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Biochemical Staging of Synucleinopathy and Amyloid Deposition in Dementia With Lewy Bodies

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Abstract

The primary feature of dementia with Lewy bodies (DLB) is the aggregation of alpha-synuclein into characteristic lesions: Lewy bodies (LBs) and Lewy neurites. However, in most of DLB cases, LBs are associated with neurofibrillary tangles and amyloid plaques (both Alzheimer disease [AD]-related lesions). We wanted to determine if this overlap of lesions is statistical, as a result of the late onset of both diseases, or results from a specific physiopathological synergy between synucleinopathy and either tauopathy or amyloid pathology. All patients with DLB from our prospective and multidisciplinary study were analyzed. These cases were compared with cases with pure AD and patients with Parkinson disease and controls. All cases were analyzed thoroughly at the neuropathologic and biochemical levels with a biochemical staging of aggregated α-synuclein, tau, and Aβ species. All sporadic cases of DLB were associated with abundant deposits of Aβ x-42 that were similar in quality and quantity to those of AD. Amyloid precursor protein (APP) dysfunction is a risk factor for AD as demonstrated by pathogenic mutations and Aβ accumulation. The constant and abundant Aβ x-42 deposition in sporadic DLB suggests that synucleinopathy is also promoted by APP dysfunction. Therefore, we conclude that APP is a therapeutic target for both AD and DLB.

Key Words: Alzheimer disease, Amyloid, Alpha-synuclein, Biomarkers, Dementia with Lewy bodies.

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INTRODUCTION

Dementia with Lewy bodies (DLB) is the second most frequent neurodegenerative dementing illness after Alzheimer disease (AD). This disease is now well described at the clinical and neuropathologic levels (1, 2). The main pathologic feature at the molecular level is the aggregation of alpha-synuclein (3) into characteristic neuronal lesions observed in the limbic system and select neocortical areas: Lewy bodies (LBs) and Lewy neurites. Like Parkinson disease (PD) and multiple system atrophy, DLB is a synucleinopathy (4). Other than the temporal course of the disease, DLB and PD with dementia share most of the same clinical and neuropathologic features (1, 5, 6) and are often considered as belonging to a spectrum of the same disease.

However, Lewy bodies are rarely the only lesions in the brain of patients with DLB. Amyloid deposits, one of the characteristic hallmarks of AD, are frequently observed in DLB (7), leading to confusing classifications such as Lewy body variant of AD (8, 9), common and pure forms of diffuse Lewy body disease (10), Lewy body dementia (11), senile dementia of Lewy body type (12), or dementia associated with cortical Lewy bodies (13). Furthermore, neurofibrillary tangles (whose main component is tau protein), the second hallmark of AD, are also found in DLB, but generally at low levels (14). Both AD pathology and Lewy pathology are likely to contribute to cognitive decline in these patients (15). Some studies have demonstrated that in the presence of overlap between AD and DLB lesions, AD tangle pathology is dominant at the clinical level and especially when tau pathology extends in neocortical areas (Braak stages IV–VI) (14, 16, 17). Thus, typical cases of clinical DLB selected for study are likely to be from a spectrum ranging from pure cortical forms of synucleinopathy to forms with amyloid deposits but limited extension of tau pathology.

A crucial question is to find the physiopathologic significance of this extremely frequent overlap of AD and DLB lesions. It could be the result of a statistical overlap between AD and DLB, because age is the main risk factor for these 2 diseases. A synergy or potentiation between different neurodegenerative processes could also be suggested. Indeed, in sporadic AD, we have shown that tau and amyloid precursor protein (APP) pathologies develop strictly in parallel as if there was a direct link or a synergy between the 2 degenerating processes (18). A synergy between tau and synuclein has also already been suggested (19).

We have been able to collect a large bank of brain tissue from patients prospectively followed up with a clinical, neuropathologic, biochemical, and genetic approach (Lille network). From this brain bank, we selected without

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bias enough cases to consider all stages of each illness represented from preclinical (as revealed by the neuropathologist) to the different stages of cognitive impairment (as revealed by clinicians). A biochemical analysis and brain mapping was also used to quantify each degenerative process. This approach, complementary to the neuropathologic one, allowed a better typing of tau pathology (20) and Aβ pathology (18). The biochemical identification and staging of a synucleinopathy, which were necessary for our study, have been set up and are described here. This multidisciplinary approach resulted in a new perception of these diseases.

MATERIALS AND METHODS

Patients

Approximately 100 patients came to autopsy between 1998 and 2003 and their brains were stored in our brain bank. Among these, 9 patients (mean age 73.1 ± 8.1 years) had cortical Lewy bodies. All patients had a complete clinical record file and follow up but were not necessarily clinically diagnosed as probable DLB. Among the 9 patients, 8 had additional AD lesions. A family history of PD with dementia was noted in the only patient with DLB who had a pure form of the disease (aged 66 years at onset). No mutations on the alpha-synuclein gene were found for this patient. Positive and negative controls were also included that had been chosen randomly among the cases of our brain bank with a complete record: 8 patients with typical sporadic AD (mean age 74.5 ± 10.9 years) with no cortical Lewy bodies, 2 patients with PD without any dementia or cortical LB, and 2 young controls with no AD lesions. The clinical diagnosis of possible or probable AD was established according to the criteria of the NINCDS-ADRDA (21) and those of DLB following McKeith et al criteria (1). Clinical and neuropathologic records for each patient are summarized in Tables 1 and 2. All patients were diagnosed prospectively at the Lille Memory Clinic. For all patients attending the clinic, a comprehensive standardized file is fulfilled, including neurologic, neuropsychologic, psychiatric, imaging, and biologic assessments. Most patients are followed up on a regular basis (every 6–12 months) either at the Lille center or at one of the 17 other memory centers in the area that are organized in a network using the same files and database (22). The diagnosis may be reconsidered during the follow up.

Brain Tissue

AQ3 All autopsies were performed at the Lille University Hospital and the brain was recovered in the department of neuropathology. Postmortem delay for each patient is shown in Table 1. The right hemisphere was deep-frozen for biochemical and molecular analyses. The left hemisphere was formalin-fixed for examination in the Department of Neuropathology of the University Hospital of Lille. All dissections were done by the same investigator (CAM).

Neuropathologic Examination

Lewy bodies were quantified in multiple brain areas and subcortical nuclei following the recommendations of Kosaka (1) and modified by Braak et al (23). Neuropathologic diagnosis of DLB was made according to the criteria of Kosaka established in accordance with the consensus for the clinical and neuropathologic diagnosis of DLB (1). For diagnostic examination, LBs were detected using a monoclonal antibody against alpha-synuclein (Clone LB 509, 1/5000e; Zymed CliniSciences, Montrouge, France). For quantification purposes, LBs were counted in an area of 0.5 mm^2, and adjacent areas from the pia mater to the white matter were analyzed. Five adjacent cortical columns were selected and the volume occupied by synucleinopathy was determined using an ocular grid.

The diagnosis of AD was established according to the criteria of the National Institute on Aging and Reagan Institute working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer Disease (24). Amyloid deposition and neurofibrillary tangles (NFTs) were classified in cortical and subcortical areas. Amyloid plaques were detected using Congo red and antibodies against Aβ. NFTs were semiquantified by counting their numbers per square millimeter using AD2 antibody directed against phosphorylated tau protein (26), and silver stained by the Braak-modified Gallyas' stain method. Other neuropathologic features were studied such as vascular involvement, atrophy, and glial reaction. The presence of NFTs in neocortex was determined according to the stages of Braak and Braak (25).

Biochemical Isolation of Insoluble Alpha-Synuclein

Two hundred milligrams of brain tissue was homogenized on ice in 2 mL Tris-buffer (10 mM Tris-HCl, pH 6.8), plus complete protease inhibitor mixture, using a glass-sintered potter. The homogenate was ultracentrifuged at 100,000 × g at 4°C for 1 hour and the supernatant was collected (S1). Two milliliters of Tris-buffer containing 0.5% (v/v) of Triton X-100 were added to the pellet, sonicated, and further homogenized with the glass-sintered potter. Following the same parameters, the homogenate was ultracentrifuged, the supernatant (S2) was collected, and the pellet was processed in Tris-buffer containing 2% Triton X-100. Following the same parameters, the homogenate was ultracentrifuged, the supernatant (S3) was collected, and the pellet was processed in Tris-buffer containing 0.5% SDS for a final ultracentrifugation at 100,000 × g at 12°C. The supernatant (S4) was collected and the pellet (P) was subsequently resuspended and sonicated in Laemmli lysis buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 20 mM DTT, 5% SDS) and heated at 100°C for 10 minutes. Supernatants S1 to S4 were treated with one volume of Laemmli lysis buffer and heated at 100°C for 10 minutes.

Antibodies

Monoclonal antibody Syn1b (3B5) (IgG2a; Innogenetics, Gent, Belgium) was raised against recombinant human alpha-synuclein. This very sensitive and specific antibody recognizes the C-terminal part of alpha-synuclein, between amino acids L113 and E126, based on epitope mapping using 14-mer overlapping peptides. Tau pathology was revealed with AD2, a monoclonal antibody against paired helical filaments that is directed against phosphorylated tau protein

TABLE 1.

Patients					Clinical Assessment				Neuropathology		Cortical Biochemical Staging				Genetic
Final Diagnosis	Code	Sex	Age (years)	Postmortem Delay (hours)	Fulfilled Clinical Criteria		Duration of disease	Familial History of DLB	Braak	Kosaka	Tau	Aβ		α-synuclein	Apo E Genotype
					AD	DLB						Aβ x-42	Aβ 40		
Pure DLB	Lc.76	M	76	8	–	Probable	10	+	2	9	5	0	0	5	E3/E4
Sporadic DLB	Ae.82	F	82	15	–	Probable	5	–	2	6	5	9	0	8	E3/E4
	Hc.80	M	80	24	Possible	Possible	9	–	4	7	5	7	0	5	E3/E4
	Bo.77	M	77	24	Possible	Probable	10	–	3	9	7	4	0	9	E3/E3
	Pa.82	M	82	6	Probable	Probable	5	–	4	7	7	6	0	9	–
	Vn.64	M	64	12	Probable	Probable	17	–	5	2	7	8	0	2	E3/E4
	Rg.61	M	61	8	Probable	Probable	7	–	5	5	10	8	0	3	E3/E3
	Gi.65	F	65	20	Probable	Possible	15	–	6	4	10	9	1	4	E3/E4
	Pr.71	F	71	6	Probable	Possible	10	–	6	9	10	9	5	8	–
AD	Fa.85	F	85	12	Probable	–	6	–	4	0	10	8	8	0	E3/E3
	Dm.68	F	68	11	Probable	–	5	–	5	0	8	7	5	0	E4/E4
	Mt.72	M	72	18	Probable	–	7	–	6	0	10	9	3	0	E3/E4
	Dn.57	M	57	28	Probable	–	5	–	6	0	10	9	4	0	E3/E4
	Fs.69	M	69	24	Probable	–	5	–	4	0	10	8	0	0	E3/E4
	Hr.92	F	92	8	Probable	–	10	–	6	0	10	6	0	0	–
	Dl.80	F	80	12	Probable	–	10	–	5	0	10	8	5	0	–
	Df.73	M	73	9	Probable	–	5	–	5	0	10	7	0	0	–
Parkinson	Ds.59	M	59	10	–	–	17	–	2	2	3	2	0	0	E3/E3
	Am.86	M	86	6	–	–	3	–	3	2	6	7	0	0	E3/E3
Control	Sf.36	M	36	23	–	–	–	–	0	0	0	0	0	0	–
	Dc.26	F	26	15	–	–	–	–	0	0	0	0	0	0	–

AD, Alzheimer disease; DLB, dementia with Lewy bodies.

TABLE 2.

Patient	MMS at Intake (/30)	DRS Mattis (/144)	DLB Criteria								AD Criteria	Vascular Lesions on Brain Imaging
			Fluctuations of Cognition	Visual Hallucinations	Parkinsonism	Falls	Delusions, Depression	Transient Confusion	Neuroleptic Sensitivity	Synthesis		
Lc.76	19	108	+	+	+	+	+	+	–	Probable	No	+
Ae.82	8	NA	+	+	+	+	+	+	–	Probable	No	+
Hc.80	15	NA	+	–	–	+	–	–	–	Possible	Possible	–
Bo.77	18	92	+	+	+	+	–	+	+	Probable	Probable	+
Pa.82	17	NA	+	+	+	–	+	–	–	Probable	Probable	+
Vn.64	10	NA	+	+	+	+	+	–	+	Probable	Probable	–
Rg.61	8	NA	+	+	–	–	+	+	–	Probable	Probable	–
Gi.65	12	NA	–	+	–	+	–	+	–	Possible	Probable	–
Pr.71	10	NA	–	+	–	–	–	+	–	Possible	Probable	–

MMS, mini mental score; DRS, Dementia Rating Scale; DLB, dementia with Lewy bodies; AD, Alzheimer disease; NA, .

(26). AD2 was used at 1 ng/mL for immunohistological staining and at 0.1 ng/mL for Western blot analyses. Amyloid plaques and aggregated Aβ peptides were detected using the rabbit polyclonal antisera, ADA40 and ADA42, generated against synthetic peptides corresponding to the 7 last carboxyterminal amino acids of Aβ 40 and Aβ 42 (18). Aβ 42 species were also specifically detected using the monoclonal antibody 21F12 that recognizes the 42, 43 carboxyterminal end of Aβ (18). Aβ 40 and 42 species were also analyzed using the monoclonal antibody WO2 against the amino acids 4 to 10 of Aβ (18).

Western Blots

Tau pathology was investigated as already described (20). Amyloid pathology was analyzed using electrophoresis in Tris-Tricine gels of formic acid-treated brain tissue homogenates as described previously (18). For alpha-synuclein analysis, an equal volume of sample (15 μL) was loaded on 10% Bis-Tris Criterion gels and processed following the manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France). To quantify alpha-synuclein, an equal amount (10 ng) of recombinant human alpha-synuclein (Innogenetics) was loaded on all gels. The proteins were transferred to 0.2-μm nitrocellulose membranes (Hybond; Amersham Biosciences, Orsay, France) using a Criterion Blotter transfer unit according to the manufacturer's instructions (Bio-Rad). The membrane was blocked in Tris-buffer saline added with 0.1% Tween-20 (TBS-T) and 5% nonfat dried milk for 30 minutes. Syn1b (3B5) monoclonal antibody was incubated overnight at 4°C diluted in TBS-T. Following 3 rinses of 10 minutes each in TBS-T, the membrane was incubated with antimouse antibody coupled to horseradish peroxidase (Sigma, Lyon, France). The immunoreactive complexes were visualized using the ECL Western Blotting Detection Reagents and Hyperfilm ECL (Amersham Biosciences). Quantification was performed using the ImageMaster Elite 1D software (Amersham Biosciences).

Two-Dimensional Gel Electrophoresis

For the characterization of synuclein, 2-dimensional gel electrophoresis (2-DE) was performed as already described (27). Equal amounts of protein (500 μg) of final pellet were used for 2-DE. The sample was sonicated in 2-DE buffer (7 M urea, 2 M thiourea, 4% [v/v] Triton X-100, 20 mM DTT, and 0.6% [v/v] Pharmalytes pH 3-10) and an IPG strip pH 4–7 (Bio-Rad) was equilibrated with the sample for 15 hours. Isoelectrofocusing was performed using the Protean IEF cell following the manufacturer's (Bio-Rad) instructions. Proteins were resolved on Tris-Tricine gels as described previously (27). The gels were transferred for immunodetection according to the manufacturer's instructions, or they were stained with Coomassie Brilliant Blue G250 (Sigma) for mass spectrometric analyses.

Mass Spectrometry Characterization

Coomassie blue-stained polypeptide spots were cut into 1-mm^2 gel pieces and washed twice with 50% (v/v) CH_3CN in 25 mM Tris-HCl pH 9. Gel pieces were dehydrated in a

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Speed-Vac and then in-gel-digested overnight with 10 ng of trypsin (EC 3.4.21.4; Promega) in 3 μL of Tris-HCl pH 9. The resulting digested peptides were recovered in 10 μL of 50% (v/v) CH3CN and 1% trifluoroacetic acid (TFA). Samples were then prepared by the dry-droplet method. One milliliter of the peptide mixture was mixed with freshly dissolved β-cyano-4-hydroxycinnaminic acid 0.5 mL (5 mg/mL in 50% [vol/vol] CH3CN and 0.1%TFA), and spotted on the sample plate. The dry spot was then washed with 5 μL of 0.1%TFA. Mass spectrometry was performed with a MALDI-TOF Voyager-DE-STR (Applied Biosystems, Palo Alto, CA) set to the following parameters: positive mode, reflector, voltage 20 kV, grid 61%, delayed extraction 90 ns, low mass gate 500 amu. The laser energy required to desorb/ionize samples was kept to a low value, compatible with a good signal/noise ratio. Spectra were calibrated externally using the $[M+H^+]$ monoisotopic ions from trypsinized lysozyme.

Statistical Analyses

Statistical analyses were performed with StatView software (StatView SE+Graphics; Abacus Concepts Inc., Meylan, France).

RESULTS

Isolation of the Insoluble Pool of Alpha-Synuclein in Human Brain Tissue

Alpha-synuclein is located in the cytosol, enriched in presynaptic terminals, and has been shown to be associated with synaptic vesicles (28). Nonetheless, alpha-synuclein behaves as a soluble protein by differential centrifugation and gradient fractionation (29–31). The pathologic accumulation of alpha-synuclein fibrils in LB and LN suggests that its biochemical properties, in particular solubility, are modified. Solubility of normal synuclein from the postmortem brain tissue from control subjects was tested by different steps of extraction in buffers with increasing percentages of nonionic and ionic detergents. TBS-soluble (S1), Triton-soluble (S2 and S3), SDS-soluble (S4), and SDS-insoluble (P) fractions were used (see "Material and Methods"). A second wash in more concentrated Triton buffer was used to verify that all Triton-soluble alpha-synuclein was recovered. In control brain tissue, alpha-synuclein was detected in S1, S2, and S4 (Fig. 1). No additional detection of alpha-synuclein was observed in S3. Thus, 3 pools of alpha-synuclein were isolated. The major pools correspond to Tris-buffer-soluble fraction and Triton-soluble fraction. The third pool is Triton-insoluble and SDS-soluble and could represent raft-associated alpha-synuclein (32). Alpha-synuclein was not detected in the final pellet fraction. Our results showed that alpha-synuclein was distributed into 2 major fractions, suggesting that it was essentially cytosolic (highly soluble) and associated with membrane (Triton-soluble fraction). The same methodology was applied to the brain tissue of DLB, AD, PD, and control patients. In both familial and sporadic DLB, alpha-synuclein was detected at 18 kDa in the SDS-soluble fraction (S4) and final pellet in addition to S1 and S2. Moreover, an additional band was detected at 36 kDa, corresponding to dimers of alpha-synuclein. Higher molecular mass aggregates were also observed in final pellet. In contrast, these fractions of alpha-synuclein were not detected in temporal cortex of the patients with AD and patients with PD. These results therefore suggest that our biochemical approach allows a specific dissociation of physiological alpha-synuclein from aggregated alpha-synuclein in DLB.

Two-Dimensional and Mass Spectrometric Analyses

To further investigate the specificity of our aggregated alpha-synuclein extraction protocol, final pellet fractions from patients with DLB and controls were analyzed by 2-dimensional electrophoresis. Antibody Syn1b (3B5) detected alpha-synuclein only in brain tissue from patients with DLB. Serial

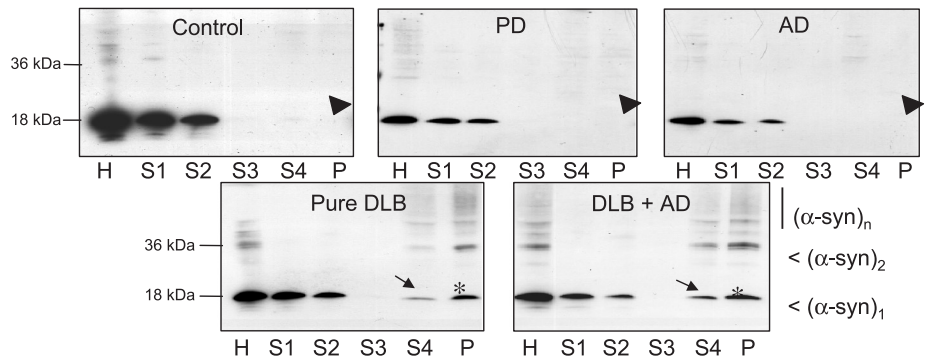

FIGURE 1. Isolation of aggregated alpha-synuclein by differential solubility. Western blot analysis of biochemically fractionated temporal cortex from controls, patients with Parkinson disease (PD), patients with Alzheimer disease (AD), patients with pure dementia with Lewy bodies (DLB), and patients with sporadic dementia with Lewy bodies. Immunoblots were incubated with Syn1b (3B5) antibody against alpha-synuclein. Fifteen microliters of total brain homogenate (H), TBS fraction (S1), Triton 0.5% fraction (S2), Triton 2% fraction (S3), SDS 0.5% fraction (S4), and final pellet (P) were loaded on 10% SDS-polyacrylamide gels. Note the presence of monomers (α-syn$_1$), dimers (α-syn$_2$), and higher molecular mass oligomers (α-syn$_n$) of alpha-synuclein in S4 (arrows) and pellet fraction (asterisks) from patients with pure DLB and patients with sporadic DLB but not from controls, patients with PD, and patients with AD (arrowhead). Our biochemical approach enables us to specifically dissociate the physiological from the aggregated alpha-synuclein in DLB.

spots at 18 kDa ranging from pI 4.3 to 4.6 (Fig. 2A, bottom panel) were observed corresponding to posttranslational-modified species of alpha-synuclein. Dimers of alpha-synuclein were also detected at 36 kDa. Three spots of monomeric species of pathologic alpha-synuclein were stained by Coomassie blue (Fig. 2, upper panel) and further analyzed by mass spectrometry (MS). An in-gel digestion with trypsin was performed and the resulting peptides were analyzed by MS. The peptide masses of the 3 spots matched the alpha-synuclein following MALDI-TOF/MS analysis (Fig. 2B, C). The immunodetected dimeric species of alpha-synuclein were not stained with Coomassie and hence not analyzed by MS. Taken together, 2-dimensional gel electrophoresis followed either by Western blotting with a specific alpha-synuclein antibody or MS analyses demonstrate that our differential solubility protocol enabled to extract only aggregated alpha-synuclein from a homogenate of DLB brain tissue.

Cortical Mapping and Biochemical Staging of Cortical Synucleinopathy

The insoluble fraction of alpha-synuclein was isolated from 5 cortical areas of the DLB-affected brains. The studied cortical areas were chosen according to the recommendation of Kosaka for the diagnosis of DLB: entorhinal cortex, anterior cingulate cortex (Brodmann area 24), temporal cortex (Brodmann area 38), frontal cortex (Brodmann area 10), and parietal cortex (Brodmann area 39). Our method allowed us to establish a cortical mapping of alpha-synucleinopathy that suggests a spatiotemporal spreading of the pathology (Fig. 3). The entorhinal cortex was affected in all DLB brains. The second affected cortical area was the anterior cingulate cortex. Eventually, neocortical areas were involved from temporal to frontal and parietal cortex. We quantified (see "Material and Methods") the amount of monomers of alpha-synuclein in the pellet fraction that represents the pool of

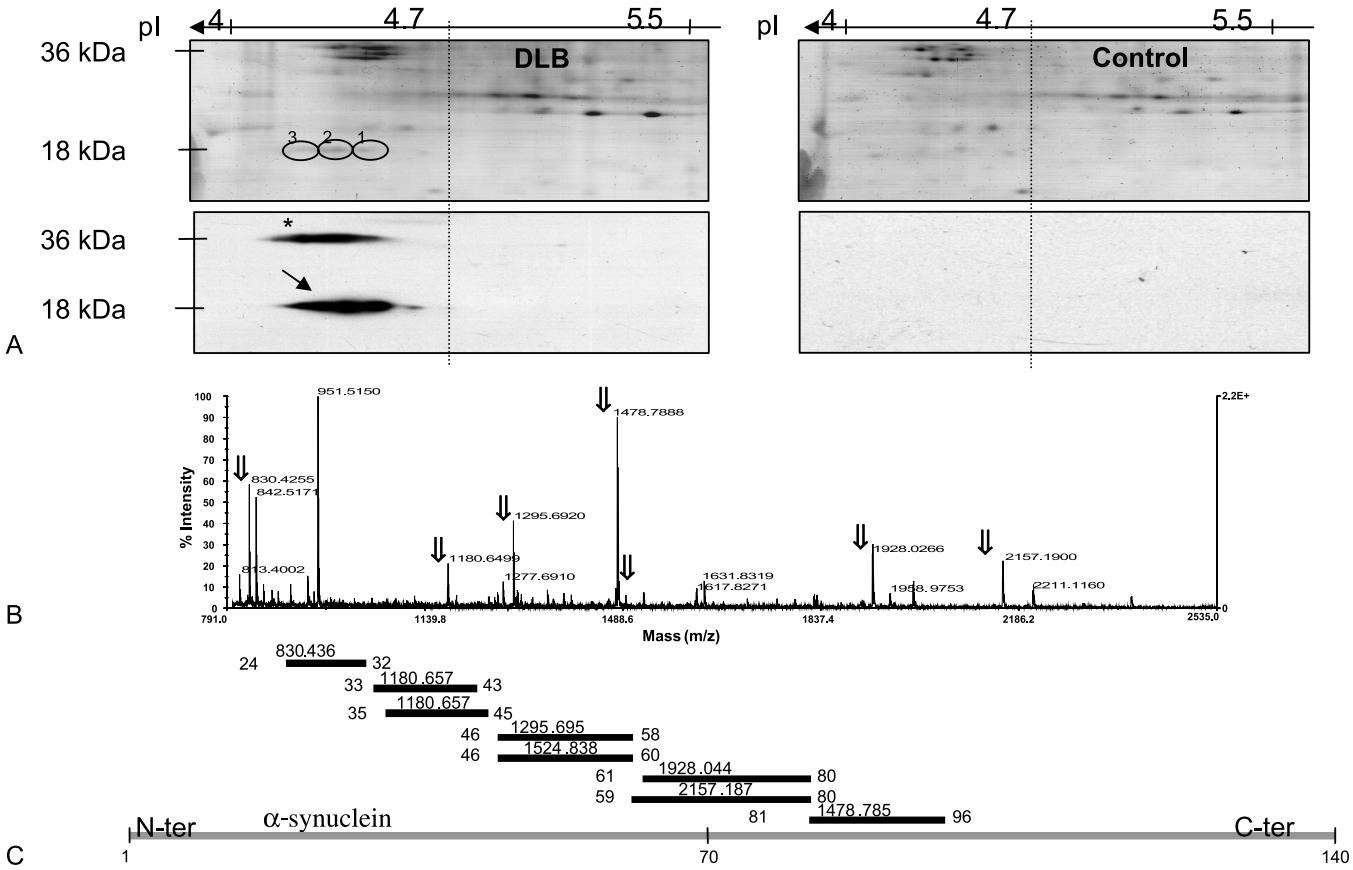

FIGURE 2. Two-dimensional and mass spectrometric analyses. **(A)** Pellet fractions from patients with dementia with Lewy bodies (DLB) and controls were subjected to separation across a pH gradient before separating the proteins according to their molecular masses. Monomeric alpha-synuclein is detected only in patients with DLB by Coomassie blue staining (top panel, spots 1, 2, and 3) and by immunoblotting with Syn1b (3B5) (bottom panel, arrow) and consists of serial spots at 18 kDa. Dimeric species of alpha-synuclein are only detected by immunoblotting with Syn1b (3B5) (bottom panel, asterisk). **(B)** Mass spectrometric analysis of 2-dimensional gels alpha-synuclein spot 1 shown in **(A)** (analysis of spots 2 and 3 revealed the same spectrum). After digestion with trypsin, resulting peptides were analyzed by MALDI-TOF MS. Masses are indicated at the top of the peaks. Peptide masses related to alpha-synuclein are indicated by an arrow. **(C)** Peptides related to alpha-synuclein are shown with their masses and sequences. The sequence of alpha-synuclein is more than 50% covered, demonstrating that the analyzed spots only contained alpha-synuclein.

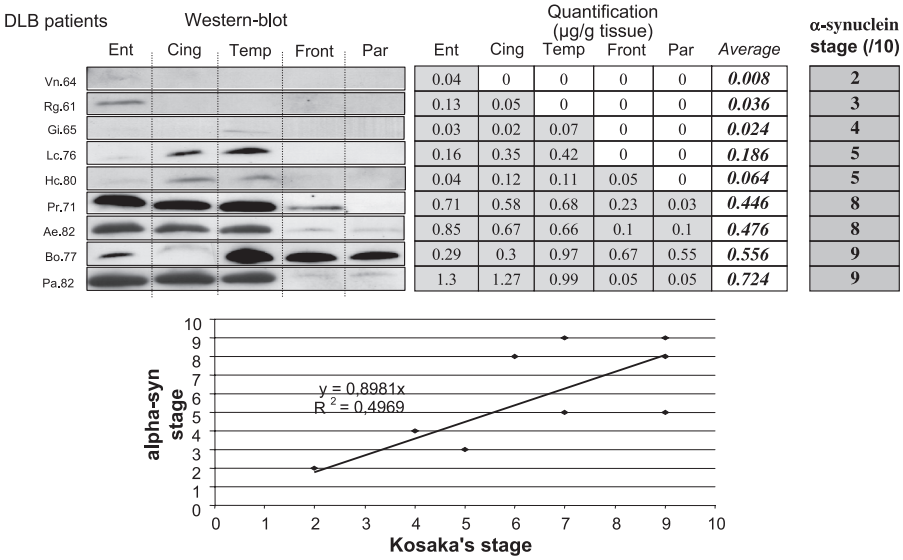

FIGURE 3. Cortical mapping and biochemical staging of synucleinopathy. Western blot analysis of aggregated alpha-synuclein isolated in pellet fraction from cortex of DLB brains. Studied areas were entorhinal cortex (ent), anterior cingulate cortex (cing), temporal cortex (temp), frontal cortex (front), and parietal cortex (par). Immunoblots were incubated with Syn1b (3B5) antibody and the amount of monomers (18 kDa) of alpha-synuclein was quantified. Patients were classified according to our synucleinopathy staging from the least (Vn.64, stage 2) to the most affected brains (Pa.82, stage 9). Note the progressive cortical spreading of synucleinopathy from limbic areas to neocortical areas. On the bottom diagram, each patient with DLB is presented according to its Kosaka's stage (x-axis) and alpha-synuclein stage (y-axis). Linear regression shows good correlation between our biochemical staging and Kosaka staging.

least soluble aggregated alpha-synuclein. We proposed a staging in which the amount of aggregated alpha-synuclein and its spatial distribution were scored from zero to 10. Five points were given as a function of the average amount of alpha-synuclein among the 5 studied areas and 5 points according to the number of affected cortical areas (Table 3).

According to this staging, all our patients with DLB ranged from stage 2 to stage 9 (mean 5.9 ± 2.7) (Fig. 3). One patient was found at stage 2, one at stage 3, one at stage 4, two at stage 5, two at stage 8, and two at stage 9. All other patients, including AD, PD, and controls, were scored at stage zero. Furthermore, the distribution of the patients with DLB among our synucleinopathy scale was strongly correlated with Kosaka stages (Fig. 3).

Biochemical Evaluation of the Overlap of tau, Aβ, and Alpha-Synuclein Pathologies in Dementia With Lewy Bodies

In addition to the quantification of alpha-synuclein pathology, we investigated tau pathology and amyloid deposits in all our brain samples according to our previous studies (18, 20). To simplify the interpretation of the results, we have proposed a staging for the quantification of either Aβ 40 or Aβ x-42 aggregates that resulted from our biochemical analysis of neocortical Aβ species (18) (Table 4).

The main characteristics of AD and DLB brains are shown in Figure 4. For AD brains, tau stages ranged from 8 to 10 (mean 9.7 ± 0.7) and Braak stages from IV to VI (mean 5.1 ± 0.8). For DLB brains, tau stages ranged from 5 to 10 (mean 7.3 ± 2.2) and Braak stages from II to VI (mean stage 4.1 ± 1.5). Eight of 9 patients with DLB had Aβ x-42 deposits (mean stage 7.5 ± 1.8). Only 2 of them had small amounts of Aβ 40 aggregates (mean stage 0.5 ± 1.1), as already described (33). All AD brains had Aβ x-42 aggregates (mean stage 7.7 ± 1). Five of 8 AD brains also had Aβ 40 aggregates (mean stage 3.1 ± 2.9). These findings suggest that most patients with DLB have concomitant AD pathology, i.e. tau and Aβ x-42 aggregates. All our cases of sporadic DLB had additional AD lesions. Pure DLB seems to be a rare event: the only patient in this study with pure DLB had a family history of PD with dementia. Eventually, tau pathology was found to be milder in DLB than in age-equivalent patients with AD.

TABLE 3. Alpha-Synuclein in Cortical Areas

Average Amount of Alpha-Synuclein in Pellet Fraction (µg/g tissue)	Points	Number of Affected Cortical Areas (entorhinal cortex, cingulate cortex, temporal cortex, frontal cortex, parietal cortex)	Points
Trace < 0.1	1	1	1
0.1–0.25	2	2	2
0.25–0.5	3	3	3
0.5–0.75	4	4	4
Over 0.75	5	5	5

The alpha-synucleinopathy stage is obtained by addition of the 2 scores.

Aggregated Aβ x-42 Species Are Identical in Alzheimer Disease and in Sporadic Dementia With Lewy Bodies

Amino-truncated Aβ species represent 60% of all Aβ species not only in full-blown AD but also at the earliest stage of AD pathology (27). Two-dimensional gel electrophoresis followed by Western blotting with 21F12 antibody enabled the characterization of the human brain tissue Aβ x-42 species solubilized with pure formic acid. We applied this approach to all the patients with AD and patients with

TABLE 4. Staging of Aβ Aggregates

Aβ Quantification (μg/g tissue)	Stage
Trace < 2.5	1
2.5–5	2
5–10	3
10–25	4
25–50	5
50–100	6
100–200	7
200–400	8
400–800	9
Over 800	10

Results for each patient are summarized in Table 1.

sporadic DLB of our series. In all cases, 9 monomeric species of Aβ x-42 were detected (Fig. 5). These species have already been fully characterized using mass spectrometric analyses (27) and the same nomenclature was used: spot 1 and 2 correspond to full-length Aβ 42 peptides, and spots 3–7 and 9–10 correspond to amino-terminal truncated and posttranslationally modified variants of Aβ 42. The 2-dimensional pattern of Aβ x-42 as revealed by 21F12 in patients with sporadic DLB strictly overlapped the pattern in patients with pure AD. These results suggest that aggregated species of Aβ x-42 are exactly the same in both pathologies.

DISCUSSION

The aim of this study was to clarify further the overlap of brain lesions of AD and DLB, the 2 major dementias with a proteinopathy. Brain lesions can now be precisely quantified at the biochemical level using specific immunologic tools. These methods allow a reproducible and more precise typing of tau pathology (20) and Aβ deposition (18) in AD. For DLB, simple and direct biochemical protocols are lacking for the very reason that, in the absence of specific antibodies against "pathologic alpha-synuclein," it is currently impossible to distinguish between aggregated and normal alpha-synuclein. Identification of aggregated alpha-synuclein requires fractionation protocols based on differential solubility of proteins in detergents. To visualize and quantify alpha-synuclein aggregates, a prerequisite was to set up a reliable protocol for the isolation of insoluble alpha-synuclein. Previous biochemical studies showed that abnormal filaments of alpha-synuclein can be isolated using ultracentrifugation in detergents such as Triton X-100 or Sarcosyl (34–39). However, none of these confirmed the results by mass spectrometric analysis. Here, we were able to specifically isolate the aggregated pool of alpha-synuclein using a 3-step protocol extraction. The biochemical synucleinopathy with our protocol was in good correlation with the presence of LB or LN at neuropathologic examination. Aggregated alpha-synuclein mainly consists of monomers detected at 18 kDa and dimers at 36 kDa. Using multiple biochemical approaches, we identified in this pool most of already described posttranslational modifications of aggregated alpha-synuclein such as phosphorylation (38), ubiquitination (40), or nitration (41) (data not shown). Furthermore, our protocol allows a reproducible quantification of the most insoluble pool of alpha-synuclein that includes LB- and LN-associated alpha-synuclein and establishes a qualitative and quantitative biochemical diagnostic test for cortical synucleinopathy with good sensitivity and specificity.

In this study, cortical synucleinopathy was graded into 10 stages based on both quantitative evaluation and cortical spreading of alpha-synuclein aggregates. Heterogeneity of the distribution of synucleinopathy lesions is described in Kosaka's assessment of DLB pathology (1, 7). According to LB distribution, LB diseases are divided into 3 main subtypes: brainstem predominant, limbic (transitional), and neocortical. We detected insoluble alpha-synuclein in the cortex from any subtype of our DLB brains. Even brainstem-predominant-type brains had pathologically undetected cortical accumulation of insoluble alpha-synuclein. All the DLB brains had LB in brainstem nuclei (dorsal motor nucleus of the vagal nerve, locus ceruleus, substantia nigra) at neuropathologic examination. Braak et al (23) showed in a large PD cohort that synucleinopathy lesions first occur in the lower brainstem and then assume an upward course, eventually reaching neocortical areas. In corroboration with published data (23, 42), we showed that cortical Lewy pathology does not develop randomly but progresses along a specific and hierarchical pathway of vulnerable neuronal populations. It appears first in limbic cortex and progressively invades neocortical areas from temporal to parietal

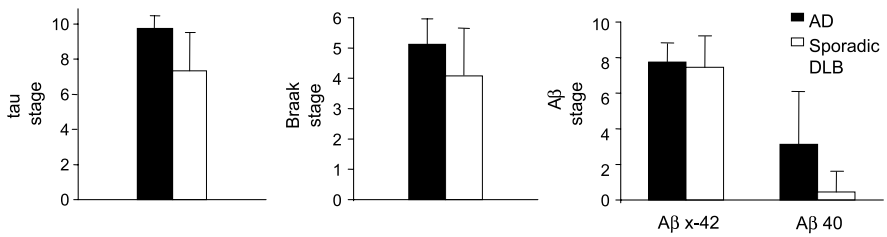

FIGURE 4. Biochemical evaluation of tau, Aβ, and alpha-synuclein overlap. All brain samples in this study were fully characterized for tau, Aβ, and alpha-synuclein pathologies. Average scores and standard deviations are shown. Compared with age-matched patients with Alzheimer disease (AD) (black bars), patients with sporadic dementia with Lewy bodies (DLB) (white bars) show lesser tau pathology evaluated either by tau staging (left diagram) or Braak staging (middle diagram). Patients with sporadic DLB had as much Aβ x-42 deposits as patients with AD (right diagram).

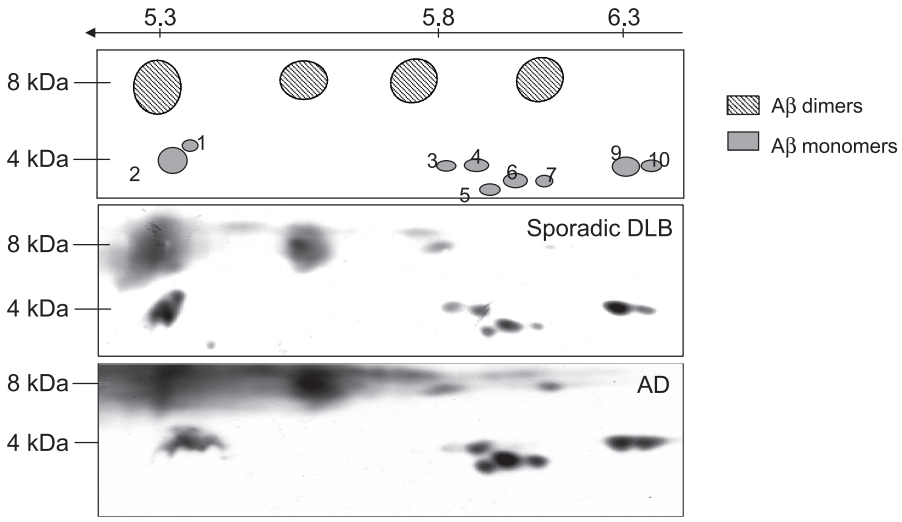

FIGURE 5. Two-dimensional electrophoretic analysis of Aβ x-42 species in Alzheimer disease (AD) and in sporadic dementia with Lewy bodies (DLB). Aβ aggregates solubilized with formic acid were resolved by 2-dimensional gel electrophoresis. Aβ x-42 monomers (4 kDa) and dimers (8 kDa) were detected with 21F12. The 9 characteristic species of aminotruncated Aβ x-42 are exactly the same in patients with AD and in patients with sporadic DLB. We used the nomenclature described by Sergeant et al (27) to label the different spots (top panel).

cortices with a good correlation with neuropathologic staging. Interestingly, there is a similarity with the biochemical pathway of neurofibrillary degeneration observed in AD (20), conferring one more homology between tau and alpha-synuclein pathologies. However, cingulate cortex, which belongs to the limbic system, is more precociously affected by Lewy pathology in DLB than by tangle pathology in AD.

All brain samples in this study were fully characterized for tau, Aβ, and alpha-synuclein pathologies. Although the coexistence of AD lesions with synucleinopathy lesions in DLB was largely reported, it was surprising to note that all sporadic DLB cases of our prospective study had AD lesions and in huge numbers.

Tau pathology was present in all the patients with DLB of this series. Nevertheless, as already described (14), when compared with age-matched AD cases, tau pathology in sporadic DLB was less severe. Patients with sporadic DLB whose tau pathology did not exceed stage 6 according Delacourte et al (20) did not fulfill the clinical criteria for probable AD. In these patients, tau pathology could be attributed to concomitant preclinical AD, and the clinical presentation was often that of typical DLB. Over stage 7, tau pathology was associated with a clinical expression of probable AD. As already suggested (16), there is a confounding role of high tangle pathology that makes clinical diagnosis of DLB less probable.

Strikingly, all sporadic cases of DLB had a large amount of Aβ x-42 aggregates. This was not the result of an enrichment of this population with the apoE E4 genotype (Table 1). We have already described a synergetic effect of tau and Aβ pathologies in sporadic AD, introducing the concept of a synergy of the amyloid burden on the progression of tau pathology (18). Indeed, tauopathy and amyloid deposition progress in parallel in sporadic AD, showing a strong link between these 2 degenerating processes. However, the mechanism by which APP and/or β-amyloid exerts its effects on tauopathy is still a matter of debate. By analogy with sporadic AD, we suggest that APP dysfunction, resulting in amyloid deposition, is a risk factor of synucleinopathy in DLB as well for several reasons.

First, it is well known that Aβ deposition is frequent in DLB (7). In fact, our study shows that they are more than frequent, because they are constant in sporadic DLB. In addition, they are also similar to AD amyloid deposition in quantity and in quality. Indeed, their biochemical profile comprises mostly heterogeneous Aβ species at the C-terminal ending (mainly x-42 species as demonstrated by 21F12 monoclonal antibody) and N-truncated species as demonstrated by 2-D electrophoresis (27) as observed in AD. Our data suggest that AD and sporadic DLB share a common biomarker, namely aggregation of Aβ x-42 species and likely a common physiopathological process.

Second, pathogenic mutations of APP resulting in familial forms of AD demonstrate that a defect in APP metabolism is unambiguously a risk factor of AD. These mutations rule out the fact that amyloid could be the common byproducts of AD tauopathy and DLB synucleinopathy.

Pletnikova et al recently showed that amyloid deposition was associated with enhanced alpha-synuclein lesions in LB diseases (43). Beyer et al suggested that alpha-synuclein accumulation is under the influence of the KPI isoform of APP (44). Together, our biochemical findings led us to conclude that APP dysfunction is also a risk factor of synucleinopathy in sporadic DLB.

Our hypothesis fits well with the demonstration that β-amyloid can enhance alpha-synuclein accumulation and neuronal deficits in a transgenic mouse model expressing both human alpha-synuclein and amyloid precursor protein (APP) (45). Moreover, the same authors showed in an additional cell-free assay that alpha-synuclein oligomerization was promoted by Aβ 42 and not by Aβ 40. In the same way, LB formation has been observed in Down syndrome (46) and familial forms of AD resulting from mutation of presenilin or APP (47), linking further alpha-synuclein and amyloid pathologies.

In our study, the only case of DLB without amyloid deposits was associated with a family history of PD with

dementia and should be considered as a particular case. A mutation of the alpha-synuclein gene (E46K) has been identified in a Spanish family presenting with autosomal-dominant parkinsonism, dementia, and visual hallucinations mimicking DLB (48). The pathologic findings in this kindred were unambiguously consistent with DLB and no AD-type (Aβ nor tau) lesion was observed.

Taken together, our results suggest that β-amyloid, or a loss of APP function associated with amyloid formation, is likely to play a "catalytic" role to stimulate the progression of cortical synucleinopathy in sporadic DLB, as observed for the progression of tau pathology in AD. Indeed, in familial forms of DLB, the synucleinopathy burden generated by a mutation or a genetic defect is so high that the pathology develops by itself. This explains that most familial cases of DLB do not exhibit any amyloid deposition.

In conclusion, the constant presence of amyloid deposits in sporadic DLB strongly suggests that APP dysfunction is a risk factor for synucleinopathy, similar to the way it is observed in AD for the progression of tauopathy. Our hypothesis makes clear that mixed disease (DLB + AD) is the norm. For the clinician, this pathologic overlap results in diagnosis difficulties because of overlapping clinical features. The clinical phenotype of the dementia depends on the relative amount of Lewy-type and AD-type pathologies. The more tau pathology will be abundant in association with synucleinopathy, the more the clinical diagnosis of DLB will be difficult; DLB clinical features will be masked by AD-related features such as memory impairment, language disturbance, or apraxia. The low accuracy of the clinical diagnosis of DLB in case of concurrent tangle pathology emphasizes the need for better diagnostic tools such as biomarkers to help in identifying the synucleinopathy. Identification of specific posttranslational modifications of aggregated alpha-synuclein would help in the biochemical diagnosis of DLB and to better understand the physiopathology of this disease.

The precise defect on APP generating the burden on "alpha-synuclein-dependent" neurons still has to be determined. It could be upstream of Aβ formation and linked to a loss of the neurotrophic or neuroprotective activity (49). If our findings are corroborated by other groups, correcting this APP dysfunction could delay AD as well as DLB.

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