Incidence and Extent of Lewy Body–Related α-Synucleinopathy in Aging Human Olfactory Bulb

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Abstract

We investigated the incidence and extent of Lewy body (LB)–related α-synucleinopathy (LBAS) in the olfactory bulb (OB) in 320 consecutive autopsy patients from a general geriatric hospital (mean age, 81.5 ± 8.5 years). Paraffin sections were immunostained with anti-phosphorylated α-synuclein, tyrosine hydroxylase, phosphorylated tau, and amyloid β antibodies. LBAS was found in 102 patients (31.9%) in the central nervous system, including the spinal cord; the OB was involved in 85 (26.6%). Among these 85 patients, 2 had LBAS only in the anterior olfactory nucleus, 14 in the peripheral OB only, and 69 in both areas. In 5 patients, Lewy bodies were found only in the OB by hematoxylin and eosin stain; 3 of these patients had Alzheimer disease, and all had LBAS. Very few tyrosine hydroxylase–immunoreactive periglomerular cells exhibited LBAS. All 35 LBAS patients with pigmentation loss in the substantia nigra had LBAS in the OB. LBAS in the amygda1a was more strongly correlated with LBAS in the anterior olfactory nucleus than with that in the OB periphery. LBAS did not correlate with systemic tauopathy or amyloid β amyloidosis. These results indicate a high incidence of LBAS in the aging human OB; they also suggest that LBAS extends from the periphery to the anterior olfactory nucleus and results in clinical manifestations of LB disease.

Key Words: α-Synuclein, Anterior olfactory nucleus, Dementia with Lewy bodies, Incidental Lewy bodies, Olfactory bulb, Parkinson disease.

INTRODUCTION

Olfaction is a major target of neuroscience research, and numerous genes are expressed for olfactory receptors (1,000 in mouse and 300 in human) (1–3). Patients with schizophrenia exhibit olfactory dysfunction (4–7) and loss of volume of the olfactory bulb (OB) (8). Olfactory dysfunction is also an early sign of Alzheimer disease (AD) and Lewy body (LB) diseases (i.e. Parkinson disease [PD], PD with dementia [PDD], and dementia with LB [DLB]) (9–19) and has been used to differentiate these disorders from progressive supranuclear palsy (20). Atrophy of the OB as revealed by magnetic resonance imaging has been reported to be useful diagnostic tools for AD (21) and PD (22). Recently, neuroprogenitor cells that migrate from the subventricular zone were identified in the human OB (23). Twice as many tyrosine hydroxylase (TH)–immunoreactive neurons were reported in the OBs of PD patients compared with those of age- and sex-matched controls (24). Thus, it is of interest as to whether LB-related α-synucleinopathy (LBAS) involves the TH system in the OB.

Subsequent to the staging system for diffuse LB disease proposed by Kosaka (25), Braak et al (26) suggested a neuropathologic staging procedure for idiopathic PD in an aged cohort that included PDD patients but not DLB or other patients with dementia and LB pathology. The latter subgroup included the amygda1a variant of LB disease (27) complicated by AD (28, 29) or other tauopathies (29). According to Braak et al, the LBAS in the central nervous system (CNS) first affects the medulla oblongata, rostrally extends to the locus coeruleus, and reaches the substantia nigra. Braak et al (30) also reported a pattern of extension from the OB to the amygdala, but neither Kosaka et al nor Braak et al (30) examined possible statistical correlations between these 2 patterns in the extension of LBAS.

In light of these previous studies, we began to include the OB in routine neuropathologic examinations and noticed that LBAS in the OB is dominant either in the anterior olfactory nucleus (AON) or in the peripheral OB. Among several anatomical nomenclature systems of OB (31–34), we adopted that of Price (34) as follows: The axons of the bipolar receptor cells (approximately 6 million per nose) in the olfactory epithelium (the primary olfactory structure) ramify in the most superficial layer of the OB, the olfactory nerve layer. They...
then form synapses with the dendrites of the secondary olfactory structure that consists of mitral and tufted cells in the glomerular layer. The external plexiform layer contains the dendrites of the mitral cells and the somata of the tufted cells. The mitral cell layer is formed by the somata of the mitral cells. The axons of the mitral cells run through the internal plexiform layer and reach the granule cell layer (Fig. 1). These secondary olfactory structures are the major anatomical regions that exhibit LBAS in the OB periphery. The AON is located in the OB and olfactory peduncle; it includes several groups of pyramidal-like cells and is termed tertiary olfactory structures.

The goal of this study was to clarify the significance of LBAS involving the OB in human aging. The OB periphery (including axon terminals of the primary structure and the soma, dendrites, and axons of the secondary structure) and AON (the axon terminals of the secondary structure and the soma, dendrites, and axons of the tertiary structure) were evaluated separately to study the extension pattern of LBAS.

### MATERIALS AND METHODS

#### Tissue Source

Tissue samples were collected at the Tokyo Metropolitan Geriatric Hospital, which, as previously reported (29), provides community-based medical service to the aged population. Between 2003 and 2006, 320 consecutive autopsy brains, spinal cords, and adrenal glands (29) from 180 men and 140 women were used for this study. Two hundred forty-seven of the 320 cases overlapped with our recent studies of LB pathology in the skin (35). The patients’ age ranged from 52 to 104 years old, with a mean ± SD age of 81.5 ± 8.5 years. The postmortem interval ranged from 52 to 4,210 minutes, with a mean of 753 minutes.

#### Neuropathology

Brains and spinal cords were examined as reported previously (36). In brief, 1 hemisphere was preserved for biochemical and molecular studies, and the other portions were prepared for morphological studies. After fixation in formalin, the representative areas were embedded in paraffin. Serial 6-μm-thick sections were stained with hematoxylin and eosin (H&E) and Klüver-Barrera methods. Selected sections were further examined with modified methenamine (37) and Gallyas-Braak silver (38) staining for senile changes, Congo red for amyloid deposition, and elastica Masson trichrome stain for vascular changes.

#### Immunohistochemistry

Selected sections were immunostained using a Ventana 20NX autostainer (Ventana, Tucson, AZ), as previously...
described (36). We immunostained representative sections as well as OBs from all patients with anti-phosphorylated α-synuclein (psyn; monoclonal, psyn no. 64 [39] and polyclonal PSer129 [40]), anti-phosphorylated tau (ptau; AT8, monoclonal; Innogenetics, Temse, Belgium), anti-β-amyloid 11-28 (12B2, monoclonal; IBL, Maebashi, Japan), anti-ubiquitin (polyclonal, DAKO, Glostrup, Denmark), and anti-TH (monoclonal; Calbiochem-Novabiochem Corp, Darmstadt, Germany, and Immunostar, Hudson, WI) antibodies.

**Evaluation of LBAS**

To evaluate LBAS, we used immunohistochemistry with anti-psyn antibodies to screen OB sections, bilateral adrenal glands, medulla oblongata at the level of the dorsal motor nucleus of the vagus, upper pons at the level of the locus coeruleus, midbrain, amygdala, and posterior hippocampus. If anti-psyn immunoreactivity was observed in any of these regions, further studies with anti-psyn and ubiquitin antibodies were conducted on the anatomical structures as recommended by the original and revised DLB Consensus Guidelines (41, 42), PD staging by Braak et al (26), and our own previous work (39), which includes staining of CA2 of the hippocampus and intermediolateral column of the thoracic spinal cord (43), for staging of LBAS (42, 44). Our revised LB staging system (29) was applied to all patients as follows: Stage 0, no anti-psyn immunoreactive structure; Stage 0.5, Lewy dots or neurites only or fine granular cytoplasmic staining without any focal aggregates; Stage I, a few LBs confirmed by H&E staining, without neuronal loss (incidental LB disease); Stage II, abundant LBs with loss of pigmentation in the substantia nigra but without attributable parkinsonism or dementia (subclinical LB disease); Stage III, PD without dementia; Stage IV, DLB or PDD, transitional (limbic) form (DLBT or PDDT); and Stage V, DLB or PDD, neocortical (diffuse) form (DLBN or PDDN). Parkinson disease with dementia was differentiated from DLB by applying the “12-month” rule noted in the Consensus Guidelines (i.e. “dementia appears more than 1 year after the onset of parkinsonism”) (41, 42). We subcategorized our Stage II patients into brainstem (B), transitional or limbic (T), and neocortical (N) forms based on Lewy score (41) and involvement of the intermediolateral column of the spinal cord (43) or the amygdala variant (A), as previously reported (27, 28). Stage 0.5 and Stage 1 patients were also subcategorized to the extent of LBAS localized in the brainstem (B), spreading to the limbic system (T) and neocortex (N) or preferentially present in amygdala (A), as previously reported.

**Evaluation of Other Senile Changes and Neuropathologic Diagnosis**

Neurofibrillary tangles (NFTs) were classified into Braak and Braak’s (45) 7 stages (0–VI) and senile plaques (SPs) into 4 stages (0–C). Argyrophilic grains were classified into our 4 stages (0–III), as reported previously (46). The

**TABLE 1. LBAS in the CNS and OB**

<table>
<thead>
<tr>
<th>BBAR LB Stage*</th>
<th>No.</th>
<th>OB LBAS Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Periphery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0  1  2  3</td>
</tr>
<tr>
<td>0</td>
<td>218</td>
<td>0  0  0  0</td>
</tr>
<tr>
<td>0.5</td>
<td>30</td>
<td>15 10 3 2</td>
</tr>
<tr>
<td>0.5B</td>
<td>8</td>
<td>5  2 0 1</td>
</tr>
<tr>
<td>0.5T</td>
<td>8</td>
<td>3  3 1 1</td>
</tr>
<tr>
<td>0.5A</td>
<td>14</td>
<td>7  5 2 0</td>
</tr>
<tr>
<td>I</td>
<td>37</td>
<td>4  4 17 12</td>
</tr>
<tr>
<td>IB</td>
<td>16</td>
<td>3  2 7 4</td>
</tr>
<tr>
<td>IT</td>
<td>14</td>
<td>1  1 8 4</td>
</tr>
<tr>
<td>IA</td>
<td>7</td>
<td>0  1 2 4</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>0  1 4 3</td>
</tr>
<tr>
<td>IIB</td>
<td>3</td>
<td>0  0 3 0</td>
</tr>
<tr>
<td>IIT</td>
<td>4</td>
<td>0  1 1 2</td>
</tr>
<tr>
<td>IIA</td>
<td>1</td>
<td>0  0 0 1</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>0  2 0 0</td>
</tr>
<tr>
<td>IIIT</td>
<td>1</td>
<td>0  1 0 0</td>
</tr>
<tr>
<td>IIIN</td>
<td>1</td>
<td>0  1 0 0</td>
</tr>
<tr>
<td>IV</td>
<td>11</td>
<td>0  2 7 2</td>
</tr>
<tr>
<td>V</td>
<td>14</td>
<td>0  2 10 2</td>
</tr>
<tr>
<td>Total</td>
<td>320</td>
<td>237 21 41 21</td>
</tr>
</tbody>
</table>

Correlation between OB periphery and AON grade in BBAR LB Stages IB, IT, and IA (bold and italic group).

Correlation between BBAR LB Stages IV and V (bold group), p = 0.005.

*BBAR LB stage (29, 39, 44).
†p < 0.01.
‡A, amygdala variant; AON, anterior olfactory nucleus; B, brainstem; BBAR, Brain Bank for Aging Research; LB, Lewy body; LBAS, LB-related α-synucleinopathy; N, neocortical; OB, olfactory bulb; T, transitional.
The neuropathologic diagnosis of AD was based on our definition (47), which proposes a modification of the National Institute on Aging and Reagan Institute criteria (48). The diagnoses of dementia with grains and NFT-predominant forms of dementia were based on the definitions of Jellinger (49) and Jellinger and Bancher (50). The diagnosis of progressive supranuclear palsy was based on the National Institute of Neurological Disorders and Stroke criteria (51). The diagnosis of vascular dementia was based on the criteria of the National Institute of Neurological Disorders and Stroke-Association Internationale pour la Recherche et l’Enseignement en Neurosciences (52). With respect to combined pathologies, the diagnosis of AD plus DLB was based on Braak NFT Stage equal to or more than IV and SP Stage C (47), in combination with a Lewy score equal to or more than 4 with involvement of the CA2 of hippocampus and intermediolateral column of the spinal cord, as previously reported (29, 43).

**Neuropathology of the OB**

Both OBs were sampled at autopsy. One was snap frozen, and the other was fixed in 4% paraformaldehyde for 48 hours. A sagittal section was embedded in paraffin, and 6-μm-thick serial sections were stained with H&E, with Klüver-Barrera, or by immunohistochemistry with the same panel of antibodies previously described.

In addition, a double labeling immunofluorescence study was performed on selected sections of the OB. Deparaffinized sections were incubated simultaneously with polyclonal p.syn (PSer129) and monoclonal anti-TH or polyclonal ptau (AP422, polyclonal, a gift from Dr Y. Ihara) (53) and monoclonal p.syn no. 64. Primary antibodies were visualized with anti-rabbit Alexa 546 Fluor and anti-mouse immunoglobulin G Alexa 488 (Molecular Probes, Eugene, OR) with 4’,6-diamidino-2-phenylindole (DAPI) staining for the nucleus under a confocal laser microscope (LSM5, PASCAL, Carl Zeiss, Jena, Germany).

**LB Grade of the OBs**

The OB periphery and the AON (Fig. 1) were separately evaluated. The secondary olfactory structure included the soma and cell processes of mitral cells, tufted cells, granule cells, and periglomerular cells. Grading of α-synuclein pathology followed the revised DLB Consensus

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**FIGURE 2.** Lewy body (LB)-related α-synucleinopathy (LBAS) in different neuron populations in the olfactory bulb (OB).

(A) Tissue from a patient with LBAS preferentially involving the periphery of the OB and surrounds the anterior olfactory nucleus (AON). Scale bar = 500 μm.

(B) Mitral cell. Scale bar = 10 μm.

(C) Tufted cell. Scale bar = 10 μm.

(D) Granule cell. Scale bar = 10 μm.

(E) Tissue from a patient with LBAS preferentially involving the AON. Scale bar = 500 μm.

(F) AON neuron. Scale bar = 10 μm.

(A–F) Immunohistochemistry with anti-phosphorylated α-synuclein antibody (p.syn no. 64) counterstained with hematoxylin.
Guidelines (42). The classification of Tsuboi et al (54) for NFTs was applied. For SPs, we developed the following criteria: Grade 0, none; Grade 1, sparse dots; Grade 2, definite SPs, scattered; and Grade 3, abundant SPs.

Clinical Information

Clinical information, including the presence or absence of parkinsonism and cognitive state, was obtained from medical charts. The Mini-Mental State Examination (55) or the Hasegawa Dementia Scale (or its revised version [56]), and the Instrumental Activities of Daily Living (57) were used to evaluate cognitive function. The Clinical Dementia Rating (CDR) (58) was retrospectively determined by 2 independent board-certified neurologists. If the resulting ratings were in agreement, the score was accepted. If not, the neurologists reconciled their differences in the score after interviews with the patients’ attending physicians and caregivers. Information about parkinsonism, bradykinesia, resting tremor, rigidity, and postural instability was obtained from neurological examinations. The presence of more than 2 of these clinical signs was interpreted as indicative of parkinsonism. The clinical diagnosis of AD was based on the criteria of the National Institute of Neurological and Communication Disorders and Stroke–Alzheimer Disease and Related Disorders Association (59).

Apolipoprotein E Genotype Analysis

We extracted genomic DNA from a freshly frozen kidney, measured the quantity of DNA with a spectrophotometer (Hitachi U2000, Tokyo, Japan), and adjusted it to 100 μg/mL. Tissues from all patients except for one (who had Creutzfeldt-Jakob disease) in the series were genetically examined in our laboratory. After polymerase chain reaction amplification, apolipoprotein E (APOE) genotyping was conducted with restriction enzyme HhaI, as described previously by Hixson and Vernier (60).

Statistical Analysis

Statistical comparison of LBAS grades in the AON and the OB periphery among patients at the Brain Bank for Aging Research (BBAR) (61) LB Stages IB, IT, IN, and IA (I, incidental LB disease; with extension of B, brainstem; T, transitional; N, neocortical; and A, amygdala as previously stated) was performed by the Friedman test. The Friedman test was used for comparison of categorical data. The Mann-Whitney U test was used for comparison of age at death. The relationships between LBAS grade in the OB and LBAS in the adrenal gland were assessed with the Mann-Whitney U test. Independent-sample t-tests were used for comparison of mean LBAS grade in the AON or the OB periphery and between Braak stages for SP and NFTs and the BBAR LB stage. The correlation between LBAS grade of the amygdala and of the AON or the OB periphery was assessed with Spearman rank correlation coefficient. Statistical comparison of LBAS grades, tau grades, and β-amyloid grades in AON and the periphery between APOE ε4 carriers and noncarriers using the Wilcoxon test. All statistical analyses were

<table>
<thead>
<tr>
<th>LBAS Grade: AON</th>
<th>0</th>
<th>1</th>
<th>2 ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBAS grade: Periphery</td>
<td>0</td>
<td>235</td>
<td>2*</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>2 ≤</td>
<td>4</td>
<td>13</td>
<td>45</td>
</tr>
</tbody>
</table>

p = 0.004.

* complicated by coexistent Alzheimer disease pathology.

AON, anterior olfactory nucleus; LB, Lewy body; LBAS, LB-related α-synucleinopathy; OB, olfactory bulb.

<table>
<thead>
<tr>
<th>LBAS Grade: Periphery</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AON</td>
<td>0/0</td>
<td>0/10</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>1</td>
<td>0/2</td>
<td>0/4</td>
<td>0/10</td>
<td>0/3</td>
</tr>
<tr>
<td>2</td>
<td>0/0</td>
<td>1/2</td>
<td>3/7</td>
<td>1/8</td>
</tr>
<tr>
<td>3</td>
<td>0/0</td>
<td>2/2</td>
<td>5/11</td>
<td>0/3</td>
</tr>
<tr>
<td>4</td>
<td>0/0</td>
<td>3/3</td>
<td>9/11</td>
<td>2/5</td>
</tr>
</tbody>
</table>

The ratio represents the number of patients with clinical LB disease/the number of patients with either subclinical or clinical LB disease for each LBAS grade as described in the Materials and Methods section.

AON, anterior olfactory nucleus; LB, Lewy body; LBAS, LB-related α-synucleinopathy.

![FIGURE 3](http://jnen.oxfordjournals.org/)

FIGURE 3. Correlations between Lewy body (LB) stage of the Brain Bank for Aging Research (BBAR) and grade of LB-related α-synucleinopathy (LBAS) in the anterior olfactory nucleus or the periphery of the olfactory bulb (OB). All patients with BBAR LB Stage ≥ II had LBAS in the OB.
performed using SPSS 15.0J for Windows (SPSS, Inc, Chicago, IL). The statistical significance level was set at p < 0.05.

RESULTS

Clinical Information

Among the 320 consecutive autopsy patients, 47 fit clinical criteria for parkinsonism (41). The CDR could retrospectively be assessed in 251 patients as follows: CDR 0, 95 patients; CDR 0.5, 41 patients; CDR 1, 31 patients; CDR 2, 15 patients; and CDR 3, 69 patients. The percentage of CDR equal to or greater than 0.5 was 62.2%.

Neuropathologic Diagnosis

The neuropathologic diagnoses consisted of AD (n = 25), vascular dementia (n = 20), DLB (n = 13), dementia with grains (n = 10), progressive supranuclear palsy (n = 6), PDD (n = 5), NFT-predominant form of dementia (n = 5), amyotrophic lateral sclerosis (n = 5), idiopathic hippocampal sclerosis (n = 3), PD (n = 2), and 1 case each of PD with Pick bodies, spinocerebellar ataxia 3/Machado-Joseph disease, multiple sclerosis, Kennedy-Alter Seng disease, Huntington disease, frontotemporal lobar degeneration with ubiquitinated inclusions, and Creutzfeld-Jakob disease. Patients with combined pathologies included AD plus DLB (n = 6), AD plus vascular dementia (n = 2), dementia with grains plus NFT-predominant form of dementia (n = 2), and PDD plus progressive supranuclear palsy plus dementia with grains (n = 1). The remaining patients did not fulfill clinical and/or pathological criteria for neurodegenerative diseases.

The BBAR Staging for LBAS in the CNS, Including Spinal Cord

Lewy body–related α-synucleinopathy was found in 102 (31.9%) of the 320 patients (Table 1); the BBAR LB stages (29, 39, 44) were as follows: Stage 0, 218 patients; Stage 0.5, 30 patients; Stage I, 37 patients; Stage II, 8 patients; Stage III, 2 patients; Stage IV, 11 patients; and Stage V, 14 patients. The Stage IV patients included 4 of PDDT and 7 of DLBT; with 5 of the 7 DLBT patients having parkinsonism. The Stage V patients included 2 with PDDN and 12 with DLBN; 3 of the 12 DLBN patients had parkinsonism (42).

Incidence, Distribution, and Extent of LBAS in the OB

Lewy body–related α-synucleinopathy was detected in the OB of 85 (26.6%) of the 320 patients. The most frequent psyn-immunoreactive neuronal cells in the periphery were granule cells, followed by mitral cells, tufted cells, and periglomerular cells (Fig. 2). Lewy bodies in the OBs usually showed cortical-type morphological features, as reported previously (11); a few had halos.

Patients with LBAS in the OBs could be classified into 2 groups: one in which LBAS predominated in the AON (Fig. 2E) and the other in which LBAS predominated in the periphery of the OB (Fig. 2A). Very few psyn-positive neurites or dots were present in the olfactory nerve layer (where ramified axons of the bipolar receptor cells are present in the olfactory epithelium); therefore, LBAS in the periphery was found to reside mainly in the secondary olfactory structure. Lewy body–related α-synucleinopathy in

TABLE 4. Correlations Between LBAS Grades in the OB and Degenerative Senile Changes in Other Areas of the CNS

<table>
<thead>
<tr>
<th>LBAS Grade</th>
<th>BBAR LB Stage</th>
<th>Braak SP Stage</th>
<th>BBraak NFT Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5- II</td>
<td>III- V</td>
<td>p</td>
</tr>
<tr>
<td>AON</td>
<td>1.53</td>
<td>3.35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Periphery</td>
<td>1.98</td>
<td>1.88</td>
<td>0.538</td>
</tr>
</tbody>
</table>

AON, anterior olfactory nucleus; BBAR, Brain Bank for Aging Research; LB, Lewy body (0.5- II, subclinical LB disease; III- V, Parkinson disease, Parkinson disease with dementia, and dementia with LBs); LBAS, LB-related α-synucleinopathy; NFT, neurofibrillary tangle; OB, olfactory bulb; SP, senile plaque.

TABLE 5. Demography of 5 Patients With LB Identified Only in the OBs by H&E Stain

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Clinical Dx</th>
<th>CDR</th>
<th>BW, g</th>
<th>Np Dx</th>
<th>NFT</th>
<th>SP</th>
<th>BBAR LB Stage</th>
<th>AON</th>
<th>Periphery</th>
<th>OB Grade</th>
<th>Other Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72/M</td>
<td>HCC</td>
<td>0.5</td>
<td>1351</td>
<td>unremarkable</td>
<td>I</td>
<td>A</td>
<td>0.5T</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1 1 0 0</td>
</tr>
<tr>
<td>2</td>
<td>78/F</td>
<td>CRF dementia</td>
<td>2</td>
<td>1147</td>
<td>CVDE, VD</td>
<td>II</td>
<td>A</td>
<td>0.5B</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1 1 0 0</td>
</tr>
<tr>
<td>3</td>
<td>89/M</td>
<td>Lung carc. post radiation therapy</td>
<td>1</td>
<td>1246</td>
<td>early AD</td>
<td>IV</td>
<td>C</td>
<td>0.5A</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0 1 0 1</td>
</tr>
<tr>
<td>4</td>
<td>74/M</td>
<td>COPD</td>
<td>0.5</td>
<td>1413</td>
<td>AC</td>
<td>V</td>
<td>C</td>
<td>0.5T</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1 1 1 0</td>
</tr>
<tr>
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<td>82/F</td>
<td>AD</td>
<td>3</td>
<td>1123</td>
<td>AD</td>
<td>V</td>
<td>C</td>
<td>0.5A</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0 0 1 0</td>
</tr>
</tbody>
</table>

a, amygdala; AC, Alzheimer disease changes; AD, Alzheimer disease; adr, adrenal gland; AON, anterior olfactory nucleus; BBAR, the Brain Bank for Aging Research; BW, brain weight; ca2, ca2 of the hippocampus; CDR, clinical dementia rating; COPD, chronic obstructive pulmonary disease; CRF, chronic renal failure; CVDE, clinically significant embolic cerebral vascular disease; dm, dorsal motor nucleus of vagus; Dh, diagnosis; F, female; H-E, hematoxylin and eosin; HCC, hepatic cell carcinoma; LB, Lewy body; LBAS, LB-related α-synucleinopathy; lc, locus caeruleus; M, male; NFT, Braak’s stages for neurofibrillary tangles; Np, neuropathologic; OB, olfactory bulb; sc, spinal cord; sn, substantia nigra; SP, Braak’s stages for senile plaques; VD, vascular dementia.

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the AON was graded from 0 to 4 and LBAS in the OB periphery from 0 to 3. The AON Grades 3 and 4 patients were combined and analyzed with the Periphery Grade 3 patients.

Among the patients with LBAS in the OB, 14 were affected in the periphery alone and 2 in the AON alone. The latter 2 patients had AD and had very few psyn-immunoreactive granules in neuronal perikarya. In the earliest stage of LBAS in the OB, the periphery was more frequently involved than the AON (Table 2; p = 0.004).

Correlations Between LBAS in the OB and the CNS, Including Spinal Cord

Lewy body–related α-synucleinopathy in the OB was compared with LBAS in other locations of the CNS (Table 1; Fig. 3). The percentages of OB LBAS–positive patients at each BBAR LB stage were as follows: Stage 0, 0%; Stage 0.5, 56.7% (0.5B, 37.5%; 0.5T, 62.5%; 0.5A, 64.3%); Stage I, 89.2% (IB, 81.3%; IT, 92.9%; IA, 100%); and Stages II to V, 100% (Table 1; Fig. 3).

Among the 35 patients at BBAR LB Stage II or higher, 31 (88.6%) had LBAS in the adrenal glands. The 4 patients who lacked adrenal LBAS all had AD pathology. The average LBAS grade in the OB periphery of the 31 adrenal LBAS–positive patients was significantly greater (p = 0.029) than that of the 4 adrenal LBAS–negative patients. In contrast, the average LB grade in the AON of the 4 adrenal LBAS–negative patients was greater than that of the 31 adrenal LBAS–positive patients, although the difference was not significant (p = 0.054).

We further analyzed patients categorized as having BBAR LB Stage I. In the IB subgroup, the average LBAS grade of the periphery was significantly larger (p < 0.01) than that of the AON, but this difference was not significant in the IT and the IA subgroups (p = 0.75; p = 0.13; Table 1).

FIGURE 4. Tissue from a patient with Lewy bodies (LBs) only in the olfactory bulb (OB) (Patient 1 in Table 5). (A) LB-related α-synucleinopathy in the periphery of the OB (antero-olfactory nucleus; immunohistochemistry (IH) with anti-phosphorylated α-synuclein antibody, visualized with diaminobenzidine. Scale bar = 200 μm. (B) LB in the periphery of the OB (H&E stain). Scale bar = 10 μm. (C) Intraneuronal perikaryal aggregates are stained by IH with anti-phosphorylated α-synuclein antibody (psyn no. 64). Scale bar = 200 μm. (D) IH with psyn no. 64 in the amygdala. Scale bar = 200 μm. (E) A single Lewy neurite is stained. Scale bar = 10 μm. (F) CA2 in the hippocampus shows almost no IH staining with psyn no. 64. Scale bar = 200 μm. (G) A fine granule is psyn no. 64 immunopositive in a neuron perikaryon. Scale bar = 10 μm.

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All of the patients at BBAR LB Stage III or higher (PD/PDD/DLB) demonstrated high-grade LBAS in the AON. The mean grade of LBAS in the AON among patients at Stage V was significantly higher than that of Stage IV patients (p = 0.005; Table 1). One of 11 Stage IV patients and 5 of 14 Stage V patients fulfilled our pathological criteria for AD (47), and all of them (6/6, 100%) showed LBAS Grade 4 in the AON. In contrast, only 7 (36.8%) of 19 patients at Stages IV and V who did not fulfill our pathological criteria for AD showed LBAS Grade 4 in the AON.

We also compared patients with subclinical (≤Stage II) and clinical (≥Stage III) LB disease with regard to LBAS grade of the AON and the periphery of the OB (Table 3). The average LBAS grade in the AON, but not in the periphery, was significantly higher in clinical than in subclinical patients (Table 4).

**LBAS in Amygdala and OB**

Ninety-four (29.4%) of the 320 patients had LBAS in the amygdala. Among the 85 patients with positive LBAS in the OB, 83 had LBAS in the amygdala; the remaining 2 had Grade 1 LBAS in the OB but not in the AON or amygdala. Five had Grade 1 LBAS in the amygdala but LBs in the OB (see later). All of the PD/PDD/DLB (BBAR LB Stages III, IV, and V) patients had Grade 4 LBAS in the amygdala, in accordance with the grading paradigm of the revised DLB Consensus Guidelines (42). The LBAS grade of the amygdala correlated more strongly with that of the AON than with that of the OB periphery (Spearman correlation coefficient, 0.853 and 0.521, respectively).

**Correlations Between Braak’s Stages for NFTs or SPs and LBAS in the OB**

The mean LBAS grade of the AON was higher in Braak’s SP Stages B and C than in Stages 0 and A (p = 0.051), although this difference was not statistically significant. Comparisons of other Braak stages did not reveal any statistical differences (Table 4).

**Influence of APOE ε4 on Olfactory LBAS**

The results of APOE genotyping of the 319 patients (excluding one with Creutzfeldt-Jakob disease) were as follows: 2 patients with the ε2/ε2 genotype; 12 with ε2/ε3; 1 with ε2/ε4; 247 with ε3/ε3; 51 with ε3/ε4; and 6 with ε4/ε4. The 57 APOE ε4 carriers had a significantly higher grade than the 262 noncarriers for tauopathy of the AON (p = 0.011) and anti-β amyloid amyloidosis of both the AON (p < 0.001) and the periphery (p = 0.001), but not for LBAS of the AON (p = 0.80) or the periphery (p = 0.28).

**Correlation Between Incidence of LBAS of the OB and Age at Death**

The mean age at death was 84.1 ± 8.1 years in OB-positive patients and 80.6 ± 8.6 years in OB-negative patients (p = 0.014). Although not statistically significant, the percentage of LBAS in the OB among those patients with LBAS involving the CNS also tended to increase with age: 73% (22/30) in the eighth decade, 87% (34/39) in the ninth decade, and 93% (25/27) in the 10th decade. No significant difference was noted between OB-positive and OB-negative patients with respect to sex.

**Analysis of Data on the 5 Patients Who Had LBs Only in the OB by Routine H&E Stain**

Among the 320 consecutive autopsy patients, 5 (1.6%) who were categorized as having BBAR LB Stage 0.5 had LBs recognized by H&E staining only in the OB (Table 5). No adrenal LBAS was found in these 5 patients. The group consisted of 3 men and 2 women, with a mean age at death of 79 years. One patient (Patient 1) had pure LBAS not markedly complicated by other senile changes or vascular

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**FIGURE 5.** Colocalization of phosphorylated α-synuclein and tau in a neuron of the anterior olfactory nucleus visualized by confocal microscopy. There is little overlap in the staining. Patient 3 in Table 5. Scale bar = 10 μm. (A) Epitope of AP422 visualized with Alexa 546 Fluor (red). (B) Epitope of psyn no. 64, visualized with Alexa 488 Fluor (green). (C) Merged image. Nuclear stain with 4’,6-diamidino-2-phenylindole (DAPI) (blue).
lesions. Lewy body–related α-synucleinopathy preferentially involved the periphery of the OB (Fig. 4A), with a significant number of LBs (Figs. 4B, C). There were only a few anti-psyn–immunoreactive dots in the AON (Fig. 4A). Anti-psyn immunoreactivity was also present (but sparse) in the locus coeruleus, substantia nigra, amygdala (Figs. 4D, E), and CA2 of the hippocampus (Figs. 4F, G). Three of these patients had AD pathology, and all had colocalization of the epitopes of anti-psyn and ptau antibodies in neurons of the AON (Figs. 5A–C).

Correlation Between Anti-TH–Immunoreactive Neurons and LBAS

The epitopes of anti-TH antibodies were localized to periglomerular cells and very few granule cells in addition to the stratum album, as reported previously (62). The locations of the epitopes of anti-psyn antibodies were different from those of anti-TH antibodies and preferentially involved inner structures (Fig. 6). Very little colocalization of the epitopes of anti-psyn and TH antibodies could be detected (Figs. 7A–C).

DISCUSSION

There are 5 major findings in this study: 1) 26% of the consecutive autopsy patients from a general geriatric hospital had LBAS in the OB (peripheral OB, AON, or both); 2) LBAS always involved the OB in the advanced subclinical and clinical stages of LB disease; 3) in this aging population, LBs first appeared in the OB of 2% of patients; 4) LBAS in the OB appeared to extend from the periphery of the OB (secondary olfactory structure) to the AON (tertiary olfactory structure); and 5) LBAS in the amygdala was strongly correlated with LBAS in the OB; this correlation was more pronounced in the AON than in the periphery.

Subsequent to Kosaka et al (63), Braak et al (26) examined a cohort that consisted of incidental cases without
dementia and clinical PD patients with and without dementia, excluding AD and DLB by immunohistochemistry with anti-α-synuclein antibodies. They proposed a staging paradigm for LBAS, starting from the medulla oblongata, including the dorsal motor nucleus of the vagus, extending rostrally in the brainstem, spreading to the limbic system, and reaching the neocortex (26). Braak et al more recently reported findings in the OB and amygdala (30). The correlation between the Kosaka-Braak rostral extension pathway and the olfactory-amygdala pathway has yet to be determined. Three patients (Patients 1, 3, and 5 in Table 5) had LBAS in the OB but not in the dorsal motor nucleus of the vagus and did not follow the original staging of Braak et al (26). A difference between the study of Braak et al (30) and the present study is that although they evaluated both tertiary and secondary olfactory structures for the presence of LBAS, our examination of the various cell types and layers in the peripheral OB enabled us to conclude that the secondary olfactory structure is preferentially involved in early-stage LBAS.

Olfactory dysfunction is one of the initial manifestations in PD and occurs before motor dysfunction (64). An LB-type rapid eye movement sleep behavior disorder is also recognized to develop into PD, and a decrease in olfactory discrimination ability is also useful for the diagnosis of this disorder (65). We found that LBAS always involves the OB when LBAS includes degeneration of the substantia nigra, irrespective of the presence or absence of clinical parkinsonism or dementia; this suggests the morphological correlate of these clinical observations. Olfactory dysfunction is rarely recognized as a medical problem in the elderly in Japan, and we did not find descriptions of an impaired sense of smell in the retrospective investigation of medical charts.

Our findings that LBAS in the OB may start in the periphery and extend to the AON are in agreement with the results of Hubbard et al (66) who studied 79 OBs, 193 AON, and 201 amygdalae. Their specimens were independently registered in the Cambridge Brain Bank, and most of them had AD pathology. These studies may support the hypothesis of Hawkes et al (67) and Hawkes (68) that a neurotropic pathogen, probably viral, enters the brain through the olfactory pathway. Intranasal administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine to rats reduced the enzyme activity of TH in the OB and substantia nigra, resulting in a significant reduction in dopamine concentration in the OB and reproducing the clinical features of PD (69). In contrast, Huisman et al (24) reported that the total number of TH-immunoreactive neurons in the OB was twice as high in PD patients as in age- and sex-matched controls; they suggested that increased dopaminergic activity in the OB may lead to the suppression of olfactory information because of the inhibitory effect of dopamine on the transmission between olfactory receptor cells and mitral cells within the olfactory glomeruli (24). In the present study, the anatomical locations of LBAS and anti-TH-immunoreactive neurons rarely matched. The role of dopamine in olfactory dysfunction should, therefore, be further investigated to clarify these issues.

Our study also indicated a strong correlation between LBAS in the AON and LBAS in the amygdala. Three of 5 patients with LBs only in the OB also had AD pathology of NFT Stage IV or higher and we confirmed colocalization of the epitopes of anti-ptau and anti-psyn antibodies in the perikarya of AON neurons. Colocalization of these 2 epitopes has been reported in the amygdala, entorhinal cortex, and CA2 and 3 of hippocampus (70, 71), as well as in the OB (72). Fujishiro et al (72) first reported colocalization of α-synuclein and tau filaments in the OB with double enzyme immunocytochemistry and immunoelectron microscopy in the amygdala variant of LB disease complicated by AD. Because they screened AD patients with a considerable burden of LBAS in the amygdala and did not separately observe the changes in the periphery of the OB and AON, they could not determine in which direction(s) the LBAS spreads (Hiroki Fujishiro, the 49th Annual Meeting of the Japanese Society of Neuropathology, personal communication, Tokyo, May 2008). In contrast, Hubbard et al (66) proposed the
hypothesis that the spread of LBAS from the periphery of the OB to the AON and amygdala was independent. Taken together with these previous studies (66, 72), our present observations make it reasonable to conclude that in AD, LBAS first affects the periphery of the OB, spreads to the AON, and then reaches the amygdala. We also provide the first morphological evidence that the colocalization of tau and α-synuclein can first appear in the AON in AD with very little burden of α-synucleinopathy in the amygdala. Thus, we propose that the amygdala variant of LB disease should be renamed as the olfactory-amygdala variant.

The chief original observation of the present study is that we confirmed this pattern of spread in patients with pure Lewy pathology without AD. Moreover, our study clearly shows that the involvement of the periphery of the OB alone does not result in either Lewy body–associated parkinsonism or dementia. This suggests that the spread of LBAS in the OB and then into the amygdala may be required for the clinical manifestations of LB disease.

Our study also confirms the biologic significance of pale or LB seen in sections stained by H&E that corresponds to α-synuclein. In the DLB revised Consensus Guidelines (42), which we followed, the presence of pale or LBs was determined to be equal to or more than Grade 2, whereas the presence of immunohistochemically visualized Lewy neurites or diffuse granular perikaryal neuronal staining alone, lack ing pale or LBs, indicated Grade 1. Our results also indicate that the presence of LBs or pale bodies in AON (LBs ≥ Grade 2) was correlated with a clinical presentation of LB disease (Table 3).

Thus, it is likely that the extension of LBAS from the OB periphery to the AON is essential for extension of LBAS from the OB to other areas of the CNS, including the amygdala. Recently, the presence of LBs in grafted fetal tissues in the striatum has been reported (73, 74), indicating the transmission of an LBAS pathogen through the neuronal network (67, 75, 76). Our present results are consistent with these new observations.

In conclusion, we show here that the OB is one of the initial anatomic sites affected by LBAS, and that its functional and morphological evaluation is useful for the neuropathologic diagnosis and clinical evaluation of LB disease.

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