangles and the Campbell–Switzer and thioflavine-S methods for senile plaques [15]. Braak's neurofibrillary tangle stages and CERAD neuritic plaque densities were assigned as described [16, 62].

Table 1 Body sites, organs and tissue types investigated with an immunohistochemical method for phosphorylated α-synuclein histopathology

Body region	Sites investigated within region
Spinal cord	Cervical (C4–5), thoracic (T6–7), lumbar (L3–4) and sacral (S1–5) spinal cord
Sympathetic ganglia	Middle cervical ganglia, middle ganglia of thoracic chain
Vagus nerve	Alongside the common carotid artery at the level of the larynx
Sciatic nerve	Overlying the external iliac artery in the pelvic cavity
Gastrointestinal system	Upper third and lower third of esophagus, stomach (body), duodenum, jejunum, ileum, transverse colon, sigmoid colon, rectum, submandibular gland, liver, pancreas (head), gallbladder
Respiratory system	Larynx, primary bronchus, lung
Endocrine system	Adrenal gland, thyroid gland, parathyroid gland, ovary, testis
Cardiovascular system	Thoracic and abdominal aorta, left and right ventricle and epicardium of heart at apex
Genitourinary tract	Kidney, urinary bladder, uterus, vagina
Musculoskeletal system	Rib (bone and associated muscle and soft tissue), psoas muscle, diaphragm
Skin	Abdominal skin, scalp
Miscellaneous	Spleen, lymph nodes (parabronchial), small bowel mesentery, breast

Statistical analysis

Statistical analyses consisted of, for comparing group means, analysis of variance (ANOVA), or, for non-parametric data, Kruskall–Wallis ANOVA. Proportional measures were compared using χ^2 tests.

Results

Ninety-two subjects had a full autopsy as well as neuropathological diagnoses within the targeted groups. All data from the study are given within the supplementary online table, for brevity some of the data are grouped for summary here. Descriptive measures of these groups are given in Tables 2, 3 and 4. The subjects were all of advanced age (Table 2) and the group means differed significantly ($P < 0.0001$), with the youngest group (PD) having a mean age of 79.3 while the oldest group (ILBD) mean was 86.7. As with all subjects of this advanced age, all had at least some neurofibrillary tangles in the brain [13] and even the elderly control group had a mean Braak stage of 2.6. All diagnostic groups had more male subjects and this was pronounced in the PD and DLB groups. The median postmortem intervals were uniformly short, ranging from 2.7 to 5.9 h and the group means were not significantly different.

The distribution of PASH within the brain is summarized in Table 3 using the Unified Staging System for Lewy Body Disorders and the mean densities of PASH within the ten scored brain regions are given in Table 4. An estimate of the aggregate CNS load of microscopic disease was also determined, using the sum of the mean PASH density scores in all ten evaluated brain regions (Table 4).

Table 2 General characteristics of the study subjects, by neuropathologic diagnosis, age, gender, postmortem interval (PMI), Braak neurofibrillary stage and CERAD neuritic plaque (NP) density

Diagnosis (*N*)	Age[a]	Gender (% male)	PMI (h)	Braak stage[a]	CERAD NP density[a]
Normal (23)	81.0 (14.1)	56.5	3.9 (2.5)	2.6 (1.3)	1.0 (1.3)
ILBD (7)	86.7 (8.5)	57.1	3.8 (2.9)	3.0 (1.0)	1.3 (1.4)
PD (17)	79.3 (7.5)	76.5	4.9 (4.1)	2.5 (1.1)	0.8 (1.2)
DLB (9)	83.2 (9.5)	67	2.7 (0.4)	4.4 (0.7)	3.0 (0.0)
ADLB (19)	84.0 (5.0)	53.0	4.3 (3.8)	5.2 (1.0)	2.7 (0.7)
ADNLB (17)	84 (5.9)	53.0	5.9 (10.2)	4.4 (1.2)	2.8 (0.4)

Means and standard deviations (SD) are given

Subjects did not differ significantly in terms of gender distribution or PMI but differed significantly in terms of age, Braak stage and CERAD neuritic plaque density

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

Table 3 Classification of subjects with phosphorylated α-synuclein histopathology by the Unified Staging System for Lewy body disorders

Diagnosis	Olfactory bulb only (I)	Brainstem predominant (IIa)	Limbic predominant (IIb)	Brainstem and limbic (III)	Neocortical (IV)
ILBD[a]	0	1 (17%)	2 (33%)	3 (50%)	0
PD[a]	0	2 (12.5%)	0	10 (62.5%)	4 (25%)
DLB	0	0	0	1 (11%)	8 (89%)
ADLB	2 (10%)	3 (16%)	11 (58%)	3 (16%)	0

Number and percentage of subjects in each stage are given

[a] One subject was not classifiable due to missing brain regions needed for staging

Table 4 Mean (SD) of phosphorylated α-synuclein histopathology regional brain density scores for all subjects by diagnostic classification, with an aggregate total brain load (last row) given by the sum of the mean regional density scores

Brain region	ILBD	PD	DLB	ADLB
Olfactory bulb	2.5 (1.0)	2.6 (0.9)	3.6 (1.1)	3.0 (0.9)
Medulla	1.6 (1.6)	3.2 (0.7)	3.4 (0.7)	1.0 (1.2)
Pons	1.1 (1.5)	3.0 (0.9)	3.1 (1.0)	0.3 (0.6)
Midbrain	0.4 (0.8)	3.0 (0.9)	2.9 (1.5)	0.2 (0.7)
Amygdala	2.5 (1.6)	3.1 (0.9)	3.9 (0.3)	2.4 (1.7)
Transentorhinal	0.8 (1.8)	2.3 (1.1)	3.6 (0.7)	2.4 (1.8)
Cingulate cortex	0.3 (0.8)	1.8 (1.0)	2.9 (1.2)	0.3 (0.4)
Mid. temp. gyrus	0.3 (0.5)	1.2 (1.1)	2.6 (0.9)	0.2 (0.4)
Mid. front. gyrus	0.3 (0.5)	1.1 (0.9)	1.7 (1.1)	0.05 (0.2)
Inf. par. lobule	0.3 (0.5)	1.0 (1.0)	1.8 (1.0)	0.05 (0.2)
Sum of mean scores	10.1	22.3	29.5	9.9

More than 80% of subjects with PD and DLB were in the two highest stages, involving either brainstem through limbic regions (Stage III) or brainstem through neocortical regions (Stage IV). Most subjects with ILBD were in the brainstem-predominant (IIa) or limbic-predominant (IIb) stages while the most frequent stage for ADLB subjects was limbic predominant (IIb). Subjects with ILBD and ADLB had similar overall low aggregate PASH load scores while subjects with PD and DLB had overall high aggregate PASH load scores.

Figure 1 shows photomicrographs of the immunohistochemical staining for α-synuclein in the spinal cord, sympathetic ganglia and vagus nerve. Generally, sections with positive staining contained fibers, puncta and neuronal perikaryal staining (a, c, e, f, h) but occasionally sections or regions contained just fibers (i) or just perikaryal staining. The perikaryal staining was either diffusely distributed in the cytoplasm (e) or condensed into defined inclusions (h), only a subset of which resembled classical Lewy bodies. Within the vagus and peripheral nerves, and generally within nerves seen elsewhere in other body regions, positive staining was only in the form of nerve fibers (i). Within the spinal cord, positively-stained structures were found in all gray matter regions, including posterior horn (b, c), anterior horn (d, e), intermediolateral region (a) and adjacent to the central canal (f). Although not formally analyzed here, the most frequent regions affected appeared to be the thoracolumbar intermediolateral horn and the base of the posterior horn of the sacral cord.

Figure 2 shows photomicrographs of the immunohistochemical staining for α-synuclein in multiple bodily organs and tissue types. As described for the spinal cord and sympathetic ganglia above, sections with positive staining contained either fibers, puncta or neuronal (ganglion cell) perikaryal staining, but ganglion cell staining was much less frequently seen, with the great majority of immunoreactive elements being fibers and puncta. A much more detailed morphologic delineation was seen in 80-μm thick sections of submandibular gland and lower esophagus (Fig. 3), where extensive fiber networks and moderately frequent ganglion cells were often visualized, especially in subjects with PD or DLB. Generally, immunoreactive structures in the gastrointestinal tract were concentrated in the myenteric plexus of Auerbach and the submucosal plexus of Meissner. In the submandibular gland and pancreas, positively stained elements tended to be concentrated in nerve bundles within the connective tissue stroma. In the submandibular gland, 80-μm section staining occasionally showed nerve fibers apparently investing arterioles (Fig. 3d). Adrenal gland staining was exclusively seen in the medulla and in nerve bundles in the surrounding fatty tissue, with no staining seen in the adrenal cortex.

The regional distribution of PASH, as evaluated in single microscopic slides from each body site, is shown in Table 5 and Fig. 4. No immunoreactive elements were present in any subject that had previously been classified, on the basis of the brain examination, as being free of PASH. Subjects with DLB and PD had similar profiles (Fig. 4b, c) and will be discussed together. In both the groups, the most frequently affected body region was the spinal cord, with 25 of 26 subjects involved, followed by the sympathetic ganglia (19/24), the vagus nerve (15/21), the gastrointestinal system (16/26), the sciatic nerve (10/22) and endocrine system (5/14), with other organ systems and tissues following at generally much lower frequencies.

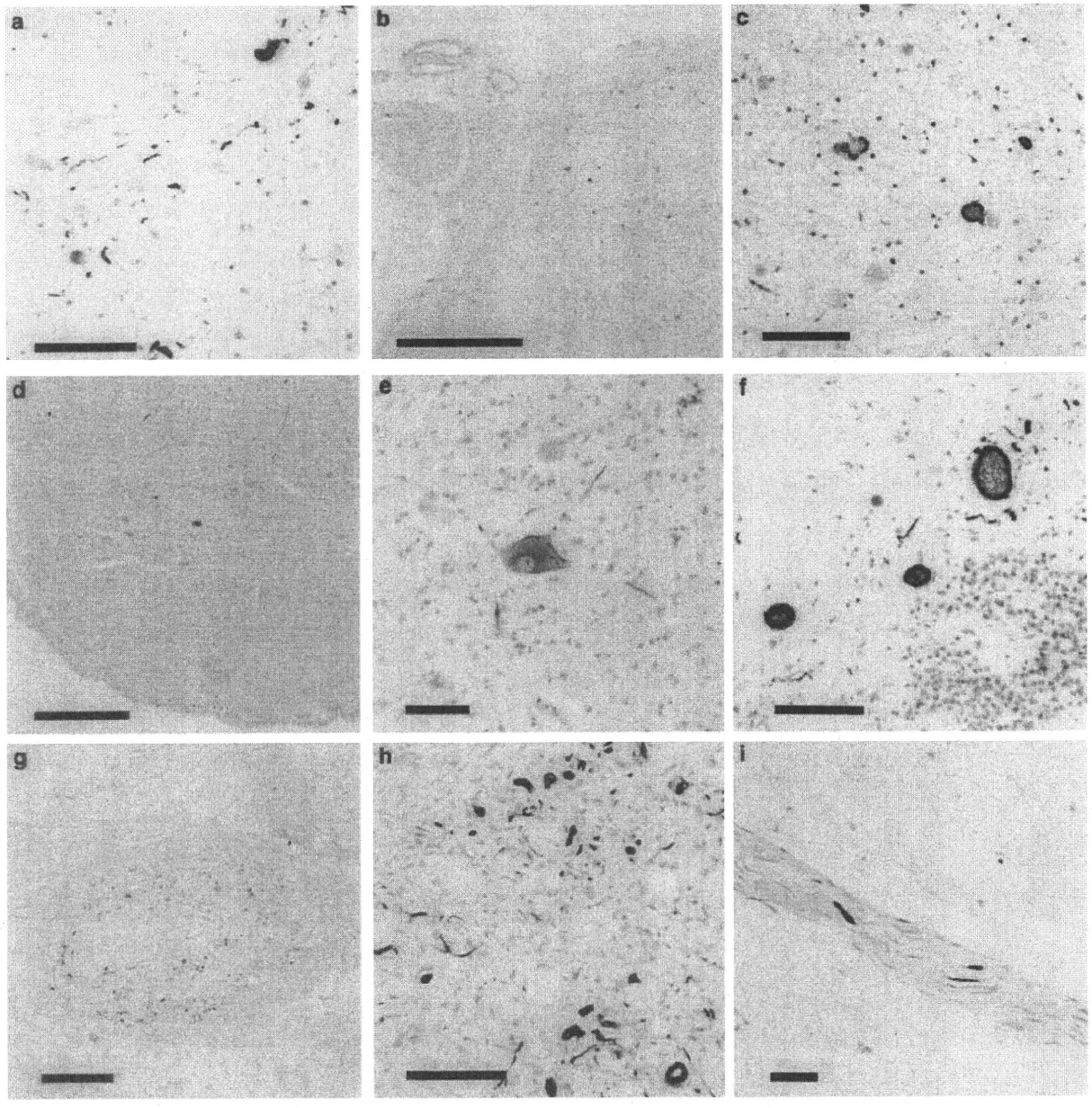
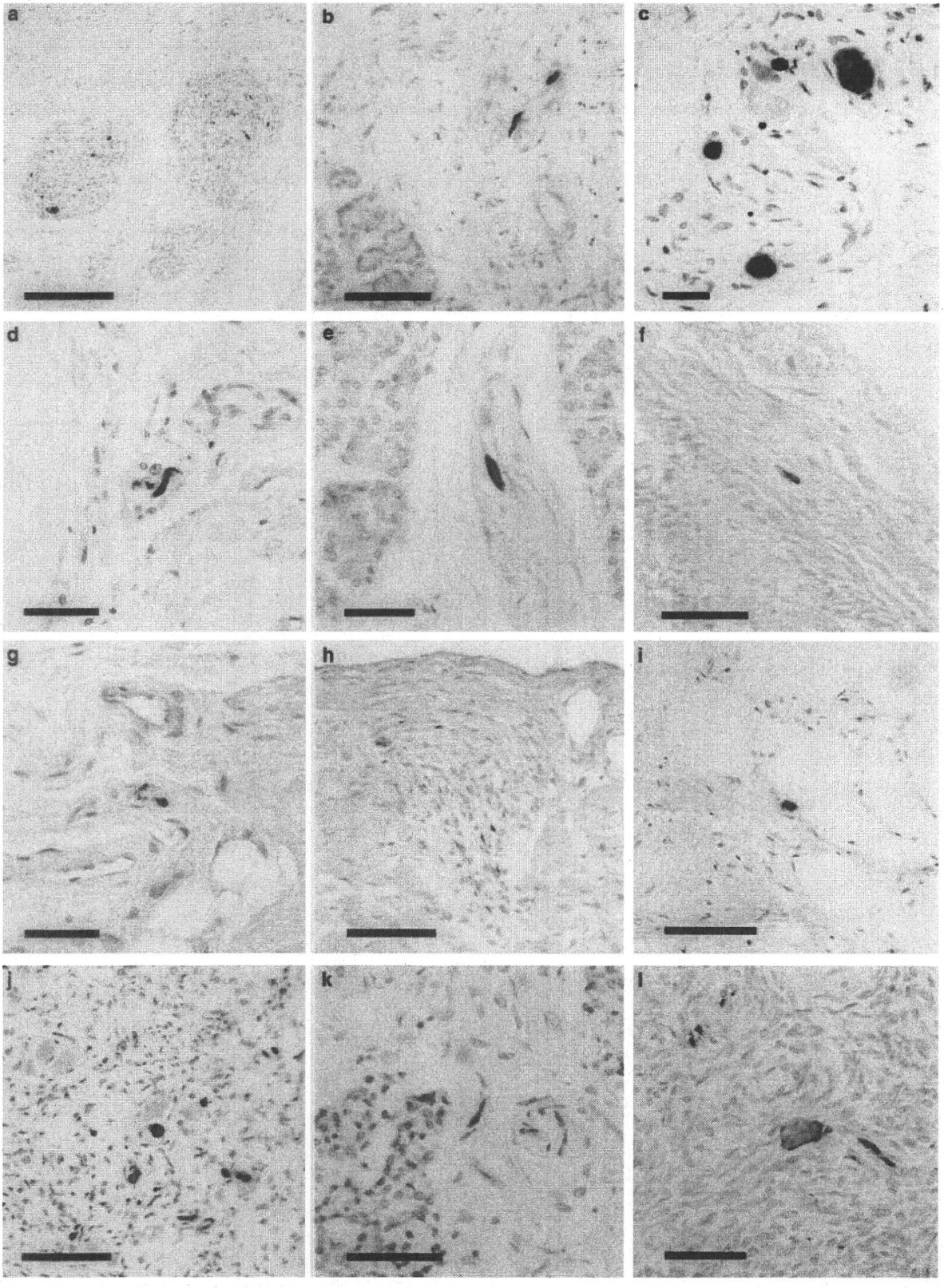


Fig. 1 Photomicrographs of slides of immunohistochemical staining for phosphorylated α-synuclein histopathology (*PASH*) in paraffin sections of the spinal cord, sympathetic ganglia and vagus nerve. Positive staining is *black*, the counterstain is neutral red. **a** The intermediolateral horn of the thoracic spinal cord of a subject with PD, showing immunoreactive fibers, puncta and perikaryal cytoplasmic inclusions. *Calibration bar* 80 μm. **b**, **c** Low and higher magnification images of the posterior root entry into the sacral spinal cord of a subject with DLB, showing immunoreactive neuronal perikaryal staining. *Calibration bar* in **b** 0.3 mm; in **c** 80 μm. **d**, **e** Low and higher magnification images of the anterior horn of the sacral spinal cord in a subject with PD, showing diffuse cytoplasmic perikaryal immunoreactivity of a large motorneuron. *Calibration bar* in **d** 0.5 mm; in **e** 60 μm. **f** Sacral spinal cord adjacent to the central canal of a subject with PD, showing immunoreactive swollen degenerating neurons or neurites. *Calibration bar* 80 μm. **g**, **h** Low and higher magnification images of a middle cervical sympathetic ganglion of a subject with DLB, showing frequent immunoreactive dystrophic neurites, puncta and neuronal perikaryal cytoplasmic inclusions. *Calibration bar* in **g** 0.1 mm; *calibration bar* in **h** 100 μm. **i** Small branch of the vagus nerve in a subject with PD, showing several immunoreactive nerve fibers. *Calibration bar* 60 μm

No positive staining was observed in the abdominal skin in any of the 14 subjects with DLB or PD. Examination of additional sets of immunostained serial paraffin and 80-μm sections of submandibular gland and esophagus from DLB and PD subjects showed positive staining in 22 of 23 subjects for which the extra sections were stained.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◀ **Fig. 2** Photomicrographs of immunohistochemical staining for phosphorylated α-synuclein in paraffin sections of bodily organs and tissue. Positive staining is *black*, the counterstain is neutral red. **a**, **b** Low and higher magnification images of the submandibular gland in two subjects with PD, showing immunoreactive nerve fibers within the stroma of the gland. *Calibration bar* in **a** 0.2 mm; in **b** 100 μm. **c** The submucosa of the lower esophagus of a subject with PD, showing immunoreactive puncta, fibers and perikaryal cytoplasmic inclusions in ganglion cells. **d** Immunostaining of fibers in the duodenal submucosa of a subjects with DLB. *Calibration bar* 20 μm. **e** A single immunoreactive fiber in the stroma of the pancreas from a subject with PD. *Calibration bar* 40 μm. **f** A single immunoreactive fiber in the submucosa of a primary bronchus of a subject with PD. *Calibration bar* 40 μm. **g** A few immunoreactive fibers in the submucosa of the larynx of a subject with PD. *Calibration bar* 20 μm. **h** Several immunoreactive fibers in an epicardial nerve twig entering the myocardium in a subject with DLB. *Calibration bar* 100 μm. **i** A single immunoreactive fiber in the intermyenteric plexus of the urinary bladder of a subject with DLB. *Calibration bar* 20 μm. **j** Frequent immunoreactive fibers, puncta as well as cells with diffusely stained perikaryal cytoplasm in the adrenal medulla of a subject with PD. *Calibration bar* 100 μm. **k** A single immunoreactive fiber in the stroma of the parathyroid gland of a subject with PD. *Calibration bar* 10 μm. **l** The ovary of a woman with PD showing diffuse perikaryal immunostaining of a neuron-like cell with adjacent immunoreactive fibers and puncta. *Calibration bar* 10 μm

Subjects with ILBD and ADLB had much lower frequencies of positive staining in body regions (Fig. 4a, d). In subjects with ADLB, positive staining was limited entirely to the spinal cord and sympathetic ganglia, with only 1/18 and 2/18 subjects affected, respectively. Subjects with ILBD showed higher frequencies of positive staining than ADLB subjects in the spinal cord, sympathetic ganglia, vagus nerve and gastrointestinal tract while all other areas, as with the ADLB cases, showed no positive staining. When several microscopic slides from each spinal cord subdivision were stained, 5/6 of the ILBD cases had PASH present while on single-slide analysis, only 1 of 6 subjects had been positive. For the ADLB subjects for which serial paraffin and 80-μm sections of submandibular gland and esophagus were examined, 3/15 were found to be positive, whereas examination of single paraffin sections had found no positively stained structures. Figure 4e depicts the regional staining distribution, derived from analysis of a single slide per region, when data from all subjects were combined. This shows generally the same pattern as for PD and DLB subjects.

Table 6 and Fig. 5a and b show the regional distribution and density of PASH within paraffin sections on single slides of the major subdivisions of the spinal cord as well as cervical and thoracic sympathetic ganglia, for all subjects combined. The frequency of positive staining between different cord regions is similar, with the cervical cord being the least-often affected (Fig. 5a). In terms of the density scores, the thoracic and sacral cord regions have higher mean scores than the cervical and lumbar regions. Cervical sympathetic ganglia had the highest frequency and mean density scores of any region.

Table 7 and Fig. 5c and d show the regional distribution and density of PASH within major sites within the gastrointestinal (GI) system. There is a marked trend for a diminishing rostrocaudal gradient. The submandibular gland and lower esophagus have the highest frequency of PASH, followed by the stomach, small bowel regions, large bowel regions and rectum. The data shown for submandibular gland and esophagus do not include, to provide an unbiased comparison between GI regions, results from the serial paraffin or 80-μm sections. As mentioned previously, when staining from these extra sections was considered, a higher percentage of subjects were graded as positive. A striking finding was the complete absence of positive staining in upper esophagus and therefore all data for esophagus shown are derived from analysis of the lower esophagus. Although not quantitatively assessed here, it was apparent that PASH was more frequently present in the myenteric plexus than in the submucosal plexus.

Discussion

The results of this investigation support prior studies [12, 14, 17–19, 29, 40, 48, 81] that have indicated that PASH is widespread throughout the spinal cord and peripheral nervous system of subjects with PD. The present study adds to this body of knowledge by providing data for other Lewy body disorders including DLB, ILBD and ADLB, by surveying many more sites than had previously been investigated, and using large enough sample sizes to provide preliminary estimates of the relative frequency and density of α-synuclein histopathology at these locations. In addition, the use of a method that is specific and sensitive for phosphorylated α-synuclein, which is detected only in pathological structures [34] effectively eliminates the ambiguity of staining results derived using antibodies that recognize normal α-synuclein.

For spinal cord and sympathetic ganglia, subjects with PD as well as DLB invariably have PASH in the regions of the cord containing preganglionic autonomic neurons as well as within sympathetic ganglia. Because these neurons project widely throughout the body, it is highly probable that, for subjects with PD and DLB, that α-synuclein histopathology is also very widely present within the end-organ targets of the autonomic nervous system, although a full test of this hypothesis will require multiple-section examination of each of the many sites [49]. This study has found that the relative frequency of PASH in end-organs was generally much lower than in the spinal cord or sympathetic ganglia, but when sampling was expanded

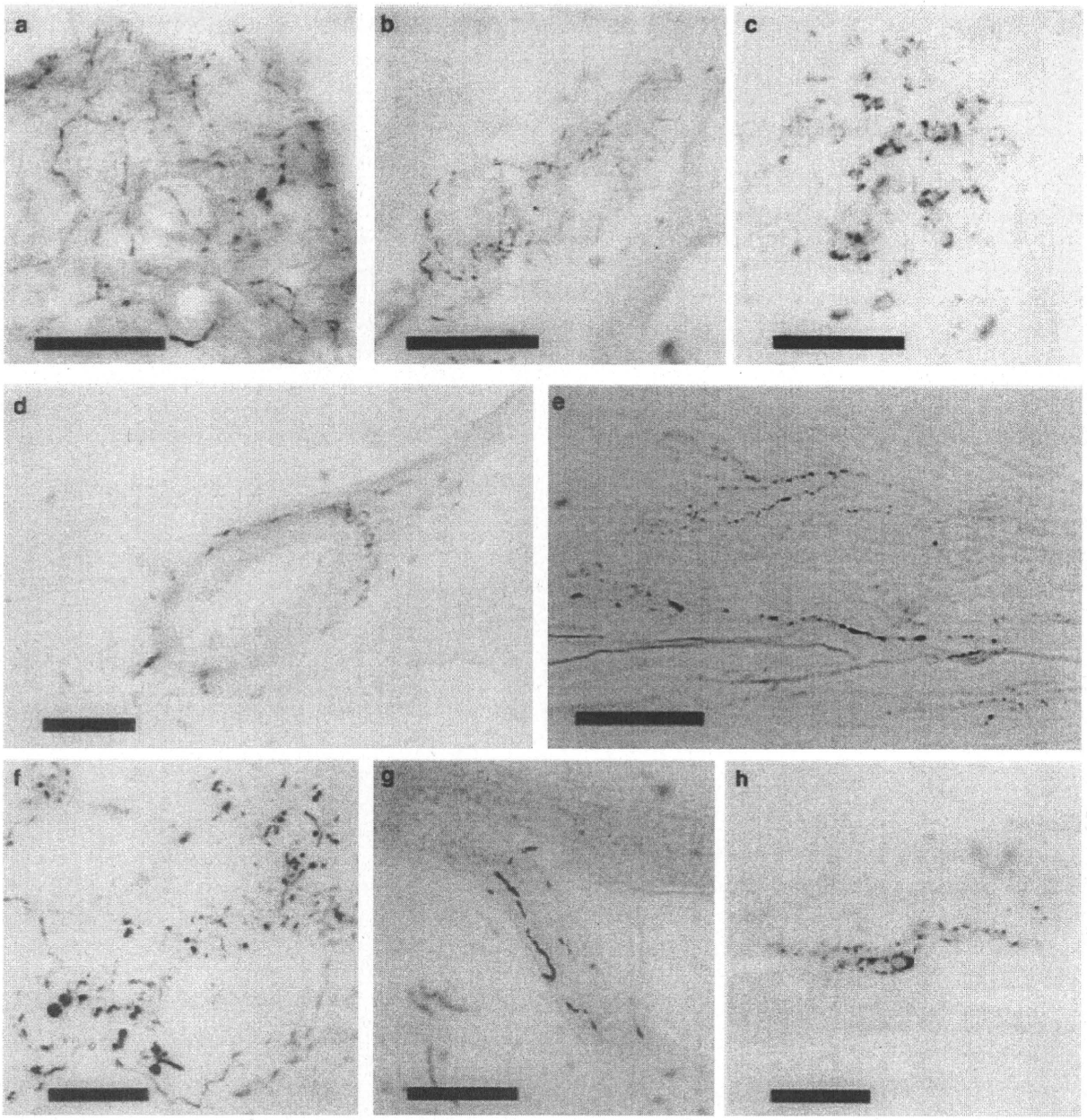

Fig. 3 Photomicrographs of immunohistochemical staining for phosphorylated α-synuclein in 80-μm thick frozen sections of formalin-fixed, cryoprotected tissue blocks of submandibular gland and lower esophagus. Positive staining is *black*. There is no counterstain. Submandibular gland from subjects with DLB (**a–c**) and PD (**d**) showing frequent immunoreactive fibers in the gland parenchyma (**a**), stroma (**b**) and around a small artery (**d**), while frequent immunoreactive puncta are seen in **c**. Lower esophagus from subjects with PD (**e**, **g**, **h**) and DLB (**f**) showing immunoreactive nerve fibers in **e**, nerve fibers and puncta in **f**, a thickened nerve fiber adherent to a bundle of smooth muscle fibers in **g** and a ganglion cell with diffuse perikaryal cytoplasmic staining in **h**

considerably through the use of multiple paraffin or 80-μm sections, such as was done for the submandibular gland and esophagus, the frequency of positive staining was increased.

For subjects with ILBD, PASH has a more limited distribution, being more likely to be confined to the spinal cord, sympathetic ganglia, vagus nerve and a subset of end-organs. Given the wide projections of the involved spinal preganglionic and sympathetic ganglion neurons, a wide but sparse involvement of the peripheral nervous system is likely to exist in ILBD, although again a more definitive investigation with multiple-section analysis will be

Table 5 Regional frequency of phosphorylated α-synuclein histopathology in single slides of different body regions, with subjects grouped by neuropathological diagnosis

Dx	SpCd	Sym	Vagus	Sciat	GI	Resp	Endo	Cardio	GU	MSK	Skin
ILBD	1/6[a]	3/6	2/7	1/6	1/7	0/4	1/4	0/6	0/4	N/A	0/2
PD	16/17	12/15	11/15	8/16	11/17[b]	1/8	2/9	0/9	1/8	0/6	0/8
DLB	9/9	7/9	4/6	2/8	5/9[c]	1/4	3/5	1/4	2/5	0/3	0/3
ADLB	1/19	2/15	1/15	0/17	1/19[d]	0/10	0/11	0/12	0/10	0/7	0/9
All	27/51	24/45	18/43	11/47	18/52	2/26	6/29	1/31	3/29	0/16	0/22

See Table 1 for listing of individual sites sampled and Fig. 4 for graphic representation; see supplementary online table for frequency of PASH within individual sites

Dx diagnosis, *SpCd* spinal cord, *Sym* sympathetic ganglia, *Vagus* vagus nerve, *Sciat* sciatic nerve, *GI* gastrointestinal system, *Resp* respiratory tract, *Endo* endocrine system, *Cardio* cardiovascular, *GU* genitourinary tract, *MSK* musculoskeletal

[a] 6/7 when multiple slides of paraffin-embedded spinal cord were examined

[b] 14/15 when multiple slides of paraffin-embedded and 80-μm frozen sections of esophagus and submandibular gland were examined

[c] 8/8 when multiple slides of paraffin-embedded and 80-μm frozen sections of esophagus and submandibular gland were examined

[d] 3/15 when multiple slides of paraffin-embedded and 80-μm frozen sections of esophagus and submandibular gland were examined

required to confirm this. Subjects with ADLB have a very restricted frequency and distribution of PASH, with very sparse involvement outside the spinal cord and sympathetic ganglia, detectable only when multiple sections are examined.

Other groups have reported higher frequencies for PASH in selected body regions including skin, adrenal medulla, urinary bladder and cardiac epicardium [35, 43, 45, 61]. Each of these earlier studies stained multiple slides from each site, however, probably accounting for most of the differences from the present work, in which for most of these regions only a single slide was stained. Differing sites of sampling may also have been responsible for differing results, for example Iwanaga et al. [45] sampled the heart from around the coronary arteries while in the present study the cardiac apex was sampled. Differences in staining methods may also have contributed to the different findings. Minguez-Castellano et al. [61] used an antibody against normal (unphosphorylated) α-synuclein while in the present study the antibody was specific for α-synuclein phosphorylated at serine 129, which is found only in pathological α-synuclein deposits [34]. Most other groups have used formic acid pretreatment for epitope exposure while the present study used proteinase K pretreatment [10, 41]. Proteinase-K would theoretically destroy normal α-synuclein and thus further eliminate non-specific (non-pathological) staining.

A critical question has been whether or not α-synuclein histopathology begins in the brain or within elements of the peripheral nervous system [20, 38, 55]. The stimulus for this intriguing hypothesis has come largely from clinical studies of PD that have found a wide range of non-motor signs and symptoms that accompany the disease [4, 78]. Many of these non-motor accompaniments are related to dysfunction of the peripheral autonomic system. These may occur early in the motor progression and there is suggestive evidence that some may even occur in the premotor prologue [2, 3, 47, 66, 74]. The description of Lewy bodies within the sympathetic and parasympathetic ganglia, adrenal medulla and GI tract within autopsied subjects with PD [17, 21, 29, 35, 39, 50, 75] has shown that peripheral nervous system α-synuclein histopathology is certainly present but there has been insufficient data regarding the findings in prodromal phases of disease. Autopsy studies of relatively small numbers of subjects with ILBD have demonstrated a high prevalence of α-synuclein histopathology within the spinal cord, sympathetic ganglia, adrenal medulla and upper GI tract [12, 17, 21, 48, 61], consistent with accumulating reports of premotor autonomic dysfunction in PD [78], but only two subjects, out of more than a thousand examined in recent studies, have had α-synuclein histopathology in the spinal cord or peripheral nervous system in the absence of brain involvement. Fumimura et al. [35] reported one case out of 783 with adrenal medulla as the only site with α-synuclein histopathology, but the olfactory bulb was not examined. Miki et al. [60] reported a single subject with α-synuclein histopathology restricted to the heart and stellate ganglion; in this case, the olfactory bulb was examined. The present work is in general agreement with these prior studies as, of the 40 subjects without brain and olfactory bulb PASH, none had PASH within the spinal cord or peripheral nervous system sites sampled. However, owing to the relatively small sample size and single-section analysis at many sites, it cannot be excluded that α-synuclein pathology may rarely begin in the peripheral nervous system prior to CNS involvement.

A derivative of the "body-first" hypothesis has been the conjecture as to whether an exogenous pathogen might be the cause of disease and gain entry through peripheral

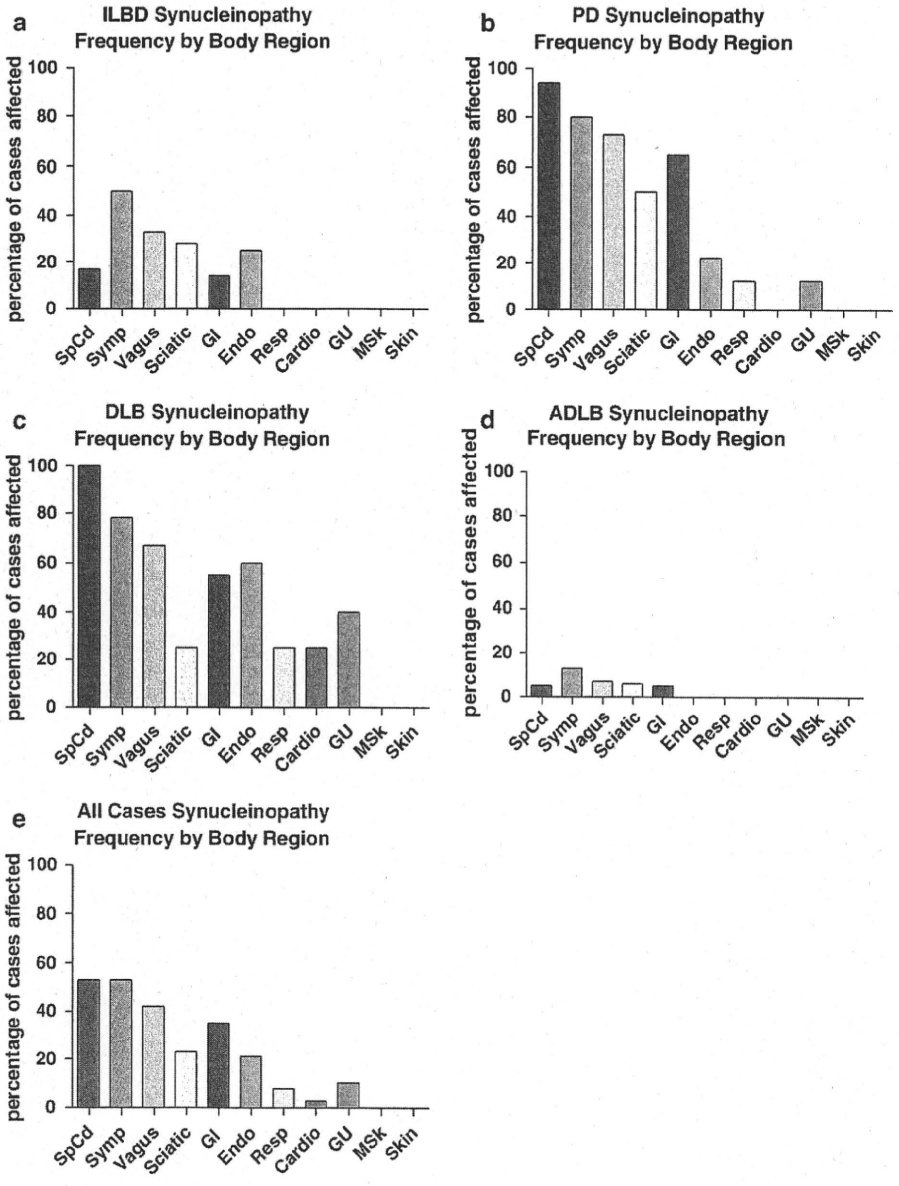

Fig. 4 Relative frequency of PASH by diagnostic group for phosphorylated α-synuclein in different body regions including spinal cord, sympathetic ganglia, vagus nerve, sciatic nerve and multiple organs and tissues. Relative frequency is the percentage of subjects that showed immunoreactive tissue elements of any kind (fibers, puncta, perikaryal diffuse staining, perikaryal cytoplasmic inclusions) in single slides from each of the sites evaluated. For list of individual sites within each body region or organ system, see Table 1 and supplementary online table. Frequency was investigated further for some sites (see text)

Table 6 Frequency and mean (SD) density of with phosphorylated α-synuclein histopathology in single slides from major spinal cord and sympathetic nervous system subdivisions (all cases considered together)

	Cervical	Thoracic	Lumbar	Sacral	CSymp	ThSymp
Frequency	23/49 (47%)	26/47 (55%)	25/49 (51%)	25/48 (52%)	14/20 (70%)	19/36 (53%)
Density score	0.7 (0.9)	1.2 (1.3)	0.9 (1.1)	1.4 (1.5)	2.1 (1.6)	1.5 (1.6)

See Fig. 5 for a graphic representation

CSymp cervical sympathetic, *ThSymp* thoracic sympathetic

nerve endings [20, 38], either through the olfactory epithelium or GI mucosa. The findings of the present study are not incompatible with a GI entry for PD, ILBD and DLB, but as subjects with ADLB have relatively rare or sparse involvement of the caudal neuraxis this seems unlikely for that group. The universal and primal involvement of the olfactory bulb in all of the Lewy body disorders [8, 11] is highly compatible with the exogenous pathogen

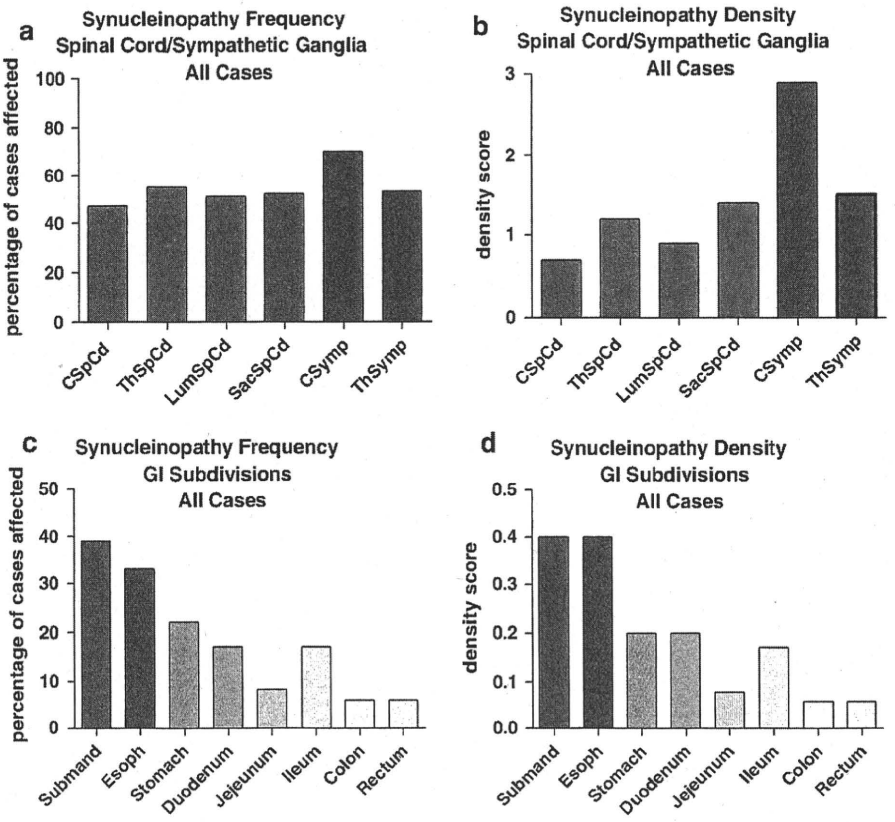

Fig. 5 Relative frequency and density of PASH by diagnostic group in different spinal cord regions and sympathetic nervous system subdivisions. Relative frequency is the percentage of subjects that showed immunoreactive tissue elements of any kind (fibers, puncta, perikaryal diffuse staining, perikaryal cytoplasmic inclusions) in single slides taken from each of the sites evaluated. Density was calculated only for sites with immunoreactivity, negative or "zero" scores were not included

Table 7 Frequency of and mean density of with phosphorylated α-synuclein histopathology in single slides of subdivisions of the gastrointestinal system (all cases considered together)

	Submand	Esoph	Stomach	Duodenum	Jejunum	Ileum	Colon	Rectum
Frequency	11/37 (39%)	17/51 (33%)	7/31 (22%)	5/30 (17%)	2/24 (8.3%)	4/24 (17%)	3/50 (6%)	3/50 (6%)
Density score	0.4 (0.7)	0.4 (0.7)	0.2 (0.4)	0.2 (0.6)	0.08 (0.28)	0.17 (0.4)	0.06 (0.2)	0.06 (0.2)

See Fig. 5 for a graphic representation

Submandib submandibular gland, *esoph* lower esophagus

hypothesis. If an exogenous pathogen is involved, whether it be a virus, micro-organism or toxin, if it was able to induce aggregation of α-synuclein in exposed neurons, this change could then be propagated throughout the remainder of the PNS and CNS, generating the observed brain and spinal cord regional pattern of α-synuclein histopathology through trans-synaptic transmission. Recently, there have been reports of PD subjects that have developed Lewy bodies within non-host neurons transplanted into the striatum more than a decade earlier, supporting the possibility that α-synuclein histopathology might be acquired and passed along from neuron to neuron [51–53, 57]. In relation to this, experimental studies have recently shown that aggregated α-synuclein may be transferred between neurons by endocytosis [26, 27, 30].

The rostrocaudal gradient of PASH within the gastrointestinal system is an interesting finding of the present work and confirms a previous report by Wakabayashi et al. [81] who mapped Lewy bodies in the alimentary tract of seven PD subjects using classical stains. The reason for this rostrocaudal gradient may be of interest. It could be due to the known distribution of vagal innervation, which extends only as far as the proximal colon and which has been documented to be more heavily distributed to the lower esophagus and stomach than to the small bowel or proximal colon [25, 42]. If so, this would suggest that, for the gastrointestinal tract, α-synuclein histopathology within vagal efferents predominates over α-synuclein histopathology originating from sympathetic ganglia or from enteric neurons. However, as Lewy bodies and α-synuclein

was used to stain sets of spinal cord and peripheral nervous system sections from 92 subjects that had previously received neuropathological diagnoses and Lewy body central nervous system (CNS) staging. The densities and frequencies of occurrence of phosphorylated α-synuclein histopathology (PASH) in multiple regions of spinal cord, sympathetic ganglia and tissue from the major organ systems were tabulated and correlated with diagnostic category. The results are presented below.

Materials and methods

Human subjects

Deceased human subjects were autopsied at Sun Health Research Institute (SHRI), a division of the not-for-profit health care provider Banner Health, located in the Sun Cities retirement communities of northwest metropolitan Phoenix, Arizona. All subjects had volunteered for the SHRI Brain and Body Donation Program (BBDP) [5, 9]. The majority of BBDP subjects are clinically characterized at SHRI with annual standardized test batteries that include functional, neuropsychological and neuromotor components, including the Mini Mental State Examination (MMSE) and Unified Parkinson's Disease Rating Scale (UPDRS). In addition, private medical records are requisitioned and reviewed for each subject and the postmortem Dementia Questionnaire [32] or an adaptation of the Clinical Dementia Rating Scale (CDR) are administered to subject contacts to help determine the presence or absence of dementia for those subjects lacking standardized antemortem evaluations. Subjects sign consent that has been approved by the Banner Health Institutional Review Board.

Subjects were chosen by searching the BBDP database for all cases that had received a whole-body autopsy, a completed neuropathologist's examination and a neuropathologic diagnosis of a Lewy body disorder, including Parkinson's disease (PD), dementia with Lewy bodies (DLB), incidental Lewy body disease (ILBD) and Alzheimer's disease with Lewy bodies (ADLB). Comparison groups were composed of subjects without evidence of dementia or parkinsonism (normal elderly subjects) and subjects with AD but no Lewy body pathology (ADNLB).

Subjects received standardized neuropathological examinations. Specific clinicopathologic diagnostic criteria were used for AD [1], PD [36], and DLB [58]. For both AD and DLB, cases received the diagnosis if they were classified as "intermediate" or "high" probabilities in their respective classification schemes. Cases with PASH, but not meeting these diagnostic criteria were designated as either ILBD, if they had no clinical history of parkinsonism or dementia, or ADLB if they had dementia, Alzheimer's disease and Lewy bodies in any brain region, but failed to meet clinicopathologic criteria for DLB or PD.

Gross and microscopic neuropathologic assessments were made by a single observer (TGB) without knowledge of the clinical history or clinical diagnosis; subsequently the clinical history was reviewed with the neurologists (CHA, JNC, HAS, MNS) in order to make an appropriate clinicopathologic diagnosis.

Histologic methods

Diagnostic histologic methods were performed on standard blocks of tissue that were fixed in 3.75% neutral-buffered formaldehyde and then either dehydrated and embedded in paraffin or cryoprotected and cut on a freezing, sliding microtome. Each case was first staged according to the Unified Staging System for Lewy body disorders with a standard set of brain sections stained with an immunohistochemical method for phosphorylated α-synuclein as previously described [8]. The Unified Staging System is a modification of the scheme first devised by the Dementia with Lewy Bodies Consortium [8, 58, 59].

Paraffin-embedded sections from multiple body sites (Table 1) were stained in an identical fashion as the brain sections, using a polyclonal antiserum raised against an α-synuclein peptide fragment phosphorylated at serine 129, after epitope exposure with proteinase K [34]. The process leading to the choice of immunohistochemical method, as well as details of the method, has been described in a previous publication [10]. In each body region, the density of α-synuclein-immunoreactive perikaryal neuronal cytoplasmic inclusions as well as puncta and neurites was scored, at the site of highest density, 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 [58]. The total number of body sites examined varied between subjects as an initially broad sampling scheme was progressively reduced to those regions showing a greater likelihood to have positive staining. To evaluate the relative frequency of immunoreactivity in the different body regions, paraffin sections on a single stained slide from each body site listed in Table 1 were used. Following this analysis, selected regions of interest were further evaluated with up to five additional paraffin sections and/or 80 μm thick formalin-fixed, frozen sections.

Alzheimer's disease histopathology was staged and graded on 40 μm thick sections stained with the Gallyas method for neurofibrillary tangles and the Campbell–Switzer and thioflavine-S methods for senile plaques [15]. Braak's neurofibrillary tangle stages and CERAD neuritic plaque densities were assigned as described [16, 62].

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IFNβ-1b may severely exacerbate Japanese optic-spinal MS in neuromyelitis optica spectrum

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ABSTRACT

Background: Interferon-β-1b (IFNβ-1b) has been used to prevent exacerbation of relapsing-remitting multiple sclerosis (RRMS) including optic-spinal multiple sclerosis (OSMS) in Japan. We encountered 2 patients with OSMS with unexpectedly severe exacerbation soon after the initiation of IFNβ-1b therapy. The experience urged us to retrospectively review the patients with RRMS who had been treated with IFNβ-1b to identify similar cases.

Methods: At neurologic departments of 9 hospitals, the medical records of 56 patients with RRMS were reviewed to identify those who showed severe exacerbation soon after the initiation of IFNβ-1b therapy.

Results: Of 56 patients with RRMS, we identified 7 who experienced severe exacerbation (exacerbation with increased scores of Expanded Disability Status Scale ≧7.0) within 90 days of the initiation of IFNβ-1b therapy. In all 7 patients, the exacerbations after the initiation of IFNβ-1b therapy were more severe than those experienced by the individual patients before the use of IFNβ-1b, and seemed to have occurred unexpectedly in a short time after the initiation of INFβ-1b therapy. A retrospective analysis revealed that all 7 patients had antibodies toward aquaporin 4, and the clinical features of all 7 patients after the exacerbation were consistent with those of neuromyelitis optica (NMO) spectrum.

Conclusions: Our study suggests that IFNβ-1b may trigger severe exacerbation in patients with the NMO spectrum. In INFβ-1b therapy, cases in NMO spectrum should be carefully excluded.
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GLOSSARY

AQP4 = aquaporin 4; **CMS** = conventional multiple sclerosis; **cRRMS** = RRMS not meeting the criteria of NMO; **EDSS** = Expanded Disability Status Scale; **IFNβ-1b** = interferon-β-1b; **LESCL** = longitudinally extensive spinal cord lesion; **MS** = multiple sclerosis; **NMO** = neuromyelitis optica; **OSMS** = optic-spinal multiple sclerosis; **RRMS** = relapsing-remitting multiple sclerosis.

In Japan, relapsing-remitting demyelinating disease with the main lesions in the optic nerve and spinal cord have long been included in relapsing-remitting multiple sclerosis (RRMS), and called optic-spinal multiple sclerosis (OSMS).[1] The clinical presentations of OSMS are characterized by optic-spinal distribution of the lesions, female preponderance, severe disability, longitudinally extensive myelitis, and prominent pleocytosis in the CSF, which are similar to those observed in neuromyelitis optica (NMO).[2-4] Recent discovery of the anti-aquaporin 4 (AQP4) antibody in patients with NMO, as well as in Japanese patients with OSMS, strongly suggests that OSMS shares common causes with NMO, and is presumably identical to NMO.[5-10] It also was revealed that anti-AQP4 antibody is positive in patients presenting with

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atypical features as NMO, including the patients with large tumefactive lesions in the cerebral hemispheres, and patients with connective tissue disease. With these as background, the notion of the NMO spectrum has been described.[11,12]

In Japan, IFNβ-1b has been used to prevent exacerbation in patients with RRMS including those with OSMS. In most cases, IFNβ-1b is effective. However, we have recently encountered 2 patients with OSMS with unexpectedly severe exacerbation soon after the initiation of IFNβ-1b therapy. The experience urged us to retrospectively review patients with RRMS receiving IFNβ-1b therapy to identify if there are any similar cases. We describe the identification of 7 patients who showed severe exacerbations soon after the initiation of IFNβ-1b, and emphasize that IFNβ-1b may become a risk for exacerbation in some patients with the NMO spectrum.

METHODS Patients. The medical records of 56 patients with RRMS who started IFNβ-1b therapy between 1997 and 2006 at 9 hospitals in Japan (Tokyo University Hospital, Teikyo University Hospital, Tokyo Postal Services Agency Hospital, Mitsui Memorial Hospital, Toranomon Hospital, International Medical Center of Japan, Japanese Red Cross Medical Center, Toshiba General Hospital, and Tokyo Metropolitan Geriatric Hospital) were reviewed. In this study, all patients who started IFNβ-1b therapy during this period at 9 hospitals were included. Patients with primary progressive multiple sclerosis (MS) and patients with monophasic NMO (without subsequent exacerbation) were excluded.

Before the initiation of IFNβ-1b therapy, all the patients had been diagnosed with clinically definite MS in accordance with the criteria,[13] and classified as OSMS, in which clinically estimated main lesions are confined to the optic nerve and spinal cord, or conventional multiple sclerosis (CMS), in which the clinically estimated main lesions are confined to the cerebrum, cerebellum, or brainstem in accordance with the criteria.[1]

In the review of the medical records by neurologists at the 9 hospitals, the subgroup of RRMS (CMS or OSMS) before the initiation of IFNβ-1b therapy, the neurologic symptoms, the duration of IFNβ-1b use, the reason for suspension of IFNβ-1b therapy if the patient had discontinued injection, MS lesions (determined by brain and spinal MRI),[14] existence of longitudinally extensive spinal cord lesions (LESCLs) longer than 3 vertebral segments, and CSF findings were assessed. For the patients who developed severe (Expanded Disability Status Scale [EDSS] ≧7.0) exacerbations within 90 days of the initiation of IFNβ-1b therapy, further detailed clinical information was collected.

After the discovery of the anti-AQP4 antibody in 2004,[5,6] several institutes, including ours, developed techniques for the assessment of the anti-AQP4 antibody, and started checking for the antibody in serum. The anti-AQP4 antibody was measured during the disease course after the initiation of IFNβ-1b therapy in 26 patients.

In the 7 patients who experienced unexpectedly severe exacerbation within 90 days, the stored sera taken at the time of exacerbation were available, and we assessed the anti-AQP4 antibody by the previously described method (appendix e-1 and figure e-1 on the *Neurology*® Web site at www.neurology.org).[5]

At the time of this study, 56 patients with RRMS were reviewed and retrospectively diagnosed with definite NMO, high risk of NMO (patients with optic neuritis and anti-AQP4 antibody, or patients with optic neuritis and LESCLs), or cRRMS (RRMS not meeting the criteria of NMO) in relation to the diagnostic criteria for NMO.[11,12]

Standard protocol approvals, registrations, and patient consents. Each institution provided institutional review board approval, and written informed consent was obtained from each patient.

RESULTS Profiles and clinical courses of patients with RRMS who received IFNβ-1b therapy. We retrospectively reviewed the clinical features of 56 patients with RRMS (male: female = 17:39, mean age ± SD = male, 35.5 ± 10.6 years; female, 41.2 ± 12.5 years) who started IFNβ-1b therapy between 1997 and 2006 in the 9 hospitals. Table 1 shows a summary of the clinical features and courses including the status of continuation of IFNβ-1b therapy in relation to NMO criteria.

Retrospectively, it was revealed that the 56 patients with RRMS who started IFNβ-1b therapy were composed of 14 patients with NMO, 6 patients with high risk of NMO, and 36 patients with cRRMS. Among the 56 patients with RRMS, 19 (13 patients with NMO, 3 patients with high risk of NMO, and 3 patients with cRRMS) had suspended injection of IFNβ-1b for the following reasons: exacerbation (9 patients), liver dysfunction (4 patients), skin ulcer (3 patients), lack of belief in the effectiveness of IFNβ-1b (3 patients), pancytopenia (1 patient), and headache (1 patient). Two patients had to suspend injection for 2 reasons.

Most importantly, we identified 7 patients, including our initial 2 patients, who were obligated to discontinue IFNβ-1b owing to the unexpectedly severe (EDSS ≧7.0) exacerbation that occurred within 90 days of initiation of IFNβ-1b therapy. All the patients developed transverse myelitis, 2 patients developed optic neuritis, and 2 patients developed cerebral lesions. In all the patients, spinal MRI revealed LESCLs and the assay for the anti-AQP4 antibody in serum showed positive results. Thus, all the patients were included in NMO.[15]

Clinical features of 7 patients with unexpectedly severe exacerbation. The 7 patients were 6 women and 1 man (appendix e-2, table e-1). Their ages ranged from 18 to 56 years. Before the initiation of IFNβ-1b therapy, 6 of the 7 patients (cases 1, 3, 4, 5, 6, and 7) had been classified as having OSMS in relation to the site of main lesions and 4 of them