Early-onset Dementia with Lewy Bodies

Masaki Takao[1,2]; Bernardino Ghetti[1]; Hirotaka Yoshida[3]; Pedro Piccardo[1]; Yolanda Narain[4]; Jill R. Murrell[1]; Ruben Vidal[1]; Bradley S. Glazier[1]; Ross Jakes[3]; Miho Tsutsui[4]; Maria Grazia Spillantini[4]; R. Anthony Crowther[3]; Michel Goedert[3]; Atsuo Koto[2,5]

[1] Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, Ind.
[2] Department of Neurology, Keio University School of Medicine, Tokyo, Japan.
[3] MRC Laboratory of Molecular Biology, Cambridge, United Kingdom.
[4] Centre for Brain Repair and Department of Neurology, Cambridge University, United Kingdom.
[5] Faculty of Nursing and Medical Care, Keio University, Tokyo, Japan.

Corresponding author:
Dr Bernardino Ghetti, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, 635 Barnhill Drive, B029D, Indianapolis, IN 46202 USA (E-mail: bghetti@iupui.edu)

The clinical and neuropathological characteristics of an atypical form of dementia with Lewy bodies (DLB) are described. The proband experienced difficulties in her school performance at 13 years of age. Neurological examination revealed cognitive dysfunction, dysarthria, parkinsonism and myoclonus. By age 14 years, the symptoms had worsened markedly and the proband died at age 15 years. On neuropathological examination, the brain was severely atrophic. Numerous intracytoplasmic and intra-neuritic Lewy bodies, as well as Lewy neurites, were present throughout the cerebral cortex and subcortical nuclei; vacuolar changes were seen in the upper layers of the neocortex and severe neuronal loss and gliosis were evident in the cerebral cortex and substantia nigra. Lewy bodies and Lewy neurites were strongly immunoreactive for α-synuclein and ubiquitin. Lewy bodies were composed of filamentous and granular material and isolated filaments were decorated by α-synuclein antibodies. Immunohistochemistry for tau or β-amyloid yielded negative results. The etiology of this atypical form of DLB is unknown, since there was no family history and since sequencing of the exonic regions of *α-Synuclein*, *β-Synuclein*, *Synphilin-1*, *Parkin*, *Ubiquitin C-terminal hydrolase L1* and *Neurofilament-M* failed to reveal a pathogenic mutation. This study provides further evidence of the clinical and pathological heterogeneity of DLB.

INTRODUCTION

Mutations (A30P, E46K and A53T) in the α-*Synuclein* gene and triplication of the region of chromosome 4 that contains the α-*Synuclein* gene cause familial forms of Parkinson's disease (PD) (33, 44, 47, 59). Moreover, α-synuclein is the major component of Lewy bodies and Lewy neurites (48,49), the defining neuropathological characteristics of PD and dementia with Lewy bodies (DLB). This has suggested that dysfunction of α-synuclein may play a central role in all cases of PD and DLB (20). An additional locus associated with Parkinson disease (PARK-3) has been mapped to chromosome 2p13 (15,57), but the underlying genetic defect is not yet known.

Mutations in the *Parkin* (31) and *DJ-1* (1) genes have been identified in cases of juvenile autosomal-recessive parkinsonism. Most cases with *Parkin* mutations appear to exhibit degeneration of dopaminergic nerve cells of the substantia nigra in the absence of Lewy bodies and Lewy neurites (24, 39,

50, 53). However, Lewy body pathology has been described in one individual with familial parkinsonism and a compound heterozygous mutation in *Parkin* (10). The neuropathological features associated with mutations in the *DJ-1* gene are not yet known. It remains to be established whether the mutant forms of α-synuclein, parkin and DJ-1 cause disease by interacting in a common pathological pathway, or whether there are separate mechanisms by which they lead to disease. Mutations in the α-*Synuclein* gene result in a gain of toxic function, whereas most mutations in the *Parkin* and *DJ-1* genes result in a loss of function. Thus far, PARK-5 has been found associated with only one mutation in the *Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1)* gene. This was found in individuals from a small family who had a clinical diagnosis of PD, but no neuropathological corroboration (37). It remains to be firmly established that *UCH-L1* is a gene for PD.

DLB is the second most common form of degenerative dementia of old age, after Alzheimer disease (AD) (26). Neuropathologically, it is characterized by the presence of large numbers of Lewy bodies and Lewy neurites in the neocortex, limbic system and brainstem. A variable amount of AD-type pathology in the form of neuritic plaques and neurofibrillary lesions is also present in the majority of patients with DLB.

Here we present the clinical, neuropathological and genetic characterization of a Japanese individual who developed rapidly progressive parkinsonism and dementia at 13 years of age. Neuropathologically, numerous widespread α-synuclein-positive Lewy bodies and Lewy neurites were present throughout the cerebral cortex, subcortical nuclei and brainstem; there was no evidence of AD-type pathology. The severity and distribution of α-synuclein deposition exceeded that of typical DLB.

MATERIALS AND METHODS

Clinical examination of the proband. The proband developed neurological signs at 13 years of age. Throughout the 2-year-long course of the disease, she underwent several neurological, neuro-ophthalmological, electrophysiological and neuroradiological examinations.

Autopsy. An autopsy of the proband was carried out. Tissue samples of the heart, lung, liver, pancreas, spleen, esophagus, stomach, small and large intestines, kidney, thyroid, adrenal glands and ovaries were fixed with 10% formalin. Following formalin fixation the tissue samples were dehydrated in graded alcohols, cleared in

Antibody	Type	Dilution	Source and Reference
Anti-α-synuclein			
PER1 (α-synuclein $_{11-34}$)	r	1:200	M. Goedert (30)
PER4 (carboxy-terminal)	r	1:1000	M. Goedert (49)
PER7 (α-synuclein $_{1-120}$)	r	1:1000	M. Goedert (30)
SYNP129	r	1:200	T. Iwatsubo (14)
α-synuclein $_{119-137}$	r	1: 300	B. Ghetti (43)
Anti β-synuclein			
PER3	r	1:200	M. Goedert (29)
Anti γ-synuclein			
PER5	r	1:500	M. Goedert (49)
Anti-parkin	m	1:200	Chemicon
Anti-DJ-1	r	1:500	M. Goedert (unpublished)
Anti-synphilin-1	r	1:400	Chemicon
Anti-ubiquitin	r	1:100	Dako Corporation
Anti-neurofilament			
SMI-31 (p-NF200kDa)	m	1:2000	Sternberger Monoclonals
SMI-32 (non p-NF200kDa)	m	1:2000	Sternberger Monoclonals
AB1981 (NF150kDa)	r	1:200	Chemicon
AB1983 (NF68kDa)	r	1:200	Chemicon
Anti-tau			
AT8(p-tau $_{Ser202/Thr205}$)	m	1:400	Polymedco, Inc.
Alz50	m	1:20	P. Davies (3)
Anti-β-amyloid 10D5	m	1:100	Elan Pharmaceuticals
Anti-prion protein 3F4	m	1:200	Signet
Anti-neuroserpin	r	1:2000	R. Davis (7, 51)
Anti-GFAP	r	1:100	Dako Corporation

Table 1. *Antibodies used for immunohistochemistry.*
Abbreviations: p-NF = phosphorylated neurofilament, non P-NF = non-phosphorylated neurofilament, p-tau = phosphorylated tau, GFAP = glial fibrillary acidic protein, m = mouse monoclonal, r = rabbit polyclonal.

xylene and embedded in paraffin. Eight-μm-thick sections were cut and stained with hematoxylin and eosin or processed for α-synuclein immunohistochemistry.

Macroscopic neuropathology. From the brain of the proband, 50 g of tissue from the superior frontal gyrus and cingulate gyrus were dissected and stored at -70°C. The remainder of the brain and the entire spinal cord were fixed in 10% formalin.

Histology and immunohistochemistry. Samples from the superior frontal gyrus, cingulate gyrus, superior temporal gyrus, precentral gyrus, postcentral gyrus, calcarine cortex, hippocampus, caudate nucleus, putamen, claustrum, globus pallidus, thalamus, amygdala, hypothalamus, cerebellar cortex, dentate nucleus, midbrain, pons, medulla and spinal cord were dehydrated in a graded alcohol series, cleared in xylene and embedded in paraffin. Eight-μm-thick sections were stained with hematoxylin-eosin, the Heidenhain-Woelcke method for myelin, the Bodian silver staining for axons, the Perls method for ferric iron and thioflavin S.

Immunohistochemical analysis of archival and newly generated paraffin-embedded tissue blocks was carried out using an antigen retrieval method, to maximize the labeling (37). Antibodies specific for α-synuclein, β-synuclein, γ-synuclein, parkin, DJ-1, synphilin-1, ubiquitin, tau, β-amyloid, prion protein, neuroserpin, neurofilaments and glial fibrillary acidic protein (GFAP) were used (Table 1). The anti-DJ-1 serum was raised in a rabbit by using a synthetic peptide corresponding to amino acids 174-189 of human DJ-1 as the immunogen. By Western blot analysis, the antibody strongly labeled recombinant GST-DJ-1 at 1:1000 dilution (not shown). The signal from polyclonal antibodies was visualized using avidin-biotin, with

goat anti-rabbit immunoglobulin as the secondary antibody, followed by horseradish peroxidase-conjugated streptavidin and the chromogens diaminobenzidine or tetramethylbenzidine. The signal from monoclonal antibodies was detected using avidin-biotin, with goat anti-mouse immunoglobulin as the secondary antibody, followed by alkaline phosphatase-conjugated streptavidin. Sections were counter-stained with hemotoxylin.

Semi-quantitative analysis. The frequency and distribution of intracytoplasmic and intraneuritic Lewy bodies were assessed semi-quantitatively. A subjective scale was used to represent the numbers of Lewy bodies in a field observed through a 10× objective (0 = none; 1 = 1-4; 2 = 5-10; 3 = >10). In addition, we evaluated the severity of Lewy neurite pathology in each area and the degree of neuronal loss and gliosis.

Electron microscopy on tissue sections. Frontal cortex, substantia nigra and locus coeruleus of the proband were fixed in 4% formaldehyde and post-fixed with 1% osmium tetroxide, dehydrated in a graded alcohol series, cleared in propylene oxide and embedded in Epon. One-μm-thick sections were stained with toluidine blue. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and scanned by electron microscopy.

Extraction and characterization of isolated α-synuclein filaments. α-Synuclein filaments were extracted from the superior frontal gyrus of the proband, as described previously for cases of DLB (49). Aliquots of the dispersed filament preparations were placed on carbon-coated 400-mesh grids and stained with 1% lithium phosphotungstate. Micrographs were recorded at a nominal magnification of ×40 000 on a Philips model EM208S microscope. Procedures for immunoelectron microscopy were as previously described (5). The following 7 antibodies were used at 1:100 dilution: PER4, which recognizes residues 121-140 of α-synuclein (49); PER1, which recognizes residues 11-34 of α-synuclein (29); SYN36, which recognizes the carboxy-terminus of α-synuclein (38); SYN303, which recognizes residues 2-12 of α-synuclein (9); SYNP129, which recognizes α-synuclein

phosphorylated at serine residue 129 (14); anti-tau antibody BR134 (19) and anti-ubiquitin antibody 1310 (Chemicon).

Sequential extraction of α-synuclein. One hundred mg of tissue of the superior frontal gyrus from the proband and from a 45-year-old control individual were extracted using a modification of the procedure described in (14). The tissues were homogenized in 400-µl buffer A (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, containing a cocktail of protease inhibitors [Complete™, Roche]). A 20-minute centrifugation at 543 000g gave supernatant TBS-sol and pellet. The pellet was homogenized in 400-µl buffer B (buffer A with 10% sucrose and 0.5 M NaCl) containing 1% Triton X-100, incubated for 30 minutes at 37°C, followed by a 20-minute centrifugation at 543 000g, giving supernatant TX-sol and pellet. The pellet was homogenized in 400-µl buffer B containing 1% sarkosyl, incubated for 30 minutes at 37°C, followed by a 20-minute centrifugation at 543 000g, giving supernatant Sark-sol and pellet. The pellet was extracted in 8M urea in buffer A and sonicated (Urea-extr). For immunoblot analysis, 5 µl of each extract was run on SDS-PAGE and labeled with anti-α-synuclein antibody SYN208, which recognizes human α-synuclein, but not β- or γ-synuclein (16). Enhanced chemiluminescence (ECL, Amersham Pharmacia) was used to detect the signal.

Genetic analysis. Genomic DNA was extracted from frozen brain tissue of the proband and blood of 50 controls from the Japanese population using the QIAamp DNA Mini Kit (QIAGEN). The DNA was analyzed by direct sequencing of exons 1-6 of the α-*Synuclein* gene (GenBank: AH005859), exons 1-6 of the β-*Synuclein* gene (GenBank: AF053134), exons 1-10 of the *Synphilin-1* gene (GenBank: AH009850), exons 1-12 of the *Parkin* gene (GenBank: XM_051780) and exons 1-3 of the *Neurofilament medium polypeptide* gene (*NF-M*) (GenBank: XM_005158). Amplification of exons 1-6 of the α-*Synuclein* gene made use of the following primers: Exon 1: 5′-GAGAA GGAGG AGGAC TAGGA GG-3′ and 5′-CGGTG TTCTC CAGGA TTTC-3′. Exon 2: 5′-CACTT TGGAG GGTTT CTCAT G-3′ and 5′-ATACC TCTGA CTCAG TCCAC

	ICLB	INLB	LN	Neuronal Loss/Gliosis
Cerebrum				
Frontal cortex	3	3	severe	severe/severe
Cingulate cortex	3	3	severe	severe/severe
Temporal cortex	3	3	severe	severe/severe
Insular cortex	3	3	severe	severe/severe
Parietal cortex	3	3	severe	severe/severe
Motor cortex	3	3	severe	moderate/severe
Sensory cortex	3	3	severe	moderate/severe
Insular cortex	3	3	severe	severe/severe
Occipital cortex	2	2	severe	moderate/severe
Amygdala	3	3	severe	severe/severe
Hippocampus	2	3	severe	moderate/severe
Dentate gyrus	1	3	mild	mild/mild
Subiculum	2	3	severe	moderate/severe
Entorhinal cortex	3	3	severe	severe/severe
Caudate nucleus	3	3	severe	severe/severe
Putamen	2	2	moderate	moderate/moderate
Claustrum	2	2	severe	moderate/moderate
Globus pallidus	0	0	mild	mild/mild
Thalamus	1	1	mild	moderate/moderate
Hypothalamus	3	2	severe	mild/mild
Substantia innominata	2	1	moderate	moderate/moderate
Suprachiasmatic nucleus	2	2	severe	moderate/mild
Cerebellum				
Cortex	0	0	none	mild/moderate
Dentate nucleus	0	0	none	moderate/moderate
Midbrain				
Periaqueductal gray	3	1	severe	moderate/moderate
Oculomotor nerve nuclei	3	1	severe	mild/mild
Substantia nigra	3	3	severe	severe/severe
Pons				
Locus coeruleus	3	2	severe	severe/severe
Raphe nucleus	3	2	severe	mild/mild
Basis pontis	1	0	mild	moderate/severe
Medulla				
Hypoglossal nerve nucleus	0	1	mild	mild/mild
Dorsal motor nucleus of vagus	3	1	severe	moderate/moderate
Vestibular nucleus	1	0	moderate	mild/mild
Lateral reticular nucleus	3	1	moderate	mild/mild
Inferior olivary nucleus	0	0	mild	moderate/moderate
Accessory olivary nucleus	0	0	mild	mild/mild
Gracile and cuneate nuclei	0	0	mild	mild/mild
Spinal cord				
Anterior gray horn	0	0	mild	mild/mild
Intermediolateral nucleus	1	0	mild	mild/mild
Dorsal nucleus of Clarke	1	0	mild	mild/mild

Table 2. *Lewy bodies, Lewy neurites, neuronal loss and gliosis.*
Abbreviations. ICLB = Intracytoplasmic Lewy body, INLB = intraneuritic Lewy body, LN = Lewy neurite. Semi quantitative scale 0 = none, 1 = 1-4, 2 = 5-10, 3 = >10. Number of lesions in a field observed through a 10x objective.
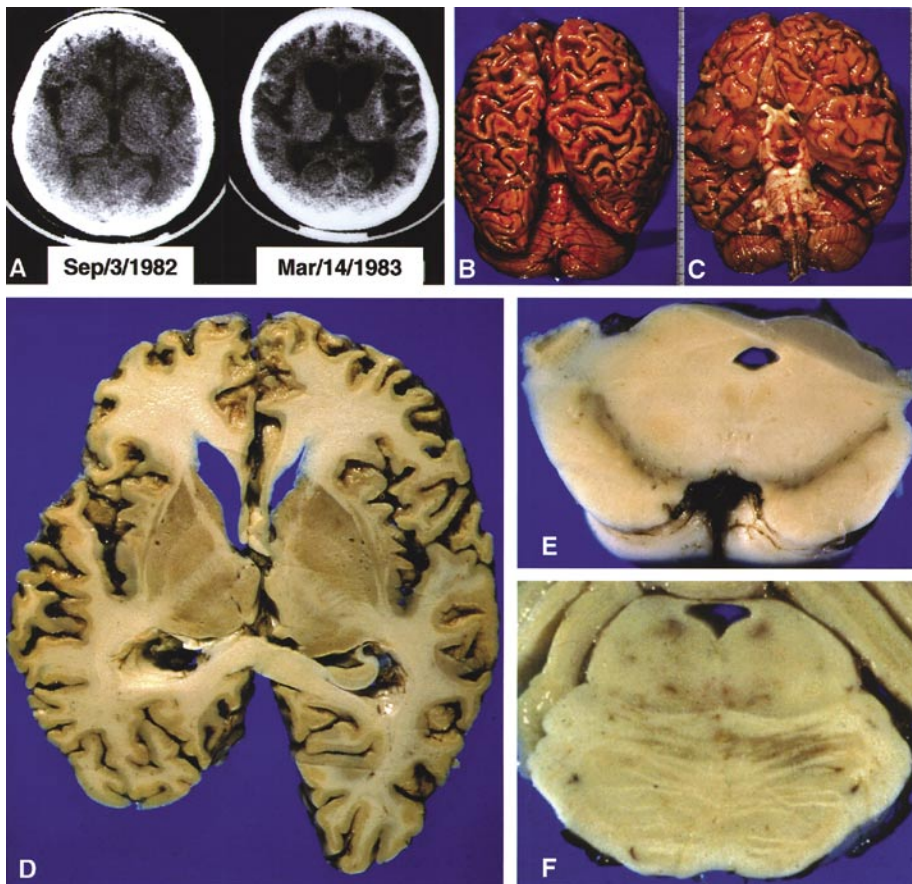
Figure 1. Proband's brain. **A.** Computed tomography scans taken 6 months apart show progressive cerebral atrophy and a marked enlargement of the lateral ventricles. **B, C.** The brain shows cortical atrophy and a widening of the sulci. **D, E, F.** Axial sections of cerebrum (**D**), midbrain (**E**) and pons (**F**). Severe cortical atrophy is present in the cerebrum; the lateral ventricles are enlarged. The substantia nigra and locus coeruleus show mild depigmentation.

C-3′. Exon 3: 5′-GTGGT GGTTA GGAGT TCCTT C-3′ and 5′-GTTCT TAGAT GCTCA GTGAT TG-3′, Exon 4: 5′-ATGGC TAGTG GAAGT GGAAT G-3′ and 5′-CACAG TAAGT ATCTT GCTCC TG-3′. Exon 5: 5′-GTGGC CAACA TCCCT ATATG-3′ and 5′-GAGAA ATGTG ACAAT GACAG G-3′. Exon 6: 5′-TTTTA GTGTA AGTGG GGAGC CATTT C-3′ and 5′-CATTG GAACT GAGCA CTTGT AC-3′.

Amplification of the exons encoding the other genes examined made use of previously described primers (11,31,34,35,56). PCR was carried out with 20 ng/µl genomic DNA as the template using the high fidelity expanded PCR system (Roche Molecular Biochemicals). PCR conditions were as follows: denaturation, 94°C, one minute; annealing, 56°C, one minute; extension, 72°C, one minute, for a total of 35 cycles. For the amplification of exon 3 of the *UCH-L1* gene (GenBank: XM-051780) the GC-rich PCR system (Roche Molecular Biochemicals) was used (96°C,

30 seconds; 55°C, 30 seconds; 72°C, 30 seconds, for a total of 40 cycles).

Total RNA was extracted from frozen brain tissue using the ToTALLY RNA kit (Ambion Inc.). Complementary DNA was produced by reverse transcription of RNA using random primers and the ProSTAR kit (Stratagene). For the amplification of α-synuclein cDNA, PCR was carried out using primers 5′-GTGTA AAGGA ATTCA TTAGC-3′ and 5′-ATCTC AAGAA ACTGG GAG-3′ (42). For the amplification of parkin cDNA, the first round PCR used primers 5′-GCCGC CACCTA CCCAG TGACC AT-3′ and 5′-AGCAC CACTC GAGCC TGCAC T-3′, corresponding to nucleotides 81-103 and 1440-1420 of the published sequence (GenBank: AB009973). Two sets of primers were used for the second round PCR: 5′-CCACC TACCC AGTGA CCATG ATA-3′ and 5′-CCACA CAAGG CAGGG AGTAG CCAA-3′, corresponding to nucleotides 85-109 and 972-949, and 5′-AACAA ATAGT CGGAA CATCA CTTGC A-

3′ and 5′-GCGGA CACTT CATGT GCATG CA-3′, corresponding to nucleotides 791-816 and 1413-1392. Both sets of primers amplified a DNA sequence containing codon 272. PCR was done at 94°C for one minute, 60°C for one minute and 72°C for 2 minutes for a total of 40 cycles.

Amplified products were gel-purified using the QIAquick gel extraction kit (Qiagen). Sequence analysis was carried out using the quick start CEQ dye terminator cycle sequencing kit on a CEQ 2000XL DNA sequencer (Beckman Coulter).

RESULTS

Clinical findings. Clinical history. The proband, a Japanese female, began to experience difficulties at school at age 13 years, as manifested by a lack of concentration and problems with dressing herself and handwriting. During the same year, she developed dysarthria, monotonous speech and tremor in her upper and lower extremities. A neurological examination documented a cognitive impairment, a stooped posture, bradykinesia, tremor and rigidity in all extremities, as well as micrographia, myoclonus and ataxia. Pharmacological treatment with L-dopa and amantadine failed to improve the neurological symptoms. The proband was hospitalized at 14 years of age because of aspiration pneumonia. At that time, she only responded to sensory stimuli and exhibited decorticate rigidity and frequent myoclonus. The tendon reflexes were normal and no pathological reflexes were noted. A neuro-ophthalmological examination failed to show conjunctival teleangiectasia, corneal or lens opacity, a cherry-red spot, macular or pigmentary degeneration, or optic atrophy. Routine blood cell counts, blood chemistry and urine analysis were normal, except for an increase in the level of creatine kinase. The level of mucopolysaccharides in the urine was normal. Vacuolated lymphocytes were not observed. Serum levels of amino acids, iron, copper, ceruloplasmin and β-lipoprotein were normal. Lysosomal enzymes involved in storage diseases, such as GM1 and GM2 gangliosidoses, Gaucher disease, α-mannosidosis and mucopolysaccharidoses IIID and IVB were normal. The titers of antibodies directed against measles virus, rubella virus, cytomegalovirus, herpes simplex virus, Japanese encephalitis virus,

poliovirus, cocksackievirus, echovirus and parainfluenza virus were normal. Examination of the cerebrospinal fluid for cells, proteins, glucose and antibodies against measles virus was also unremarkable. The patient died of bronchopneumonia at 15 years of age.

Family history. Based on clinical records, there was no neurological disease in any of the proband's relatives. There was no evidence suggesting consanguineous marriage in any relative. The karyotype of the proband was not examined.

Electrophysiology. An electroencephalogram (EEG) was carried out at 14 years of age and showed diffuse slow waves. Nerve conduction studies and a needle electromyography were carried out at 14 years of age and revealed no abnormalities.

Neuroradiology. Brain computed tomographic (CT) scans were carried out when the proband was 13- and 14-years-old. No abnormalities were reported at 13 years, but a progressive diffuse brain atrophy was observed on repeated CT scans at 14 years (Figure 1A).

Biopsy. At 14 years of age, the proband underwent biopsies of quadriceps femoris muscle, sural nerve and rectum. No Lafora bodies or deposits characteristic of storage diseases or metabolic disorders were present. At the time of this study, biopsy tissue was not available for reexamination.

Autopsy. Analysis of multiple sections of the lung revealed the presence of bronchopneumonia. No other pathologic alterations were found in extraneural tissues. α-Synuclein immunohistochemistry of sections obtained from the gastrointestinal tract yielded negative results.

Macroscopic neuropathology. The proband's brain weighed 780 g. A severe generalized atrophy and an enlargement of the sulci were present (Figure 1B, C). On horizontal slices, the cortical grey matter was much thinner than normal and the lateral ventricles were enlarged (Figure 1D). The head of the caudate nucleus was moderately atrophic. The substantia nigra and the locus coeruleus were pigmented (Figure 1E, F). The degree of pigmentation was less than that expected in an adult. It was not possible to determine how the degree of pigmentation would compare to age, gender and race-matched normal controls.
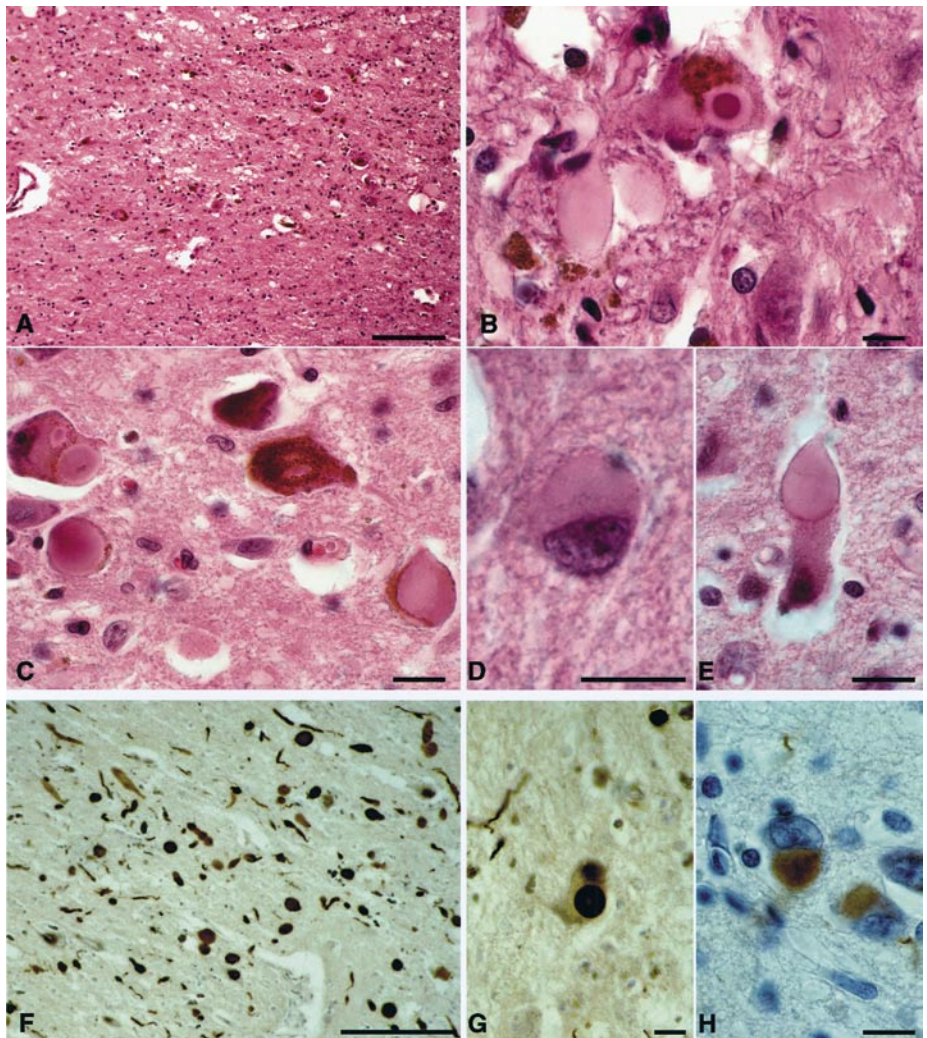

Figure 2. Sections of the substantia nigra (**A, B, F, G**), locus coeruleus (**C**), temporal cortex (**D, E**) and frontal cortex (**H**). **A.** Neuronal cell loss and gliosis are evident. **B, C.** Brainstem Lewy bodies. **D, E.** Cortical Lewy bodies. **F, G, H.** Lewy bodies and Lewy neurites. **A-E.** Hematoxylin-eosin; **F-H**, Immunohistochemistry using antibodies SYNP129 (**F, G**) and α-synuclein (119-137) (**H**). Scale bars: **A, F** = 100 μm; **B-E, G-H** = 10 μm.

Microscopic neuropathology. Three types of α-synuclein deposits were widespread throughout the cerebral cortex and the brainstem: intraperikaryal Lewy bodies, intraneuritic Lewy bodies and Lewy neurites (Figure 2; Table 2). In nerve cell perikarya, Lewy bodies had an eosinophilic and hyaline appearance with or without a dense core. Most were round, but some had a highly irregular shape. Intraneuritic Lewy bodies were eosinophilic, hyaline and mildly argentophilic. Some α-synuclein deposits extended from nerve cell bodies into processes. Most Lewy neurites were argentophilic; they had either a thread-like or torpedo-like shape. Lewy bodies and Lewy neurites were strongly labeled by anti-α-synuclein antibodies PER1, PER4, PER7, SYNP129 and α-synuclein 119-137 (Figures 2-6). Most α-synuclein-positive

structures were also ubiquitin-immunoreactive (Figure 6D). Some Lewy bodies and Lewy neurites were labeled by antibodies directed against NF-M (Figure 4C, D). Occasional deposits were weakly labeled by an anti-parkin antibody. Lewy bodies and Lewy neurites were not labeled by antibodies directed against DJ-1, β-synuclein, γ-synuclein, synphilin-1, the low- and high-molecular weight neurofilament subunits, tau, β-amyloid, prion protein, neuroserpin or GFAP (data not shown).

The neocortex showed severe neuronal cell loss and gliosis, with extensive vacuolar changes in the upper layers of the frontal, cingulate and temporal cortices (Figure 3). Intraperikaryal Lewy bodies were frequently observed in small to medium-sized neurons of the deeper cortical layers, with smaller numbers in the upper layers. How-
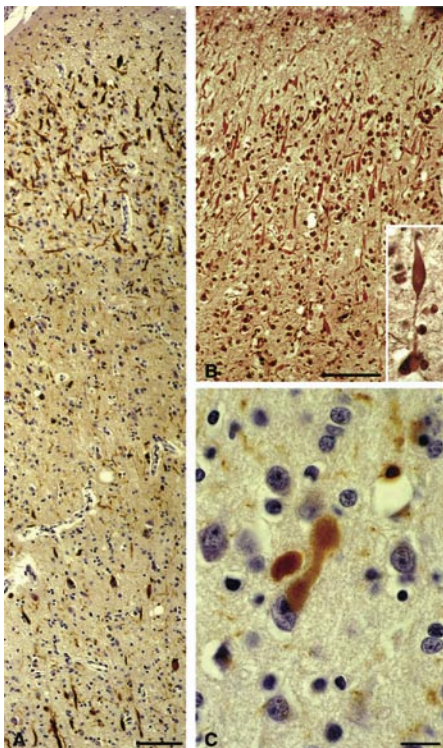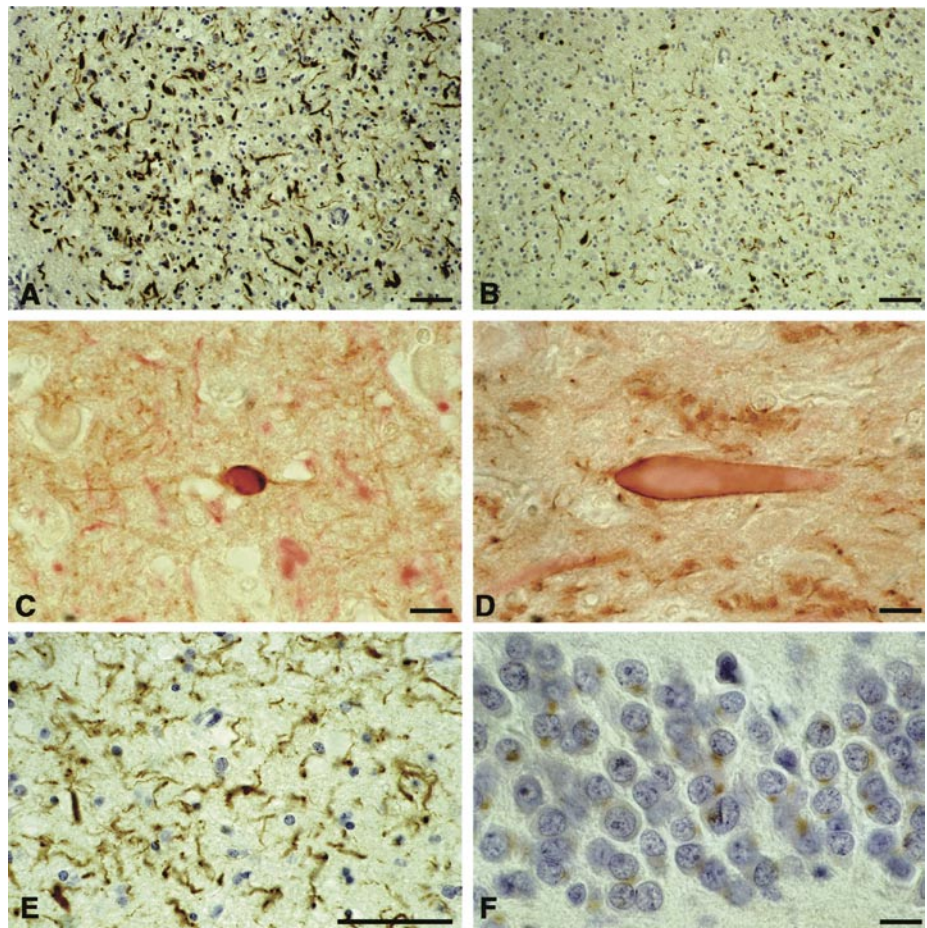
Figure 3. Sections of the frontal cortex. **A.** Layers I-VI, showing numerous α-synuclein-immunoreactive Lewy bodies and Lewy neurites. **B.** Silver-positive inclusions, with numerous segmental swellings in dendrites; the inset shows argentophilic inclusions in the soma and dendrite belonging to the same neuron. **C.** α-Synuclein-immunoreactive segmental dendritic swelling. **A, C.** Immunohistochemistry using anti-α-synuclein (119-137) antibody; **B.** Bodian silver stain. Scale bars: **A, B** = 100 μm; **C** = 10 μm.

Figure 4. Sections of the insular cortex (**A**), visual cortex (**B**), CA1 layer of the hippocampus (**C, D**), parahippocampus (**E**) and granule cell layer of the hippocampus (**F**). **A, B.** Numerous α-synuclein-positive Lewy bodies and Lewy neurites. **C, D.** Neurites are double-labeled for α-synuclein (pink) and neurofilaments (brown). **E.** Numerous α-synuclein-positive neurites. **F.** α-Synuclein deposits in granule cell bodies. **A, B, E, F.** Immunohistochemistry using anti-α-synuclein (119-137) antibody. **C, D.** Double-labeling immunohistochemistry using the polyclonal anti-α-synuclein (119-137) antibody and polyclonal antibodies specific for NF-M. Scale bars: **A, B** = 100 μm; **C-D, F** = 10 μm; **E** = 50 μm.

ever, a clear laminar distribution was not observed. Intraneuritic Lewy bodies were most numerous in cortical layers II-IV. Lewy neurites were present throughout all cortical layers. Lewy bodies were hematoxylin-eosin and silver positive. Most cortical Lewy bodies lacked a dense core.

Neuronal cell loss and gliosis were moderate in hippocampal pyramidal cells, subiculum and entorhinal cortex. By contrast, the transentorhinal cortex and the inferior temporal cortex showed severe neuronal loss, gliosis and vacuolar changes. Numerous Lewy bodies and Lewy neurites were present in the hippocampus and subiculum (Figure 4). They were particularly abundant in the stratum oriens, stratum pyramidale and stratum radiatum. Lewy bodies and Lewy neurites were also present in the dentate gyrus of the hippocampus. In the subcortical white matter, scattered α-synuclein-immunoreactive-glial-cell inclusions were present, akin to coiled bodies. A mild loss of myelinated nerve fibers

and a moderate to severe gliosis were also present.

Lewy bodies and Lewy neurites were abundant in the caudate nucleus, putamen, claustrum and thalamus (Figure 5). They were particularly numerous in the caudate nucleus, where neuronal cell loss and gliosis were also very severe. Lewy bodies and Lewy neurites were present in hypothalamic nuclei and the substantia innominata, but not the globus pallidus. The substantia nigra contained abundant Lewy bodies and Lewy neurites. Nerve cell loss and gliosis were severe and neuromelanin was found extracellularly and/or within macrophages. (Figure 2A, B). Only a few pigmented neurons remained in the medial part of the substantia nigra. Numerous Lewy bodies and Lewy neurites were present in the oculomotor nerve nucleus, a region where nerve cell loss was only minimal. The peri-

aqueductal grey matter was also affected, but not the red nucleus.

The locus coeruleus contained numerous Lewy bodies and Lewy neurites in spite of the severe nerve cell loss (Figures 2C,5D). The raphe nucleus also contained many Lewy bodies and Lewy neurites; however, there was relatively little nerve cell loss (Figure 5F). The basis pontis showed moderate nerve cell loss and gliosis (Figure 5E). Lewy bodies were only occasionally seen, but Lewy neurites were numerous in the pontine nuclei. Moderate nerve cell loss and gliosis in the dorsal motor nucleus of the vagus nerve and in the inferior olivary nucleus were noted. Varying numbers of Lewy bodies and Lewy neurites were seen in the dorsal motor nucleus of the vagus, in the lateral reticular nucleus and inferior olivary nucleus (Figure 5G, H). In other anatomical areas, Lewy bodies and Lewy neurites were also present with various

degrees of abundance, as summarized in Table 2. They were scarce in the spinal cord (Figure 5I).

Neurofibrillary lesions and neuritic plaques were not present in any of the sections sampled. Moreover, there were no tau-, β-amyloid- or prion protein-immunoreactive deposits. A few Hirano bodies were present in the hippocampus. Granulovacuolar degeneration was not seen. Iron accumulation and spheroids were absent from substantia nigra and basal ganglia.

Electron microscopy. In the cerebral cortex, most intraperikaryal and intraneuritic Lewy bodies lacked the dense core that is characteristic of brainstem Lewy bodies (Figure 7). They were composed of dense granular material and randomly arranged filaments with a diameter of 7 to 20 nm. Occasionally, cellular organelles were present among the filaments. When a core was present, its electron density was variable. Filaments of the core were more densely packed than those of the peripheral zone, which were loosely arranged in a radial pattern.

Characterization of isolated filaments. Filaments were extracted from frontal cortex tissue using a method that enriches for sarkosyl-insoluble filaments. The resuspended pellets were tested with α-synuclein, tau and ubiquitin antibodies (Figure 8). The labeled structures corresponded mostly to single filaments, though occasionally small clumps of filaments were seen. All filaments were straight and unbranched with a length of 200 to 600 nm. The width was 5 to 10 nm, with some filaments showing a variation in width along their length. The filaments were labeled all along their length by antibodies PER4, SYN36, SYN303 and SYNP129, with SYN36 labeling fewer filaments than the other antibodies. A quite different pattern was observed with antibody PER1, which only ever labeled one end of each filament. Occasional filaments were also labeled at only one end by antibody SYN36. No specific labeling was observed with anti-tau and only occasional weak labeling with anti-ubiquitin antibodies.

Sequential extraction of α-synuclein. α-Synuclein was sequentially extracted from frontal cortex tissue of a control individual
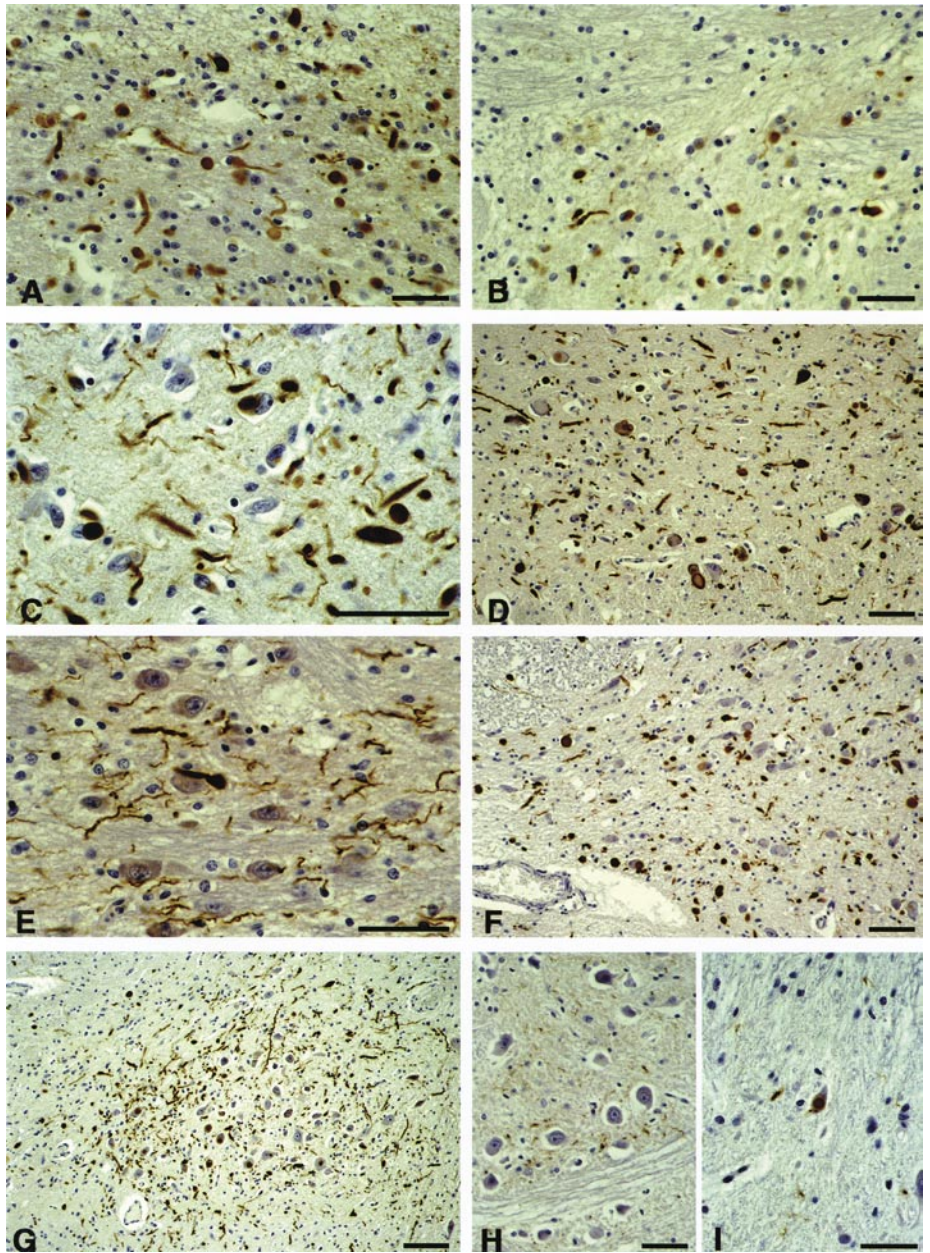

Figure 5. Sections of the caudate nucleus (**A**), putamen (**B**), suprachiasmatic nucleus (**C**), locus coeruleus (**D**), basis pontis (**E**), raphe nucleus (**F**), dorsal motor nucleus of the vagus (**G**), inferior olivary nucleus (**H**) and thoracic spinal cord (**I**). Numerous α-synuclein-immunoreactive deposits are seen. **A-I.** Immunohistochemistry using anti-α-synuclein (119-137) antibody. Scale bars: **A-I** = 50 μm.

(c) and the proband (p) using TBS, 1% Triton X-100, 1% sarkosyl and 8M urea (Figure 9). A protein with an apparent molecular mass of 17 kDa was detected with anti-α-synuclein antibody SYN208 in the TBS, Triton X-100 and sarkosyl-soluble fractions from both brains. This band, which was much stronger in the TBS- and Triton X-100-soluble fractions than in the sarkosyl-soluble fraction, corresponds to normal α-synuclein. When the sarkosyl-insoluble material was treated with urea, a major, broad band centered at 17 kDa with a smear to lower molecular weight, a weak band at 22 to 24 kDa and a high-molecular weight smear were visible in the extract from the proband's brain, but not in the extract from the control brain. This material corresponds to abnormal aggregates of α-synuclein.

Genetic analysis. No mutations or polymorphisms were found upon sequencing of exons 1-6 of the proband's α-*Synuclein* gene. A silent nucleotide change (CTT to CTC) was present on one allele at codon 38 in exon 2 of β-*Synuclein*. Sequencing of exons 1-12 of *Parkin* showed a heterozygous
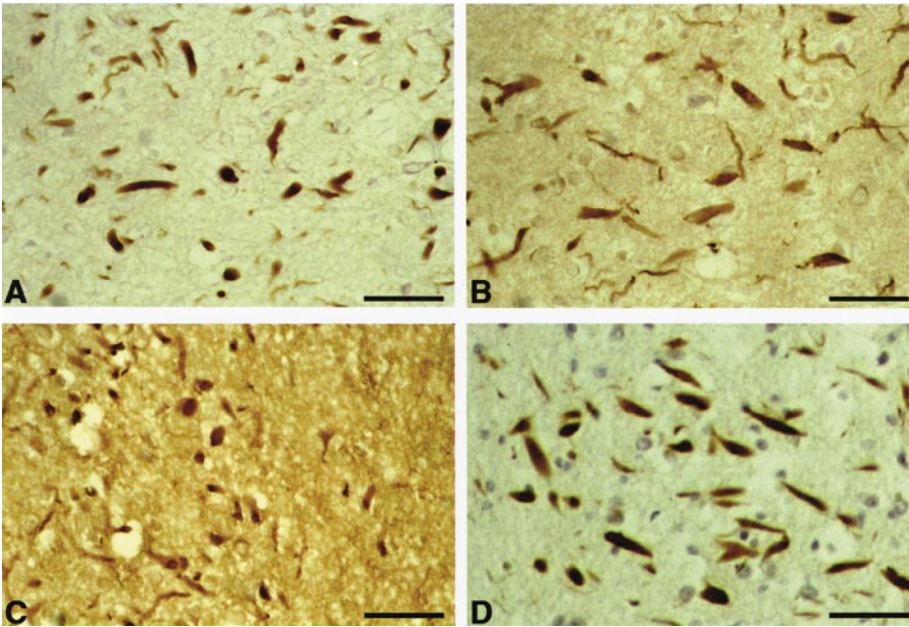
Figure 6. Sections of the frontal cortex immunolabeled with anti-α-synuclein antibodies PER1 (**A**), PER4 (**B**), PER7 (**C**) and an anti-ubiquitin antibody (**D**). Numerous α-synuclein and ubiquitin deposits are seen. Scale bars: **A-D** = 50 µm.
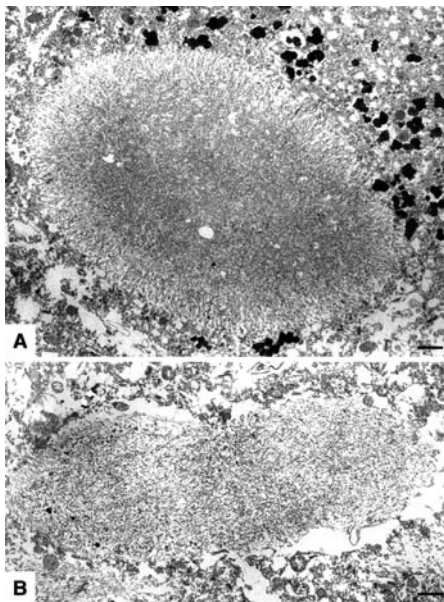
Figure 7. Electron micrographs of Lewy bodies in the locus coeruleus (**A**) and frontal cortex (**B**). Note the presence of filaments and granular material. A dense core is present in (**A**), but not (**B**). Scale bars: **A-B** = 1 µm.

nucleotide change (CTC to ATC) at codon 272 in exon 7, resulting in a Leu to Ile substitution. This substitution is probably a polymorphism, since it was also present in one out of 50 controls from the Japanese population (a 67-year-old male, with no family history of neurological disease). No additional changes were found when cDNAs encoding parkin were sequenced, ruling out deletion mutations on the other allele. The sequence of exons 1-10 of the proband's *Synphilin-1* gene was normal, except for 2 heterozygous, silent nucleotide changes at codons 144 (GAC to GAT) and 244 (AAA to AAG) in exon 3. Sequencing of the exons encoding NF-M showed a homozygous nucleotide change (GCT to GTT) at codon 482, resulting in an alanine to valine change, which has previously been reported as a polymorphism (NCBI SNP CLUSTER ID: rs1063314). No nucleotide changes were detected in the exons encoding the proband's *UCH-L1* gene.

DISCUSSION

We describe a case of DLB with progressive dementia, parkinsonism and myoclonus, and an onset of clinical signs at 13 years of age. The disease progressed rapidly until the patient died at 15 years of age, which is an earlier age of death than previously reported in cases of DLB (18, 27, 32, 58). In addition to the severe cortical atrophy, a finding of interest was that the pigment observed macroscopically in the substantia nigra was more than expected based on the severe loss of neurons observed microscopically. In a study of pigmentation of the substantia nigra in 64 Japanese individuals between the ages of 2 and 27 years, it was reported that pigmentation was visible by naked eye by the age of 16 years (40). In another study of 44 children between the ages of 34 weeks and 16 years, it was reported that the pigment could be identified macroscopically in preadolescent individuals (12). Thus, it would appear that the level of pigmentation of the substantia nigra is variable during adolescence. The pigmentation observed in the case reported here may have been due to the few remaining neurons, extracellular neuromelanin and/or the presence of numerous neuromelanin-containing macrophages.

Abundant and widespread Lewy bodies and Lewy neurites were present throughout the central nervous system; there was an absence of neuritic plaques and neurofibrillary lesions. The Lewy bodies had the typical electron microscopic appearance of brainstem and cortical Lewy bodies (13). This case was atypical because of the early age at onset of clinical signs and the severity of the α-synuclein pathology. The latter was extensive throughout the cerebral cortex and much of the brainstem. The large number of Lewy bodies in the basal ganglia, inclusions in hippocampal granule cells and Lewy neurites in the basis pontis and inferior olivary nucleus were particularly striking. Nerve cell loss and gliosis were extensive in cerebral cortex and brainstem, probably accounting for the cognitive and motor symptoms of the proband. The extent of α-synuclein pathology was more severe than in typical cases of DLB. A similarly extensive pathology has been only described in the Iowa kindred with a hereditary form of parkinsonism and dementia caused by the triplication of the region of chromosome 4 that contains the α-*Synuclein* gene (22, 47).

Intracytoplasmic and intraneuritic Lewy bodies, as well as Lewy neurites, were strongly reactive with anti-α-synuclein antibodies whose epitopes spanned the length of the protein. They were also labeled by an antibody specific for α-synuclein phosphorylated at S129. The inclusions were not labeled by antibodies specific for β- or γ-synuclein. Most inclusions were ubiquitin-immunopositive. These labeling characteristics are identical to those of the Lewy pathology of PD and DLB (14, 48, 49). Some inclusions were labeled by antibodies specific for NF-M, in accordance with the known association between neurofilament labeling and Lewy body pathology (21). Published reports on the labeling of Lewy bodies and Lewy neurites for parkin and synphilin-1 have been inconsistent, ranging from extensive to little or no co-localization

(25, 41, 46, 54). In the proband's brain, we found a small number of inclusions that were weakly immunoreactive for parkin. Inclusions were not labeled for synphilin-1. Nothing is known about the presence of DJ-1 in Lewy bodies and Lewy neurites. We show here that an antibody raised against the C-terminus of DJ-1 failed to label the inclusions. Recent studies have reported that tau staining is frequently associated with Lewy bodies and that α-synuclein assemblies can induce tau filament formation (17, 28). However, in the present case, we failed to observe co-localization of tau and α-synuclein. Furthermore, by immuno-electron microscopy, there was no evidence of tau filaments.

Isolated filaments from the proband's brain were 5 to 10 nm in diameter and strongly decorated by α-synuclein antibodies. Antibody PER4, which recognizes the C-terminus of α-synuclein, decorated filaments along their entire length. This contrasted with antibody PER1, which was raised against residues 11-34 of α-synuclein. It labeled only one end of the filaments. These findings are identical to what we have described previously for α-synuclein filaments from DLB and PD brains (6, 49). Two antibodies (SYN36 and SYN303), which are specific for the N-terminus (residues 1-10 and 2-12, respectively) of α-synuclein, decorated filaments, as did the antibody specific for α-synuclein phosphorylated at S129. In some filaments, SYN36 labeled only one end. This indicates that full-length α-synuclein was present in the filaments and that the amino- and carboxy-termini were exposed on the surface of most filaments, in agreement with reports showing that the core of synthetic α-synuclein filaments extends approximately from residues 30-110 (8, 38). Filaments were only very weakly decorated by an anti-ubiquitin antibody, indicating that ubiquitination is not necessary for assembly into filaments. This is consistent with the view that ubiquitination of α-synuclein is a late event (45, 49, 52). Sequential biochemical extraction showed the presence of insoluble α-synuclein in the proband's brain, but not in a control brain. This is consistent with previous studies on DLB (2, 52). In Western blot analysis, the insoluble α-synuclein appears as a broad band corresponding to the monomer of α-synuclein and its degra-
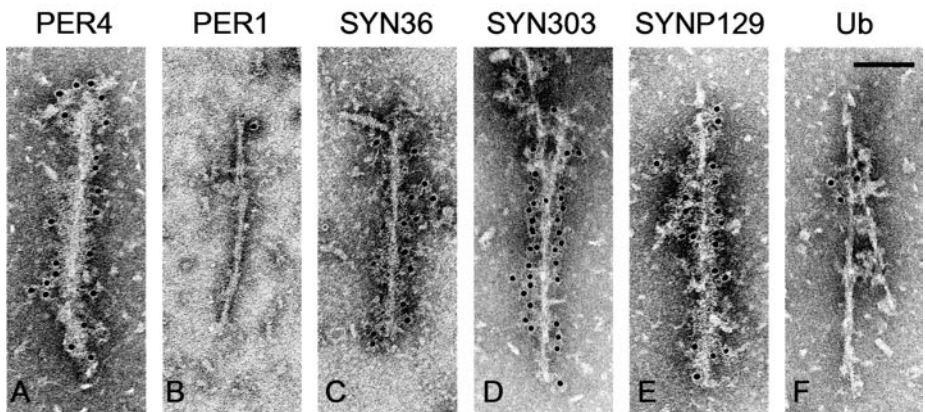
Figure 8. Filaments isolated from the frontal cortex of the proband's brain were labeled with anti-α-synuclein antibodies PER4, PER1, SYN36, SYN303 and SYNP129 (**A-E**), and an anti-ubiquitin antibody (**F**). The gold particles conjugated to the second antibody appear as black dots. Scale bar = 100 nm.

dation products, a weak 22 to 24 kDa band and a high-molecular weight smear.

The etiology of this form of DLB is unknown, since the family history was negative for neurological disease and the exon sequences of α-*Synuclein* were normal. Moreover, the exonic regions of β-*Synuclein*, *UCH-L1*, *Synphilin-1* and *NF-M* showed no pathogenic mutations. At present, we cannot exclude the possibility of gene dosage effects or the presence of mutations in the promoter regions or the introns of these genes. When exons 1-12 of *Parkin* were sequenced, a heterozygous leucine to isoleucine change was found at codon 272 in exon 7. It is located in the first ring finger domain, a functionally important part of the molecule, suggesting a possible pathogenic role. It is also located close to the R275W mutation in exon 7 of *Parkin*, which has been described in a patient with familial parkinsonism and Lewy body pathology (10). The latter patient was a compound heterozygote, with a 40 bp deletion in exon 3 of *Parkin* on the other allele. In our proband, sequencing of parkin cDNAs confirmed the L272I change, but failed to reveal a mutation on the other allele. It remains possible that a second mutation was present, but that it went undetected, because it consisted of a large inversion or duplication, or because it was located in regions that were not sequenced, such as the promoter and the introns. Although it has not been reported before (see recent listing in [55]), the L272I change is probably not pathogenic on its own, as it was also found in a 67-year-old control individual from the Japanese population, indicating that it can exist as a polymorphism. Recently, a heterozygous R271S
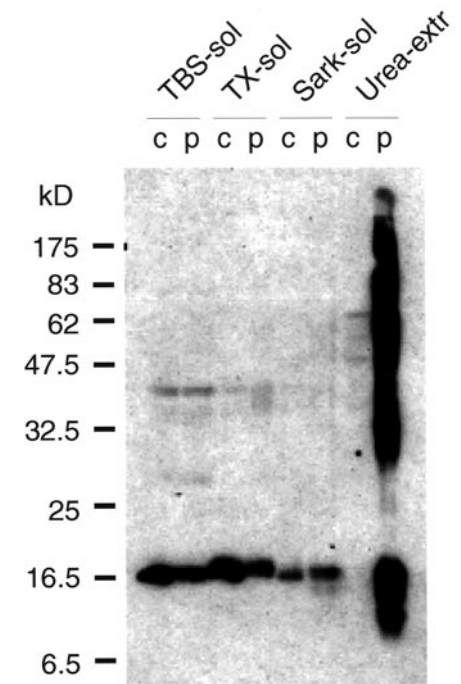
Figure 9. Sequential extraction of α-synuclein from the frontal cortex of a control brain (c) and the proband's brain (p). The tissues were sequentially extracted with Tris-HCl (TBS), Triton X-100 (Tx), sarkosyl (Sark) and urea, followed by immunoblotting with anti-α-synuclein antibody SYN208.

change in exon 7 of *Parkin* was reported in a cohort of North American individuals free of neurological signs (4). This supports the existence of polymorphisms in this functionally important region of the protein. The etiology of this form of DLB remains unresolved.

REFERENCES

1. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MCJ, Squiteri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P (2003) Mutations in the *DJ-1* gene associated with autosomal recessive early-onset parkinsonism. *Science* 299:256-259.

2. Campbell BCV, Li QL, Culvenor JG, Jäkälä P, Cappai R, Beyreuther K, Masters CL, McLean CA (2000) Accumulation of insoluble α-synuclein in dementia with Lewy bodies. *Neurobiol Dis* 7: 192-200.

3. Carmel G, Mager EM, Binder LI, Kuret J (1996) The structural basis of monoclonal antibody Alz50's selectivity for Alzheimer's disease pathology. *J Biol Chem* 271:32789-32795.

4. Chen R, Gosavi NS, Langston JW, Chan P (2003) Parkin mutations are rare in patients with young-onset parkinsonism in a US population. *Parkinsonism Rel Disord* 9:309-312.

5. Crowther RA (1991) Straight and paired helical filaments in Alzheimer disease have a common structural unit. *Proc Natl Acad Sci USA* 88:2288-2292.

6. Crowther RA, Daniel SE, Goedert M (2000) Characterisation of isolated α-synuclein filaments from substantia nigra of Parkinson's disease brain. *Neurosci Lett* 292:128-130.

7. Davis RL, Shrimpton AE, Holohan PD, Bradshaw C, Feiglin D, Collins GH, Sonderegger P, Kinter J, Becker LM, Lacbawan F, Krasnewich D, Muenke M, Lawrence DA, Yerby MS, Shaw CM, Gooptu B, Elliott PR, Finch JT, Carrell RW, Lomas DA (1999) Familial dementia caused by polymerization of mutant neuroserpin. *Nature* 401:376-379.

8. Der-Sarkissian A, Jao CC, Chen J, Langen R (2003) Structural organization of α-synuclein fibrils by site-directed spin labeling. *J Biol Chem* 278:37530-37535.

9. Duda JE, Giasson BI, Mabon ME, Lee VMY, Trojanowski JQ (2002) Novel antibodies to synuclein show abundant striatal pathology in Lewy body diseases. *Ann Neurol* 52:205-210.

10. Farrer M, Chan P, Chen R, Tan L, Lincoln S, Hernandez D, Forno L, Gwinn-Hardy K, Petrucelli L, Hussey J, Singleton A, Tanner C, Hardy J, Langston JW (2001) Lewy bodies and parkinsonism in families with *parkin* mutations. *Neurology* 50: 293-300.

11. Farrer M, Destée A, Levesque C, Singleton A, Engelender S, Becquet E, Mouroux V, Richard F, Defebre L, Crook R, Hernandez D, Ross CA, Hardy J, Amouyel P, Chartier-Harlin MC (2001) Genetic analysis of synphilin-1 in familial Parkinson's disease. *Neurobiol Dis* 8:317-323.

12. Fenichel GM, Bazeloa M (1968) Studies on neuromelanin. II. Melanin in the brainstems of infants and children. *Neurology* 18:18:817-820.

13. Forno LS (1996) Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol* 55: 259-272.

14. Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T (2002) α-Synuclein is phosphorylated in synucleinopathy lesions. *Nature Cell Biol* 4:160-164.

15. Gasser T, Müller-Myhsok B, Wszolek ZK, Oehlmann R, Calner DB, Bonifati V, Bereznai B, Fabrizio E, Vieregge P, Horstmann RD (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nature Genet* 18:262-265.

16. Giasson BI, Jakes R, Goedert M, Duda JE, Leight S, Trojanowski JQ, Lee VMY (2000) A panel of epitope-specific antibodies detects protein domains distributed throughout human α-synuclein in Lewy bodies of Parkinson's disease. *J Neurosci Res* 59:528-533.

17. Giasson BI, Forman MS, Higuchi M, Golbe LI, Graves CL, Kotzbauer PT, Trojanowski JQ, Lee VMY (2003) Initiation and synergistic fibrillization of tau and alpha-synuclein. *Science* 300:636-640.

18. Gibb WRG, Esiri MM, Lees AJ (1987) Clinical and pathological features of diffuse cortical Lewy body disease (Lewy body dementia). *Brain* 110: 1131-1153.

19. Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA (1989) Multiple isoforms of human microtubule-associated protein tau: Sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron* 3:519-526.

20. Goedert M (2001) Alpha-synuclein and neurodegenerative diseases. *Nature Rev Neurosci* 2: 492-501.

21. Goldman JE, Yen SH, Chiu FC, Peress NS (1983) Lewy bodies of Parkinson's disease contain neurofilament antigens. *Science* 221:1082-1084.

22. Gwinn-Hardy K, Mehta ND, Farrer M, Maraganore D, Muenter M, Yen SH, Hardy J, Dickson DW (2000) Distinctive neuropathology revealed by α-synuclein antibodies in hereditary parkinsonism and dementia linked to chromosome 4p. *Acta Neuropathol* 99:663-672.

23. Hasegawa M, Fujiwara H, Nonaka T, Wakabayashi K, Takahashi H, Lee VMY, Trojanowski JQ, Mann D, Iwatsubo T (2002) Phosphorylated α-synuclein is ubiquitinated in α-synucleinopathy lesions. *J Biol Chem* 277, 49071-49076.

24. Hayashi S, Wakabayashi K, Ishikawa A, Nagai H, Saito M, Maruyama M, Takahashi T, Ozawa T, Tsuji S, Takahashi H (2000) An autopsy case of autosomal-recessive juvenile parkinsonism with a homozygous exon 4 deletion in the parkin gene. *Mov Disord* 15:884-888.

25. Huynh DP, Scoles DR, Ho TH, Del Bigio MR, Pulst SM (2000) Parkin is associated with actin filaments in neuronal and nonneuronal cells. *Ann Neurol* 48:737-744.

26. Ince PG, McKeith IG (2003) Dementia with Lewy bodies. In: *Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders* (D. Dickson, ed), pp. 188-199. ISN Neuropath Press, Basel 2003.

27. Iseki E, Marui W, Kosaka K, Kato M, Yamamoto T, Ueda K (1999) Clinicopathological multiplicity of dementia with Lewy bodies. *Neuropathology* 19:386-394.

28. Ishizawa T, Mattila P, Davies P, Wang D, Dickson DW (2003) Colocalization of tau and alpha-synuclein epitopes in Lewy bodies. *J Neuropathol Exp Neurol* 62:389-399.

29. Jakes R, Spillantini MG, Goedert M (1994) Identification of two distinct synucleins from human brain. *FEBS Lett* 345:27-32.

30. Jakes R, Crowther RA, Lee VMY, Trojanowski JQ, Iwatsubo T, Goedert M (1999) Epitope mapping of LB509, a monoclonal antibody directed against human α-synuclein. *Neurosci Lett* 269: 13-16.

31. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392:605-608.

32. Kosaka K (1990) Diffuse Lewy body disease in Japan. *J Neurol* 237:197-204.

33. Krüger R, Kuhn W, Müller T, Woitalla D, Graeber M, Kösel S, Przuntek H, Epplen JT, Schöls L, Riess O (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. *Nature Genet* 18:106-108.

34. Lavedan C, Leroy E, Torres R, Dehejia A, Dutra A, Buchholtz S, Nussbaum RL, Polymeropoulos MH (1998) Genomic organization and expression of the human α-synuclein gene (SNCB). *Genomics* 54:173-175.

35. Lavedan C, Buchholtz S, Nussbaum RL, Albin RL, Polymeropoulos MH (2002) A mutation in the human neurofilament M gene in Parkinson's disease that suggests a role for the cytoskeleton in neuronal degeneration. *Neurosci Lett* 322:57-61.

36. Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, Polymeropoulos MH (1998) The ubiquitin pathway in Parkinson's disease. *Nature* 395:451-452.

37. McQuaid S, McConnell R, McMahon J, Herron B (1995) Microwave antigen retrieval for immunocytochemistry on formalin-fixed, paraffin-embedded post-mortem CNS tissue. *J Pathol* 176:207-216.

38. Miake H, Mizusawa H, Iwatsubo H, Hasegawa M (2002) Biochemical characterization of the core structure of α-synuclein filaments. *J Biol Chem* 277:19213-19219.

39. Mori H, Kondo T, Yokochi M, Matsumine H, Nakagawa-Hattori Y, Miyake T, Suda, K, Mizuno Y (1998) Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. *Neurology* 51:890-892.

40. Morimatsu Y, Satoh J, Nagata J, Kubota Y, Hayashi M, Sakamoto K, Shinohara T (1987) Pathology of neuromelanin in the development disabilities. *Kouseishour Shinkei Shikkan Kenkyu Itaku Jigyou* (Japanese) 1:268-282.

41. Murray IVJ, Medford MA, Guan HP, Rueter SM, Trojanowski JQ, Lee VMY (2003) Synphilin in normal human brain and in synucleinopathies: studies with new antibodies. *Acta Neuropathol* 105:177-184.

42. Ozawa T, Takano H, Onodera O, Kobayashi H, Ikeuchi T, Koide R, Okuizumi K, Shimohata T, Wakabayashi K, Takahashi H, Tsuji T (1999) No mutation in the entire coding region of the α-synuclein gene in pathologically confirmed cases of multiple system atrophy. *Neurosci Lett* 270:110-112.

43. Piccardo P, Mirra SS, Young K, Gearing M, Dlouhy SR, Ghetti B (1998) α-Synuclein accumulation in Gerstmann-Sträussler-Scheinker disease (GSS) with prion protein gene mutation F198S. *Neurobiol Aging* 19:S172.

44. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubinstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045-2047.

45. Sampathu DM, Giasson BI, Pawlyk AC, Trojanowski JQ, Lee VMY (2003) Ubiquitination of α-synuclein is not required for formation of pathological inclusions in α-synucleinopathies. *Am J Pathol* 163:91-100.

46. Schlossmacher MG, Frosch MP, Gai WP, Medina M, Sharma N, Forno L, Ochiishi T, Shimura H, Sharon R, Hattori N, Langston JW, Mizuno Y, Hyman BT, Selkoe DJ, Kosik KS (2002) Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. *Am J Pathol* 160:1655-1667.

47. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, Gwinn-Hardy K (2003) α-Synuclein locus triplication causes Parkinson's disease. *Science* 302:841.

48. Spillantini MG, Schmidt ML, Lee VMY, Trojanowski JQ, Jakes R, Goedert M (1997) α-Synuclein in Lewy bodies. *Nature* 388, 839-840.

49. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M (1998) α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson disease and dementia with Lewy bodies. *Proc Natl Acad Sci USA* 95:6469-6473.

50. Takahashi H, Ohama E, Suzuki S, Horikawa Y, Ishikawa A, Morita T, Tsuji S, Ikuta F (1994) Familial juvenile parkinsonism: clinical and pathologic study in a family. *Neurology* 44:437-441.

51. Takao M, Benson MD, Murrell JR, Yazaki M, Piccardo P, Unverzagt FW, Davis RL, Holohan PD, Lawrence DA, Richardson R, Farlow MR, Ghetti B (2000) Neuroserpin mutation S52R causes neuroserpin accumulation in neurons and is associated with progressive myoclonus epilepsy. *J Neuropathol Exp Neurol* 59:1070-1078.

52. Tofaris GK, Razzaq A, Ghetti B, Lilley K, Spillantini MG (2003) Ubiquitination of α-synuclein in Lewy bodies is a pathological event not associated with impairment of proteasome function. *J Biol Chem* 278:44405-44411.

53. Van de Warrenburg BPC, Lammens M, Lücking CB, Denefle P, Wesseling P, Booij J, Praamstra P, Quinn N, Brice A, Horstink MWIM (2001) Clinical and pathologic abnormalities in a family with parkinsonism and parkin gene mutations. *Neurology* 56:555-557.

54. Wakabayashi K, Engelender S, Yoshimoto M, Tsuji S, Ross CA, Takahashi H (2000) Synphilin-1 is present in Lewy bodies in Parkinson's disease. *Acta Neuropathol* 47:521-523.

55. West A, Périquet M, Lincoln S, Lücking CB, Nicholl D, Bonifati V, Rawal N, Gasser T, Lohmann E, Deleuze JF, Maraganore D, Levey A, Wood N, Dürr A, Hardy J, Brice A, Farrer M (2002) Complex relationship between parkin mutations and Parkinson's disease. *Am J Med Genet* 114:584-591.

56. Wintermeyer P, Krüger R, Kuhn W, Müller T, Woitalla D, Berg D, Becker G, Leroy E, Polymeropoulos M, Berger K, Przuntek H, Schöls L, Epplen JT, Riess O (2000) Mutation analysis and association studies of the UCHL1 gene in German Parkinson's disease patients. *Neuroreport* 11:2079-2082.

57. Wzolek ZK, Gwinn-Hardy K, Wszolek EK, Muenter MD, Pfeiffer RF, Rodnitzky RL, Uitti RJ, McComb RD, Gasser T, Dickson DW (2002) Neuropathology of two members of a German-American kindred (Family C) with late onset parkinsonism. *Acta Neuropathol* 103:344-350.

58. Yoshimura N, Yoshimura I, Asada M, Hayashi S, Fukushima Y, Sato T, Kudo H (1988) Juvenile Parkinson's disease with widespread Lewy bodies in the brain. *Acta Neuropathol* 77:213-218.

59. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atares B, Llorens V, Gomez Tortosa E, del Ser T, Munoz DG, de Yebenes JG (2004) The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann Neurol* 55:164-173.