Original Article

Localization of MAP1-LC3 in Vulnerable Neurons and Lewy Bodies in Brains of Patients With Dementia With Lewy Bodies

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Abstract

There is emerging evidence implicating a role for the autophagy-lysosome pathway in the pathogenesis of Lewy body disease. We investigated potential neuropathologic and biochemical alterations of autophagy-lysosome pathway–related proteins in the brains of patients with dementia with Lewy bodies (DLB), Alzheimer disease (AD), and control subjects using antibodies against Ras-related protein Rab-7B (Rab7B), lysosomal-associated membrane protein 2 (LAMP2), and microtubule-associated protein 1A/1B light chain 3 (LC3). In DLB, but not in control brains, there were large Rab7B-immunoreactive endosomal granules. LC3 immunoreactivity was increased in vulnerable areas of DLB brains relative to that in control brains; computerized cell counting analysis revealed that LC3 levels were greater in the entorhinal cortex and amygdala of DLB brains than in controls. Rab7B levels were increased, and LAMP2 levels were decreased in the entorhinal cortex of DLB brains. In contrast, only a decrease in LAMP2 levels versus controls was found in AD brains. LC3 widely colocalized with several types of Lewy pathology; LAMP2 localized to the periphery or outside of brainstem-type Lewy bodies; Rab7B did not colocalize with Lewy pathology. Immunoblot analysis demonstrated specific accumulation of the autophagosomal LC3-II isoform in detergent-insoluble fractions from DLB brains. These results support a potential role for the autophagy-lysosome pathway in the pathogenesis of DLB.

Key Words: α-Synuclein, Autophagy, Dementia with Lewy bodies, Endosome, LC3, Lysosome.

INTRODUCTION

Dementia with Lewy bodies (DLB) is the second most common age-related neurodegenerative dementia after Alzheimer disease (AD) and is characterized clinically by cognitive decline, parkinsonism, and psychiatric disturbance (1). Dementia with Lewy bodies is pathologically characterized by the presence of Lewy bodies (LBs) and dystrophic neurites (Lewy neurites) in the brainstem, limbic areas, and neocortex (1). Although the etiology of DLB is not known, genetic and neuropathologic analyses have provided important insights into its pathogenesis. Missense mutations or multiplication in the α-synuclein gene causes disease in some families with autosomal dominant Parkinson disease (PD) (2–6). The neuropathologic findings in these patients often consist of extensive Lewy pathology reminiscent of DLB or diffuse Lewy bodies (6–8). Importantly, α-synuclein is a major component of both brainstem-type and cortical-type LBs (9). Therefore, Lewy pathology is considered to be associated with the pathogenesis of Lewy body diseases, such as DLB and PD.

The LBs contain filamentous forms of α-synuclein protein. Under normal conditions, α-synuclein exists in mixed soluble unstructured or structured conformations (10). However, under pathological conditions, it can undergo aggregation and fibrillation with eventual deposition of insoluble α-synuclein fibrils into LBs. Indeed, mutant forms of α-synuclein tend to aggregate more rapidly than the wild-type protein (11–13). Thus, fibrillation and aggregation of α-synuclein may be a central pathogenetic mechanism in LB diseases. Two important cellular systems responsible for the selective degradation of misfolded or damaged proteins are...
FIGURE 1. (A) Schematic diagram of the autophagy-lysosomal pathway. Cytoplasmic cargo is first sequestered by an isolation membrane termed "phagophore" to form a microtubule-associated protein 1A/1B light chain 3 (LC3)-positive autophagosome that then undergoes fusion with Ras-related protein Rab-7b (Rab7B)-positive late endosomes. Finally, fusion of the LC3-positive autophagosome with the lysosomal-associated membrane protein 2 (LAMP2)-positive lysosome results in the formation of the autophagolysosome in which the contents are proteolytically degraded. (B) Evaluation of each antibody used in this study. Equivalent soluble protein lysates (20 μg/lane) derived from HEK-293T cells transiently expressing Rab7B, Lamp2A, or LC3, together with a mock transfection control, were analyzed by Western blotting with anti-Rab7B, anti-LAMP2, or anti-LC3 antibodies, as indicated. All antibodies detect the corresponding protein (arrows). (C) Triton X-100-soluble (1%) fractions of inferior temporal cortex tissue from normal control brain (Cont 3) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with anti-Rab7B, anti-LAMP2, or anti-LC3 antibodies. Asterisk denotes a nonspecific cross-reactive protein detected by the anti-LC3 or anti-Rab7B antibody.
the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP). The UPS can proteolytically degrade and remove harmful aggregated proteins that are targeted by ubiquitination. Because LBs contain abundant ubiquitin and proteasomal subunits, dysfunction of the UPS may lead to the accumulation and aggregation of ubiquitinated proteins and their deposition in LB disease brains (14). This hypothesis is supported by the identification of disease-causing mutations in genes encoding several UPS-related proteins in PD, including the E3 ubiquitin ligase parkin (15) and ubiquitin C-terminal hydrolase L1 (16). In addition, recent studies demonstrate that ubiquitination is also relevant to the ALP (17). For example, the ubiquitin-interacting protein, p62/sequestosome-1, can function in the autophagic clearance of aggregated proteins by mediating the docking of ubiquitinated proteins to the autophagosome via interaction with microtubule-associated protein 1A/1B light chain 3 (LC3) (18, 19). p62 also colocalizes with LBs, together with

### TABLE 1. Clinical and Neuropathological Data

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis (Subtype of Concomitant Alzheimer Pathology)</th>
<th>Age, years</th>
<th>Sex</th>
<th>Disease Duration, years</th>
<th>Brain Weight, g</th>
<th>Neuropathologic Stage</th>
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<td></td>
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<td>2</td>
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<td>—</td>
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<td>—</td>
<td>1260</td>
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</tr>
</tbody>
</table>

* Amyloid β (Aβ) stages (A–C) and neurofibrillary (NF) stages (I–VI) were determined according to Braak staging (33). Lewy stages (I–IV) in dementia with Lewy bodies (DLB) were assigned as described (34, 35). Consensus criteria were used to diagnose DLB (1) and Alzheimer disease (AD) (36). Subtype of concomitant Alzheimer pathology in DLB patients was estimated as previously described (35).

Cont, elderly control subject; F, female; M, male.
The inhibition of chaperone-mediated autophagy by mutant α-synuclein and ubiquitin (20). Impaired macroautophagy (hereafter referred to as autophagy) through genetic disruption of autophagy protein 5 (ATG5) or 7 (ATG7) expression in neurons causes neuronal degeneration and the appearance of ubiquitin-positive inclusions in mice, neuropathologic features that are common to many neurodegenerative disorders (21, 22). Moreover, there is accumulating evidence supporting a role for ALP dysfunction in the pathogenesis of PD, including the accumulation of autophagic vacuoles in PD brains (23), the inhibition of chaperone-mediated autophagy by mutant α-synuclein (24–26), a role for autophagy in neuronal cell death induced by mutant forms of LRRK2 that are associated with familial PD (PARK8) (27, 28), and recessive mutations in the lysosomal P-type ATPase, ATP13A2, that cause familial PD (PARK9) (29). Collectively, the ALP may play a role in neurodegenerative processes in LB diseases.

In the ALP, cytoplasmic cargo, such as harmful or damaged proteins and organelles, is first sequestered by an isolation membrane, or phagophore, to form double-membrane autophagosomes that can fuse with lysosomes to produce autophagolysosomes, with subsequent degradation of its contents (Fig. 1A). Before the delivery of autophagosomal cargo to the lysosome, autophagosomes fuse with late endosomes to undergo a maturation process that is driven by factors that include the late endosomal small GTPase Ras-related protein Rab-7B (Rab7B) (30, 31). Thus, autophagic maturation may interconnect with the endocytic pathway by sharing similar machinery required for the concomitant lysosome fusion process (32). Therefore, ALP seems to be regulated by a number of processes that are involved in vesicular trafficking.

To understand the involvement of the ALP in neuropathologic alterations in DLB brains, we investigated the localization of 3 proteins that are involved in ALP and vesicular trafficking, that is, Rab7B, lysosomal-associated membrane protein 2 (LAMP2), and the autophagosome marker LC3 (Fig. 1A) in brains from DLB, AD, and control subjects. Our data provide support for a role of autophagy in the pathogenesis of DLB.

**MATERIALS AND METHODS**

**Patients**

Postmortem human brains from 10 subjects with no history of neurological disease and no neuropathologic abnormalities, 21 DLB cases, and 9 AD cases were obtained from the PET/CT Dementia Research Center, Juntendo Tokyo Koto Geriatric Medical Center, Juntendo University School of Medicine. Studies were approved by the Juntendo Tokyo Koto Geriatric Medical Center Ethics Committee. The patients had no family history of neurological or psychiatric disorders. Clinical data and brain weights are given in Table 1. The DLB cases fulfilled the consensus criteria for a high or moderate likelihood of DLB (1), and AD cases fulfilled consensus criteria for a high likelihood of AD (36). The degree of Lewy pathology was graded from stage I to stage IV based on the number of LBs and the degree of LB-related neurites, according to our previous staging criteria (34, 35) (Table 2). Stages I to II and stages III to IV of Lewy pathology correspond to the limbic (transitional) type and diffuse neocortical type, respectively. In addition, DLB patients were classified into 3 subtypes of concomitant Alzheimer pathology based on Braak stages. The pure form, common form, and AD form corresponded to stages I to II/stages 0 to A, stages II to III/stages B to C, and stages IV to VI/stage C of Alzheimer pathology, respectively (35) (Table 1).

**Antibodies**

Antibodies used were as follows: mouse anti-LAMP2 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Rab7B (Abnova Corporation, Taipei, Taiwan), rabbit anti-LC3 (LifeSpan BioSciences, Seattle, WA), mouse anti-β-actin (AC-15, Sigma, St. Louis, MO), rabbit anti-α-synuclein (AB5038, Chemicon, Temecula, CA), mouse antiphosphorylated α-synuclein (pSer129 [37]), and rabbit antiphosphorylated α-synuclein donated by Dr. H. Akiyama (Tokyo Metropolitan Organization for Medical Research, Tokyo, Japan).

**Plasmid**

pCI-neo-LAMP2 plasmid containing human LAMP2A was prepared as previously described (38). A cDNA encoding human Rab7B (Accession no. BC017092) or LC3 (Accession no. NM_022818) in the plasmid pCMV-SPORT6 or pSPORT-nd was purchased from Open Biosystems (Huntsville, AL). The plasmid DNA was used as the template for polymerase chain reaction using the following primers: Rab7B, 5'-AAAGTTGGAC-3' and 5'-AAAAGGCGCCGCAGAAG-3'; LC3, 5'-AAGGCCTTCAAG-3' and 5'-AACGCGGCAAGCTGAGCAGCA-3'. The amplified fragment containing each cDNA was cloned into the pCI-neo vector (Promega, Madison, WI) via XhoI and NotI sites. All resulting constructs were confirmed by DNA sequencing.

**Cell Culture and Transfection**

The HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Iwaki, Tokyo, Japan). Transient transfection of cultured cells with each plasmid DNA was performed using the Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions.

<table>
<thead>
<tr>
<th>TABLE 2. Staging of Lewy Pathology Progression</th>
</tr>
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<tbody>
<tr>
<td>Stage I</td>
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<tr>
<td>--------</td>
</tr>
<tr>
<td>Amygdala</td>
</tr>
<tr>
<td>Limbic cortex</td>
</tr>
<tr>
<td>(V–IV layers)</td>
</tr>
<tr>
<td>Neocortex</td>
</tr>
<tr>
<td>(V–VI layers)</td>
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</table>

Numbers of α-synuclein-immunopositive Lewy bodies were counted and then scored from 0 to 4, and the degree of α-synuclein-immunopositive Lewy neurites was scored from (−) to (+++), as previously described (34, 35). Lewy stages were graded from stage I to stage IV based on the counts (34, 35).
Western Blotting

HEK-293T cells were seeded at 2 \times 10^5 cells in a collagen I-coated 6-well dish. At 24 hours after transfection with 2 \mu g of each expression plasmid or empty vector, cells were harvested in ice-cold lysis buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, and protease inhibitors). Lysates were rotated at 4°C for 1 hour, and soluble supernatant fractions were obtained by centrifugation at 17,500 \times g for 15 minutes at 4°C.

Triton-soluble, sodium dodecyl sulfate (SDS)–soluble and formic acid–soluble fractions from human brain tissue were prepared for Western blot analysis. Fresh-frozen ischemic temporal cortex from 2 control brains (Cont3 and Cont4) and 4 DLB brains (DLB6, DLB8, DLB20, and DLB 21) were homogenized in 10 volumes of TNE buffer (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton, 1× Complete protease inhibitor cocktail [Roche, Tokyo, Japan]. The homogenate was centrifuged at 100,000 \times g for 30 minutes at 4°C, and then supernatant fractions (Triton soluble) were collected. The resulting pelleted was washed once in TNE buffer then homogenized and further solubilized by sonication. After formic acid was evaporated, the dried sample was resuspended in sample buffer, and the pH was adjusted to neutral with NaOH (formic acid–soluble fraction).

Western blot analysis was performed as previously described (39). The protein samples were separated by SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), blocked for 1 hour, and analyzed by Western blot using antibodies against Rab7B, LAMP2, LC3 (1:1000 dilution), α-synuclein (1:2000 dilution), or β-actin (1:5000 dilution) as the primary antibodies. HRP-conjugated secondary antibodies were used at higher dilutions (Rab7B and LAMP2, 1:20,000 dilution). Sections were blocked overnight at 4°C and processed with avidin-biotin horseradish peroxidase complex (Vector Laboratories). Immunoreactivity was visualized with 0.5 mg/mL 3,3’-diaminobenzidine tetrachloride and 0.03% H2O2 (Vector Laboratories); 2 washes of 20 minutes each in phosphate-buffered saline were carried out between each step. The sections were lightly counterstained with hematoxylin, dehydrated through a graded alcohol series, cleared with xylene, and mounted in mounting medium.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described (40). Cerebral hemispheres including the amygdala, hippocampus, entorhinal cortex, and inferior temporal cortex (Brodman area 20), and brainstem, including the midbrain and pons in each brain (DLB1 to DLB19, AD 1 to AD 9, and Cont 1 to Cont 10 in Table 1) were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, embedded in paraffin, and cut into 6-μm-thick sections. After removal of paraffin, endogenous peroxidase activity was quenched for 30 minutes with 1.5% H2O2 in methanol. After rehydration, antigens were retrieved with autoclaving or treatment with 70% formic acid. Sections were blocked for 1 hour with 10% normal goat or horse serum and then incubated overnight at 4°C, with primary antibodies at the appropriate dilution (Rab7B and LAMP2, 1:200; LC3, 1:1000). Sections were then incubated for 1 hour with biotinylated secondary antibody, either goat anti-rabbit or horse anti-mouse IgG (1:500, Vector Laboratories, Burlingame, CA), and processed for 45 minutes with avidin-biotin horseradish peroxidase complex (Vector Laboratories). Immunoreactivity was visualized with 0.5 mg/mL 3,3’-diaminobenzidine tetrachloride and 0.03% H2O2 (Vector Laboratories); 2 washes of 20 minutes each in phosphate-buffered saline were carried out between each step. The sections were lightly counterstained with hematoxylin, dehydrated through a graded alcohol series, cleared with xylene, and mounted in mounting medium.

Double Immunofluorescence Labeling

For double labeling immunofluorescence, the primary antibodies were used at higher dilutions (Rab7B and LAMP2, 1:100; LC3, 1:500). After incubation overnight at 4°C in rabbit anti-phosphorylated α-synuclein or rabbit anti-LC3 together with mouse anti-Rab7B, mouse anti-LAMP2 or mouse anti-phosphorylated α-synuclein, the sections were incubated for 3 hours with AlexaFluor-488 goat anti-rabbit IgG, AlexaFluor-594 goat anti-mouse IgG and Hoechst (Molecular Probes, Eugene, OR). Immunofluorescence was visualized on an Olympus Fluoview FV1000 Confocal Microscope.

Computerized Cell Counting

For quantification of neuron immunoreactivity for each antibody in DLB brains (amygdala, 15 patients, 73.1 ± 12.2 years, 9 male, 6 female; entorhinal cortex, 19 patients, 75.4 ± 11.8 years, 11 male, 8 female), AD brains (n = 9, 78.9 ± 9.4 years, 4 male, 5 female), and age-matched control brains...
of detergent-soluble extracts derived from HEK-293T cells transiently overexpressing each protein demonstrated that each antibody detects its corresponding target protein (Fig. 1B). We also used human brain extracts (temporal lobe from Cont 3) to demonstrate the antibody specificities. Whereas anti-LAMP2 and anti-LC3 antibodies detected only a single protein species corresponding to the appropriate size of the endogenous LAMP2 or LC3 in human brain extracts, the anti-Rab7B antibody detected a protein corresponding to Rab7B in addition to several larger cross-reactive proteins.

In most of the control brains, Rab7B immunoreactivity was barely detectable in neurons of the amygdala (Fig. 2A), entorhinal cortex (Fig. 2C), hippocampus, and inferior temporal cortex (data not shown), and the expression was generally only detectable in restricted neuronal populations in these brains. In contrast, neurons containing Rab7B-positive structures with various abnormal morphologies are observed in the entorhinal cortex (Fig. 2D) and, to a lesser extent, the amygdala (Fig. 2B) and hippocampus (data not shown) in approximately half of the DLB brains. The proportions of these Rab7B-positive neurons varied among DLB brains. In brains from some DLB subjects, anti-Rab7B labeled markedly enlarged granular structures (Fig. 2B, D) that were not observed in the control brains. We determined the proportions of neurons containing these abnormal structures by counting Rab7B-positive neurons detected by computerized threshold analysis (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A207).

Statistical Analysis

Differences in median value of percentages of neurons immunoreactive for each antibody between DLB and control brains were analyzed by Mann-Whitney U test. The correlation between the percentages of neurons immunoreactive for each antibody in the amygdala or entorhinal cortex and Lewy stages was analyzed by Spearman rank correlation coefficient. Differences in the median value of percentages of neurons immunoreactive for each antibody in the amygdala or entorhinal cortex among the 3 subtypes of DLB brains classified by concomitant Alzheimer pathology (pure, common, and AD forms) were analyzed by Steel-Dwass test, a nonparametric multiple comparison test (41, 42). The value of \( p < 0.05 \) was considered statistically significant.

RESULTS

Localization of the Late Endosomal Marker, Rab7B, in DLB

We used anti-Rab7B, anti-LAMP2, and anti-LC3 antibodies to investigate the localization of late endosomal, lysosomal, and autophagosomal vesicular compartments, respectively, in DLB and control brains. Western blot analysis of detergent-soluble extracts derived from HEK-293T cells transiently overexpressing each protein demonstrated that each antibody detects its corresponding target protein (Fig. 1B). We also used human brain extracts (temporal lobe from Cont 3) to demonstrate the antibody specificities. Whereas anti-LAMP2 and anti-LC3 antibodies detected only a single protein species corresponding to the appropriate size of the endogenous LAMP2 or LC3 in human brain extracts, the anti-Rab7B antibody detected a protein corresponding to Rab7B in addition to several larger cross-reactive proteins.

In most of the control brains, Rab7B immunoreactivity was barely detectable in neurons of the amygdala (Fig. 2A), entorhinal cortex (Fig. 2C), hippocampus, and inferior temporal cortex (data not shown), and the expression was generally only detectable in restricted neuronal populations in these brains. In contrast, neurons containing Rab7B-positive structures with various abnormal morphologies are observed in the entorhinal cortex (Fig. 2D) and, to a lesser extent, the amygdala (Fig. 2B) and hippocampus (data not shown) in approximately half of the DLB brains. The proportions of these Rab7B-positive neurons varied among DLB brains. In brains from some DLB subjects, anti-Rab7B labeled markedly enlarged granular structures (Fig. 2B, D) that were not observed in the control brains. We determined the proportions of neurons containing these abnormal structures by counting Rab7B-positive neurons detected by computerized threshold analysis (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A207).

This analysis demonstrated a significant increase in the percentage of neurons with detectable Rab7B immunoreactivity in the entorhinal cortex (2-tailed \( p = 0.011 \), Mann-Whitney U test = 39), but not the amygdala (2-tailed \( p = 0.797 \), Mann-Whitney U test = 70) of DLB vs control brains (Fig. 2E). The presence of neurons containing Rab7B in each DLB brain was not significantly correlated with the degree of Lewy pathology or concomitant Alzheimer pathology (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A209).

In the substantia nigra pars compacta (SNpc), Rab7B-positive enlarged, granular structures were also occasionally observed in the remaining pigmented neurons in DLB brains but not in control brains (Fig. 2F, G). Brainstem-type and cortical-type LBs and Lewy neurites were immunonegative for Rab7B in the SNpc (Fig. 2H, I), dorsal vagal nucleus, locus coeruleus, and amygdala (data not shown).

Localization of the Lysosomal Marker LAMP2 in DLB

In control brains, LAMP2 localized to the neuronal cytoplasm and processes and to glial cells in the amygdala (Fig. 3A), entorhinal cortex (Fig. 3C), hippocampus, and

FIGURE 3. Immunohistochemical localization of the lysosomal marker, lysosomal-associated membrane protein 2 (LAMP2). Immunostaining patterns in the amygdala (A, B) and entorhinal cortex (C, D) are similar in control (A, C) and dementia with Lewy bodies (DLB) (B, D) brains. (E) Scatter plots show the percentage of neurons containing LAMP2-positive lysosomes in the amygdala or entorhinal cortex. Horizontal bars indicate the mean values. *\( p < 0.05 \). n.s., not significant (Mann-Whitney U test). (F–H) Localization of LAMP2 surrounding Lewy bodies (LBs) in the substantia nigra pars compacta (F), dorsal vagal nucleus (G), and locus coeruleus (H). (I) Double immunofluorescent labeling for LAMP2 (red) and serine-129 phosphorylated α-synuclein (p-α-syn, green) shows that LBs are surrounded by LAMP2-positive lysosomes. Panels (A–D) and (F–H) are counterstained with hematoxylin; (I) is counterstained with Hoechst (blue). Scale bars (A–D, F–H) 20 \( \mu \)m; (I) 10 \( \mu \)m.

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in inferior temporal cortex (data not shown), with a punctate or granular immunostaining pattern. The neuroanatomical distribution and morphological characteristics of LAMP2 immunoreactivity in control brains were not different from those in DLB brains (Fig. 3B, D). Comparison of the percentage of LAMP2-positive neurons between control and DLB brains revealed that in DLB cases, LAMP2 levels were significantly decreased in the entorhinal cortex (two-tailed p = 0.018, Mann-Whitney U test = 43), but not in the amygdala (two-tailed p = 0.112, Mann-Whitney U test = 46). Statistical analyses of LAMP2 levels among DLB groups classified by neuropathology did not demonstrate correlation with the degree of Lewy pathology, whereas there was a small significant difference between concomitant Alzheimer pathologies of DLB subjects in the entorhinal cortex (p < 0.05) (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A210). In general, however, the data do not demonstrate a strong correlation between LAMP2 levels and pathological stages of DLB.

Intriguingly, LAMP2 immunoreactivity occasionally (<5%) surrounded brainstem-type LBs in the SNpc, dorsal vagal nucleus, and locus coeruleus (Fig. 3F–H) of DLB brains. Double immunofluorescent analysis reveals localization of LAMP2 to peripheral external regions of α-synuclein–positive LBs (Fig. 3I).

Localization of the Autophagosomal Marker LC3 in DLB

In most control brains, LC3 immunoreactivity was barely detectable in the cortical and limbic areas, including the amygdala (Fig. 4A), entorhinal cortex (Fig. 4C), hippocampus (Fig. 4E) and inferior temporal cortex (data not shown). In contrast, in most of the DLB brains, detectable or intense LC3 immunoreactivity was observed in neuronal cytoplasm and processes in these brain regions (Fig. 4B, D, F). Cell counting analysis demonstrated that the percentage of LC3–positive neurons was significantly increased in both the amygdala (two-tailed p = 0.015, Mann-Whitney U test = 30.5) and entorhinal cortex (two-tailed p = 0.0008, Mann-Whitney U test = 21.5) and in DLB versus control brains (Fig. 4G). Although there were no significant differences in percentages of LC3–positive neurons among DLB groups classified by concomitant Alzheimer pathology (Figure, Supplemental Digital Content 4, parts C, D, http://links.lww.com/NEN/A212), there was a significant correlation with the degree of Lewy pathology in the entorhinal cortex (Spearman rank correlation coefficient [r] = 0.59, p = 0.025, Figure, Supplemental Digital Content 4, part B; http://links.lww.com/NEN/A212), but not in the amygdala (r = 0.52, p = 0.070, Figure, Supplemental Digital Content 4, part A; http://links.lww.com/NEN/A212). The localization of LC3 in the primary motor cortex, a brain region that usually exhibits only mild Lewy pathology (43), was compared with LC3 in the amygdala or entorhinal cortex to confirm whether increased LC3 immunoreactivity in neurons is specific to vulnerable regions of DLB brains. The LC3 was barely detectable in the primary motor cortex in most DLB cases compared with that in the amygdala or entorhinal cortex (Figure, Supplemental Digital Content 5, parts A–C, http://links.lww.com/NEN/A213). Cell counting analysis demonstrated that the percentage of neurons with detectable LC3 immunoreactivity was significantly decreased in the primary motor cortex versus the amygdala (two-tailed p = 0.003, Mann-Whitney U test = 54.5; Figure, Supplemental Digital Content 5, part D; http://links.lww.com/NEN/A213) or entorhinal cortex (two-tailed p = 0.0001, Mann-Whitney U test = 46.5; Figure, Supplemental Digital Content 5, part D; http://links.lww.com/NEN/A213). Thus, LC3 selectively accumulates in neurons in vulnerable regions of DLB brains.

In addition, many LC3-positive spheroids (Fig. 5A, B) and dystrophic neurites (Fig. 5C) were observed in the lateral hypothalamus of DLB brains. Double immunofluorescent analysis demonstrated that most of these LC3-positive spheroids colocalized with phosphorylated α-synuclein (Fig. 5D). However, the number of LC3-positive spheroids was less than that of α-synuclein–positive spheroids (by ~25–60%). Intriguingly, LC3 predominantly localized to the inside or the inner rim of α-synuclein–positive spheroids (arrows in Fig. 5E, F), but localized throughout α-synuclein–positive dystrophic neurites (arrowheads in Fig. 5E). In the brainstem, LC3 immunoreactivity was generally reduced because there was marked neuronal loss and/or neurodegeneration in these regions. However, LC3-positive puncta or granules (which are considered to represent the vesicular membrane-bound isoform, LC3-II) were frequently observed (Fig. 6A–C). Furthermore, there were LC3-positive brainstem-type LBs (Fig. 6D), spheroids, or dystrophic neurites (Fig. 6E, F). Similar to LC3-positive spheroids in the lateral hypothalamus, double immunofluorescent analysis demonstrated that in α-synuclein–positive brainstem-type LBs, LC3 localized in the LB core and/or the inner rim of the LB halo, whereas phosphorylated α-synuclein localized to the LB halo (Fig. 6G). In contrast, in dystrophic neurites, LC3 entirely colocalized with phosphorylated α-synuclein (Fig. 6H). In the amygdala, entorhinal cortex and inferior temporal cortex, modest or diffuse LC3 immunoreactivity was observed in cortical-type LBs (Fig. 6I, J), although the staining intensity was less in these limbic and neocortical regions than in the brainstem.

Localization of Rab7B, LAMP2, and LC3 in the Entorhinal Cortex in AD

Because the DLB and control cases exhibited varying degrees of concomitant Alzheimer pathology (Table 1), we investigated whether Alzheimer pathology in DLB cases

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**FIGURE 4.** Immunohistochemical localization of the autophagosomal marker microtubule-associated protein 1A/1B light chain 3 (LC3). (A–D) There is greater LC3-immunoreactivity (ir) in the amygdala (B), entorhinal cortex (D), and hippocampal CA region (F) in dementia with Lewy bodies (DLB) brains than in comparable regions in control brains (A, C, E). Insets highlight differences in LC3 immunoreactivity in neurons. (G) Scatter plots of percentages of neurons containing LC3-immunopositive autophagosomes in the amygdala and entorhinal cortex. Horizontal bars indicate mean values. Differences of median values between control and DLB groups in both amygdala and entorhinal cortex were significant by Mann-Whitney U test. * p < 0.05, ** p < 0.001. Panels (A–F) are counterstained with hematoxylin. Scale bars = 100 μm.
might influence the findings by determining the localization of Rab7B, LAMP2, and LC3 in the entorhinal cortex of 9 AD brains. The Rab7B-immunopositive enlarged granules were found in some AD cases (Fig. 7A), but the percentages of neurons containing detectable Rab7B immunoreactivity did not differ between control and AD brains (two-tailed p = 0.225, Mann-Whitney U test = 30.0; Fig. 7B). Lysosomal-associated membrane protein 2 immunoreactivity in neurons was only weakly detected in AD brains (Fig. 7C); levels of LAMP2 were significantly decreased in AD versus control brains (two-tailed p = 0.019, Mann-Whitney U test = 16.0; Fig. 7D). Levels of LC3 immunoreactivity tended to vary among AD cases, but a significant difference was not observed between AD and control brains (two-tailed p = 0.266, Mann-Whitney U test = 31.0; Fig. 7E, F). We also did not observe Rab7B- and LC3-immunopositive senile plaques or neurofibrillary tangles (NFTs) in the entorhinal cortex or hippocampus of AD brains (data not shown). However, LAMP2 immunoreactivity did localize to glial cells surrounding senile plaques in AD brains (Figure, Supplemental Digital Content 6, parts A-C, http://links.lww.com/NEN/A214) but was only rarely observed in NFT-bearing neurons (data not shown), consistent with a previous study describing LAMP1 localization in AD brains (44). Thus, LC3 levels are not upregulated in AD brains or appreciably localized to Alzheimer pathology, whereas decreased levels of LAMP2 are commonly observed in AD and DLB brains.

Biochemical Analysis of Rab7B, LAMP2, and LC3 in DLB

To investigate biochemical alterations of Rab7B, LAMP2, and LC3 proteins in brains of DLB cases, we carried out Western blot analysis using the inferior temporal cortex from 2 control and 4 DLB brains. In control brains, α-synuclein was detected only in the 1% Triton-soluble fractions, whereas in DLB cases, α-synuclein was present in the Triton-soluble (monomeric α-synuclein) and formic acid–soluble fractions (monomer, dimer, and high-molecular-weight species) with negligible levels in the SDS-soluble fraction (Fig. 8), consistent with previous reports (45). Rab7B was detected in both the Triton-soluble and SDS-soluble fractions in DLB and control cases; LAMP2 expression was restricted to the Triton-soluble fraction (Fig. 8). The biochemical solubility of Rab7B or LAMP2 proteins was not different between DLB cases and control subjects because LAMP2 is not detected in the SDS-soluble fraction of DLB or control brains; the relative ratio of Rab7B in the SDS-soluble fractions to Triton-soluble fractions was equally detected in the Triton-soluble and SDS-soluble fractions of control and DLB brains, the specific autophagosomal marker LC3-II (16 kd; membrane-bound isoform) was detected in the SDS-soluble fraction of DLB brains, but was absent from control brains (Fig. 8). Furthermore, in the formic acid–soluble fraction in which Rab7B and LAMP2 expression were not detected, there was accumulation of LC3-II protein and LC3-positive high-molecular-weight species in 3 out of 4 DLB brains, but not in control brains (Fig. 8). The increase of LC3-II levels in SDS-soluble and formic acid–soluble fractions of DLB brains indicates increased numbers of LC3-II-positive autophagosomes in DLB brains, which may reflect increased autophagic activity or alterations in autophagic flux (i.e. impaired autophagosome/lysosome fusion).

DISCUSSION

In this study, we demonstrate marked neuropathologic alterations of the autophagosomal marker LC3 in DLB brains by immunohistochemical analysis. Autophagic activity is normally maintained at low levels in the brain even after induction of nutrient starvation (46). In agreement with this, we demonstrate a negligible level of LC3 immunoreactivity in elderly control brains. In contrast, however, we detected significantly greater levels of neuronal LC3 in vulnerable regions of DLB brains than in control brains. Furthermore, we demonstrate that LC3 localizes to brainstem-type LBs and, albeit to a lesser extent, cortical LBs. Western blot analysis using cortical regions of DLB brains reveals that the membrane-bound autophagosomal marker LC3-II is abundantly detected in detergent-insoluble fractions (SDS-soluble or formic acid–soluble fractions). Importantly, formic acid–soluble fractions, which contain highly insoluble aggregated proteins such as α-synuclein in LBs, accumulate predominantly the LC3-II isoform. Given that in the limbic and cortical regions, LC3 localizes only to cortical LBs but not to senile plaques and NFTs, immunoreactivity of LC3 observed in cortical LBs might be, at least in part, vesicular membrane-associated LC3-II detected in the formic acid fraction. This speculation may be consistent with our observations that in the brainstem, LC3 localizes particularly in the core or inner rim of the halo of LBs, where dense vesicles are observed (47–49). Gitler et al (50) demonstrated that, in a yeast model overexpressing human α-synuclein, α-synuclein accumulated within vesicular clusters. Taken together, our results suggest that LC3-II may exist within, or interact with, membrane-bound compartments in LBs. Intriguingly, recent studies have shown that several gene products associated with familial PD, such as parkin, LRRK2, and synphilin-1, are also localized in the core or inner

FIGURE 5. Localization of microtubule-associated protein 1A/1B light chain 3 (LC3) in Lewy pathology of the lateral hypothalamus of brains from dementia with Lewy bodies (DLB) subjects. (A–C) Light microscopy. Low (A) and high (B, C) magnifications show LC3 immunoreactivity with spheroid-like morphology (arrows in [A] and [B]) and LC3-positive dystrophic neurites (arrows in [C]). (D–F) Double immunofluorescent labeling for LC3 (green) and serine-129 phosphorylated α-synuclein (p-α-syn, red). Low magnification shows colocalization of LC3 with p-α-syn (arrowheads in D). Colocalization of LC3 autophagosomes with Lewy neurites (arrowheads in [E]) and Lewy spheroids (arrows in [E] and [F]). Note that LC3 predominantly localizes to the center or the inner rim of Lewy spheroids. Panels (A–C) are counterstained with hematoxylin; (D–F) are counterstained with Hoechst (blue). Scale bars = (A, D) 100 μm; (B, C) 50 μm; (E, F) 20 μm.
rim of LBs (51–53). Parkin and LRRK2 are reported to be associated with trafficking of the membrane-bound organelles (54, 55) and autophagic vesicles (27, 56–58). Thus, autophagosomal vesicular compartments are likely to be implicated in LB formation, together with such familial PD-related proteins. Taken together, it is plausible that the accumulation of LC3-immunoreactive autophagosomal compartments in LBs is specifically implicated in the pathophysiology of DLB rather than as a consequence of the pathogenic process.

We also investigated the localization of the lysosomal marker, LAMP2, in DLB and control brains. In contrast to LC3, LAMP2 levels were significantly decreased in the entorhinal cortex from DLB brains compared with control brains, although significant differences were not found in the amygdala, suggesting that downregulation of LAMP2 may not be necessary for the pathogenic process of DLB or may represent a benign pathogenic change compared with that of LC3. In addition, we identified LAMP2 immunoreactivity surrounding α-synuclein–positive brainstem-type LBs. Recent studies have shown that the LAMP2a receptor directly interacts with α-synuclein and is required for the degradation of α-synuclein by chaperone-mediated autophagy (CMA), which suggests that CMA is the principal machinery that controls α-synuclein turnover (24, 59). Our observations demonstrate that LAMP2 localization is closely associated with α-synuclein in vivo; however, the number of LBs surrounded by LAMP2 was small, and LAMP2 did not colocalize within LBs themselves. Thus, further studies are warranted to clarify whether LAMP2 is implicated in LB formation in DLB brains.

We speculate that the increased immunostaining of autophagosomal compartments in brain regions, where LB pathology and neuronal loss are most abundant in DLB, may indicate that a resulting increase in LC3 activity (or autophagy in general) may not be sufficient to prevent LB formation and/or neuronal degeneration. Indeed, increased autophagy under these pathological conditions might even promote LB formation and neuronal loss, or perhaps the dense vesicular nature of LBs themselves might reflect stalled autophagy. Whether an increase in LC3 expression in DLB brains directly translates into increased autophagic processing is not known at present, although the autophagic pathway is likely to be altered. Previous reports support our speculation of molecular alterations in the autophagy pathway in Lewy body disease brains. Crews et al (60) demonstrated that levels of mammalian target of rapamycin (mTOR) were increased and autophagy-related protein 7 (Atg7) levels were reduced in the brains of patients with DLB and α-synuclein transgenic mice; mTOR and LC3 colocalized with neurons displaying α-synuclein accumulation and neurodegenerative changes, indicating alteration of the autophagic pathway in DLB brains (60).

In addition, intriguingly, Dehay et al (61) recently demonstrated that LC3-II levels were increased, that LAMP1 levels were decreased, and that LC3 colocalized to α-synuclein in LBs in PD brains, consistent with our results in DLB brains. Dehay et al (61) also found that in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of PD, the lysosomal breakdown induced by increased mitochondrial-derived reactive oxygen species resulted in a defective clearance and subsequent accumulation of autophagosomes (61). Inasmuch as we have also demonstrated decreased levels of LAMP2, our results may indicate impaired fusion between autophagosomes and lysosomes, resulting in the accumulation of autophagosomes containing misfolded proteins such as α-synuclein. In addition to cellular degradation, autophagy is also implicated in cell death and apoptosis based on the presence of autophagosomes in dying cells under some circumstances (62, 63). The pathological characteristics of certain neurodegenerative diseases are often associated with an autophagic morphology (64).

The accumulation of LC3-II protein in the detergent-insoluble fractions we observed may indicate increased numbers of autophagosomes and autophagic vacuoles in neurons with neurodegenerative changes. Therefore, accumulation of LC3-positive autophagosomes in DLB brains may be associated with neuronal degeneration as well as the clearance of LBs. It will be of particular interest to determine the precise role played by the autophagic pathway in the pathogenesis of DLB (65).

The immunoreactivity of the late endosomal marker Rab7B was negligible in control brains. By contrast, in some DLB brains, many large granular compartments immunoreactive for Rab7B were identified in vulnerable regions of DLB brains, including the amygdala, entorhinal cortex, and SNpc. Furthermore, the proportion of neurons exhibiting Rab7B-positive late endosomes was significantly increased in the entorhinal cortex of DLB brains versus control brains. These data are consistent with those of our previous study demonstrating that Rab7B immunoreactivity frequently colocalized to enlarged granular compartments immunoreactive for LRRK2 in DLB brains, where its volume in neurons of the entorhinal cortex was significantly increased compared with control brains (51). Recent studies have shown that α-synuclein can disrupt vesicular trafficking in yeast cells that can be rescued by overexpression of certain Rab GTPase proteins (50, 66).

**FIGURE 6.** Localization of microtubule-associated protein 1A/1B light chain 3 (LC3) within Lewy bodies (LBs) and Lewy neurites in the brainstem and limbic regions of brains from dementia with Lewy bodies (DLB) subjects. **(A–C)** The LC3-positive puncta and granules are observed in the remaining neurons in the substantia nigra pars compacta (SNpc) **(A, B)** and locus coeruleus (C). Magnified images of insets in **(A–C)** highlight LC3-puncta or granules in these neurons (arrows). **(D–F)** Localization of LC3 in the cores of LBs in neuromelanin-containing dopaminergic neurons in the SNpc (arrowheads in **[D]**), in Lewy spheroids in the dorsal vagal nucleus (arrowheads in **[E]**), and in Lewy spheroids and neurites in the locus coeruleus (arrowhead and arrow in **[F]**, respectively). **(G, H)** Double immunofluorescent labeling for LC3 (green) and serine-129 phosphorylated α-synuclein (p-α-syn, red) shows colocalization of LC3-positive autophagosomes in the LB cores (arrowheads in **[G]**) and Lewy neurites (arrows in **[H]**) in the SNpc. **(I)** Light microscopy showing weak LC3 immunoreactivity within cortical LB-like morphology in the amygdala from a DLB case. **(J)** Double immunofluorescent labeling for LC3 (green) and p-α-syn (red) shows that weak LC3 immunoreactivity localizes to a cortical LB in the amygdala in a DLB brain (arrowheads). Panels **(A–F)** and **(I)** are counterstained with hematoxylin; **(G, H, and J)** are counterstained with Hoechst (blue). Scale bars = **(A–H)** 20 μm; **(J)** 10 μm; **(I)** 50 μm.

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Thus, at least some proteins of the small Rab GTPase family can modify α-synuclein–induced toxicity in vivo. However, it should be noted that some DLB brains did not show abnormal enlargement of Rab7B-positive late endosomes; thus, these neuropathologic alterations were variable. Furthermore, significant upregulation of Rab7B-positive late endosomes was not observed in the amygdala of DLB brains. Both results suggest that upregulation of Rab7B may not be necessary for the pathogenic process of DLB. Nevertheless, given that autophagosomes fuse with late endosomes to undergo a maturation process that is driven in part by Rab7B (30, 31), it is possible that the enlargement of Rab7-positive late endosomes could play a role in the function and/or fate of autophagosomal molecules important for the etiology of DLB. Because some cross-reactive proteins were observed by Western blot analysis of human brain extracts using the anti-Rab7B antibody, further investigations using antibodies against other endosome-associated proteins would be worthwhile to clarify the role of the endosomal pathway in DLB.

In conclusion, we demonstrate here that the expression of the autophagosomal protein LC3 is significantly increased in vulnerable brain regions of DLB. The late endosomal protein Rab7B is also increased in DLB brains, but to a lesser extent than LC3. The lysosomal protein LAMP2 is decreased in DLB brains as well as AD brains. LC3 colocalizes within Lewy pathology in many affected regions of the DLB brain, whereas LAMP2 only occasionally localizes to the periphery of brainstem-type LBs. Biochemical analysis demonstrates the accumulation of the autophagosomal marker LC3-II in detergent-insoluble fractions of DLB brains. Recent studies have revealed the contribution of molecules involved in the ALP and membrane trafficking to the pathogenesis of Lewy

FIGURE 7. Localization of Ras-related protein Rab-7b (Rab7B), lysosomal-associated membrane protein 2 (LAMP2), and microtubule-associated protein 1A/1B light chain 3 (LC3) in Alzheimer disease (AD) and control brains. (A, C, E) Immunohistochemical localization of Rab7B (A), LAMP2 (C), and LC3 (E) in the entorhinal cortex from AD subjects. (B, D, F) Scatter plots showing percentages of neurons containing Rab7B-immunoreactivity (ir) (B), LAMP2-ir (D), or LC3-ir (F) detected by computerized threshold analysis in the entorhinal cortex. Horizontal bars indicate the mean values. n.s., not significant, * p < 0.05 (Mann-Whitney U test). Scale bars = 20 μm.
body disease (69), which may be consistent with our results. Because understanding of the role of the autophagic machinery in Lewy body disease is still in its infancy, our findings provide important insights into the pathogenesis of DBL and Lewy body diseases.

FIGURE 8. Biochemical analysis of Ras-related protein Rab-7b (Rab7B), lysosomal-associated membrane protein 2 (LAMP2), and microtubule-associated protein 1A/1B light chain 3 (LC3) in the inferior temporal cortex from control and dementia with Lewy bodies (DLB) brains. Triton X-100 soluble (1%), sodium dodecyl sulfate (SDS) soluble (1%), and formic acid-soluble fractions of the inferior temporal cortex tissue from 2 normal control brains and 4 DLB brains were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-LC3, anti-LAMP2, anti-Rab7B, anti-α-synuclein, or anti-β-actin (loading control) antibodies.

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