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Distinctive neuropathology revealed by α-synuclein antibodies in hereditary parkinsonism and dementia linked to chromosome 4p

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Abstract The identification of the α-synuclein gene on chromosome 4q as a locus for familial Lewy-body parkinsonism and of α-synuclein as a component of Lewy bodies has heralded a new era in the study of Parkinson's disease. We have identified a large family with Lewy body parkinsonism linked to a novel locus on chromosome 4p15 that does not have a mutation in the α-synuclein gene. Here we report the clinical and neuropathological findings in an individual from this family and describe unusual high molecular weight α-synuclein-immunoreactive proteins in brain homogenates from brain regions with the most marked neuropathology. Distinctive histopathology was revealed with α-synuclein immunostaining, including pleomorphic Lewy bodies, synuclein-positive glial inclusions and widespread, severe neuritic dystrophy. We also discuss the relationship of this familial disorder to a Lewy body disease clinical spectrum, ranging from Parkinson's disease to dementia with psychosis.

Key words Dementia · Lewy body · Neuropathology · Synuclein · Western blotting

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Introduction

The identification of the α-synuclein gene as a locus for Lewy-body parkinsonism [15, 22] and the realization that antibodies to α-synuclein stain Lewy bodies [25, 27, 30] has led to an enormous interest in the role of α-synuclein in Parkinson's disease (PD) and related disorders, such as dementia with Lewy bodies (DLB) [19]. α-Synuclein staining has been observed in Lewy bodies in PD and DLB, in some cases of familial Alzheimer's disease [18] and recently in glial cytoplasmic inclusions of multiple system atrophy (MSA) [2, 9, 26].

We have studied a large kindred previously described separately by Spellman and Muenter [21, 24] and by Waters and Miller [31], now termed collectively the "Iowa kindred". Affected individuals do not have mutations in the α-synuclein gene [11], but are linked to a genetic locus at 4p15 [12]. The clinical phenotype in the Iowa kindred ranges from that of typical PD to DLB. Some family members have essential tremor that genetically maps to the same locus [12]. A member of this kindred recently came to autopsy and was studied using methods not available at the time of study of other members of the kindred, namely, immunocytochemistry and immunochemistry with antibodies to α-synuclein. Here we describe the clinical findings and distinctive pathology revealed with α-synuclein antibodies in this individual.

Materials and methods

Clinical case description

The male subject was 47 years old at the time of his death, and had a history of levodopa (L-dopa)-responsive parkinsonism, cognitive difficulties and orthostatic hypotension. From this family 22 individuals are known to have been or be affected with parkinsonism, both with and without dementia, including the proband's mother (Fig. 1). The disorder appears to show autosomal dominant transmission, although recent linkage data suggest this disease is not fully penetrant [12].

This patient was first seen at 25 years of age during the assessment of his family for parkinsonism, at which time he had no com-

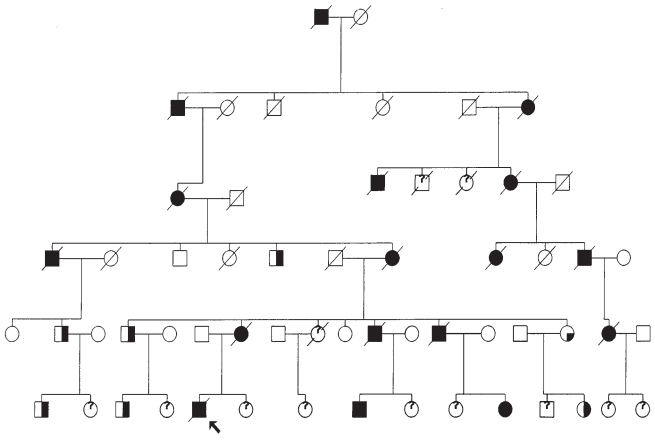

Fig. 1 Pedigree of the kindred. The *arrow* indicates the proband. Males are indicated with *squares*, females with *circles*, deceased individuals with *slashes*. *Solid black* symbols indicate parkinsonism, *half-filled* symbols indicate essential tremor. The *quarter-filled* symbol represents an individual with no neurological findings on examination but with an offspring who is affected with essential tremor. *Question marks* indicate individuals at risk or for whom a diagnostic category is not known

plaints and a normal exam. At 32 years of age, he returned with depression, rigidity and slowness in thinking and movement. Examination revealed a normal mental status, masking of the facies, hypokinetic speech, mildly slowed rapid alternating movements throughout and a decreased right arm swing. Extra-ocular movements were normal. Mild resting tremor was seen. A thorough medical and neurological work-up was negative. Over the next several months he suffered marked worsening of his rigidity and slowness, and carbidopa-levodopa (Sinemet) was initiated with good motor response. During the next year he had marked worsening of parkinsonism, orthostatic hypotension, gastrointestinal difficulties and loss of erectile function. He had a feeling of fatigue that waxed and waned. He often felt as if he could easily faint, and he had fallen several times. He had become a sleepwalker and talker. He recounted vivid dreams, although he had been a "restless" sleeper since his early twenties. He became socially withdrawn and no longer participated in his athletic hobbies. Autonomic testing revealed severe abnormalities of central origin. Fludrocortisone treatment resulted in good initial benefit in the postural orthostatic hypotension; however, this benefit persisted for only about a year. The severity of the orthostatic hypotension ultimately required discontinuation of carbidopa-levodopa.

He suffered from disturbing realistic visual hallucinations, which improved after discontinuing carbidopa-levodopa, but did not resolve. He retired on disability at the age of 34 years. By the age of 36, he had severely inarticulate speech, cognitive problems characterized by forgetfulness and confusion, and he occasionally acted inappropriately. By 42 years of age he could only utter three to four words. He was bedridden with arms flexed at the elbows and legs extended. He did not blink. He would occasionally respond with a faint smile or by turning his eyes towards the examiner. Spontaneous ocular pursuit movements were saccadic. Severe rigidity was present throughout. He did not grimace or withdraw to painful stimuli, possibly due to his severe rigidity. Occasional spontaneous myoclonic jerks, which were asynchronous, were seen in all limbs. No startle myoclonus was elicited. He died from pneumonia at the age of 47 years. An autopsy limited to examination of the brain was performed.

Histopathology

At the time of the autopsy the brain was divided in the sagittal plane, with the entire left hemibrain frozen at –70 °C and the right hemibrain fixed in 10% neutral buffered formalin. After photography and dissection, multiple sections of neocortex, hippocampus, basal forebrain, basal ganglia, thalamus, midbrain, pons, medulla and cerebellum were embedded in paraffin and sections were examined with H&E and with thioflavin-S fluorescence microscopy. Sections of cortex, hippocampus, basal forebrain and brain stem were also stained with immunocytochemical methods and antibodies to α-synuclein. Some sections were also stained with tau antibodies (PHF-1; Peter Davies, Albert Einstein College of Medicine, Bronx, N.Y.) and ubiquitin antibodies [17]. Sections were double stained with monoclonal antibodies to glial fibrillary acidic protein (GFAP; BioGenex, San Ramon, Calif.) or to complement factor 4d (C4d; Biogenesis, Poole, UK), which has been shown to label pathological oligodendroglia in a number of conditions [32, 33] and a polyclonal antibody to α-synuclein [9].

Sections, 5 μm thick, were deparaffinized in xylene and alcohols, and incubated in 3% H_2O_2 for 30 min to block endogenous peroxidase and then in 5% normal goat serum for 10 min to block nonspecific antibody binding. The primary antibodies were incubated overnight at 20 °C. Antibody binding was detected with the avidin-biotin-complex method (Vector Labs, Burlingame, Calif.). The chromogen was 3,3′-diaminobenzidine (Sigma Chemical, St. Louis, Mo.). The sections were lightly counterstained with hematoxylin, dehydrated and coverslips mounted with Permount (Fisher Scientific, Pittsburgh, Pa.). For double labeling experiments with light microscopy, sections were incubated simultaneously with both antibodies. Following extensive washing in buffer, the antibodies were detected with peroxidase- and alkaline phosphatase-labeled isotype-specific secondary antibodies (Southern Biotechnology Assoc., Birmingham, Ala.). The chromogens [3,3'-diaminobenzidine and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) nitro-blue tetrazolium (Sigma)] were developed sequentially.

Laser confocal microscopy was performed on 40-μm-thick vibratome sections of formaldehyde-fixed tissue. The sections were incubated in 0.4% Triton X-100 and 5% normal goat serum before overnight incubation with rabbit α-synuclein and mouse glial (GFAP or C4d) antibodies. After several buffer washes the antibodies were detected with rhodamine- and fluorescein-conjugated isotype-specific goat secondary antibodies. The sections were mounted on glass slides and coverslipped with Aqua-Mount (Lerner Labs., Pittsburgh, Pa.). The slides were viewed with an Olympus FluoView BX50 confocal microscope.

Antibodies to α-synuclein

The 19 amino acid peptide DQLGKNEEGAPQEGILED-C (displaying a cysteine residue at its C terminus and corresponding to α-synuclein amino acids 98 –115) was used for antisera production. Peptide was coupled to an equal amount of maleimide-activated keyhole limpet hemocyanin (KLH; Pierce Chemicals, Rockford, Ill.) as recommended by the manufacturer. Female New Zealand white rabbits were immunized essentially as described by Greens et al. [13]. Specific antibody (NACP98) was purified using affinity chromatography on peptide-immobilized sulfolink gel columns (Pierce). The specificity of this antibody was tested with immunocytochemistry [8] and with Western blots of cell lines stably transfected with α-synuclein. Two monoclonal antibodies were also used to confirm the results with the polyclonal antibody in select Western blots and immunocytochemistry. Takeshi Iwatsubo, University of Tokyo, provided a previously characterized monoclonal antibody (LB509) [3], and the other was purchased from Transduction Laboratories (Lexington, Ky.).

Immunoblotting

Small cubes of brain tissue were dissected from partially thawed frozen brain from specified neuroanatomic regions. Control brains

Table 1 Clinical information on cases examined by immunochemical studies (*LBD* Lewy body disease, *MSA* multiple system atrophy, *PMI* postmortem interval)

Diagnosis	Age (years)	Sex	PMI (h)	Other pathology
Familial LBD	48	M	24	This report
LBD – diffuse cortical type	62	F	15	Widespread neocortical Lewy bodies; early Alzheimer's disease (Braak stage IV–V); clinical history of dementia
LBD – transitional type	66	M	20	Brain stem, diencephalic and limbic Lewy bodies; no Alzheimer pathology; clinical history of Parkinson's disease
LBD – brain stem type	78	F	18	Incidental Lewy bodies in brain stem nuclei only; no Alzheimer pathology; clinically normal
MSA	49	M	14	Striatonigral degeneration
Normal	27	F	18	Clinical history of systemic lupus erythematosus
Normal	6	M	20	Cerebellar congenital vascular malformation with hemorrhage

are summarized in Table 1 and included cases without Lewy bodies of various ages, a case of MSA and several cases with Lewy bodies. The Lewy body cases spanned the disease spectrum with a case with lesions confined to the brain stem, another with a transitional form and a third with widespread diffuse cortical Lewy bodies. Cells were obtained by scraping cultures plates and pelleted with low-speed centrifugation. The brain samples and cell culture pellets were extracted by homogenization and brief sonication in 2% SDS in TRIS-buffered saline (TBS) containing complete protease inhibitors. The samples were centrifuged and equal amounts of supernatant protein (50 μg) were loaded per lane on 10–20% Tricine gels and subjected to electrophoresis. Separated proteins were transferred to polyvinylidene difluoride membranes (Millipore). Unbound sites on the blots were blocked with 5% newborn calf serum in TBS containing 0.05% Tween-20. Blots were then probed with antibody and visualized by chemiluminescence (Pierce Super Signal). Positive controls for immunoblotting consisted of extracts from human neuroblastoma [BE (2)-M17] and human embryonic kidney (HEK 293) cell lines that had been transfected with cDNAs for α-synuclein and selected for stable protein expression. Northern blots using cDNA probes for α-synuclein prepared by polymerase chain reaction, confirmed expression of α-synuclein in these cell lines.

Results

Neuropathology

Gross findings

The fixed right hemibrain weighed 620 g, and the calculated whole brain weight was 1,240 g. There was mild cortical atrophy affecting the frontal convexity, but marked atrophy affecting the temporal pole, especially the anterior two-thirds of the superior temporal gyrus and the medial temporal lobe, including the uncus. The olfactory bulb and tract were attenuated. The cortical gray mantle was thinner than normal, especially in the frontal and temporal lobes. The hippocampal formation and amygdala were both atrophic. The subjacent white matter showed no unusual features. The globus pallidus and pars reticularis of the substantia nigra had a rust color. The nucleus accumbens was attenuated and had a slightly gray discoloration. The thalamus and subthalamic nucleus were unremarkable. Transverse sections of the brain stem revealed almost total loss of pigmentation in the substantia nigra and locus ceruleus. The cerebellar sections, including the dentate nucleus and the cerebellar peduncles, showed no unusual features.

Microscopic findings

The neocortex was remarkable for neuronal loss and superficial spongiosis that was most severe in the superior temporal gyrus (Fig. 2 A), but also noted in the cingulate gyrus and to a lesser extent the frontal and parietal association areas. With thioflavin-S fluorescence microscopy senile plaques and neurofibrillary tangles were not detected in any cortical section, nor was there any evidence of amyloid angiopathy. Tau immunostaining revealed a

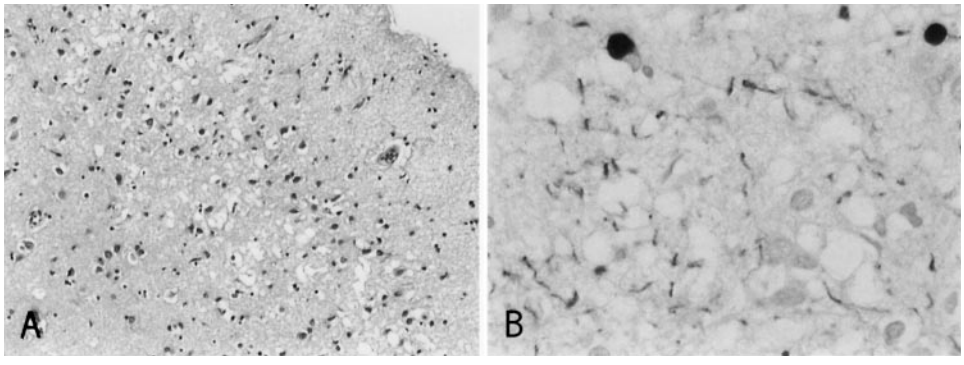

Fig. 2 A, B Temporal lobe pathology. **A** Superficial spongiosis is noted in the upper cortical lamina of the superior temporal gyrus (*arrows*) H&E staining. **B** In the same region the synuclein immunostain reveals numerous neuritic profiles in cross and longitudinal sections, as well as a few cortical Lewy bodies. **A** × 200, **B** × 400

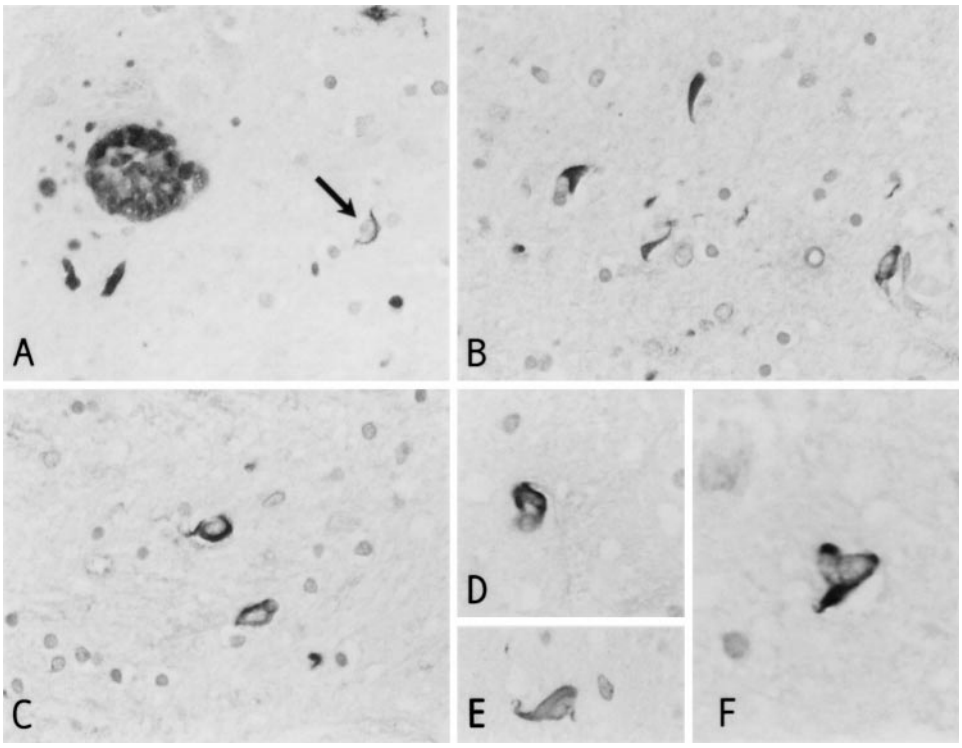

Fig. 3 A–F Synuclein-positive inclusions are noted throughout the brain. **A** Bizarre pleomorphic Lewy bodies and glial inclusions (*arrow*) are located in the basal nucleus of Meynert. **B**, **C** Many glial inclusions are detected in the globus pallidus and the internal capsule. **D–F** Widely spaced glial inclusions are present in the cerebellar white matter. Crescent-shaped or ring-like peri-nuclear filamentous aggregates are similar, but not identical, to glial inclusions in multiple system atrophy. **A** × 200, **B–F** × 400

few widely scattered thread-like processes, but no neurofibrillary tangles.

The most striking cortical pathology was revealed with α-synuclein immunostaining. The regions with spongiosis and gliosis were also rich in many thread-like dystrophic cell processes (Fig. 2B). These were accompanied by scattered glial cells with α-synuclein-immunoreactive inclusions somewhat similar to glial cytoplasmic inclusions of MSA. There were also glial inclusions in the cerebral and cerebellar white matter and in certain white matter fiber tracts, especially in the basal ganglia and basal forebrain (Fig. 3). In addition to the thread-like processes and glial lesions, there were also many round or dot-like structures in affected regions, which probably represent dystrophic neurites on cross-section. There were also α-synuclein-immunoreactive round to pleomorphic inclusions within neurons, mostly in lower cortical layers, but not showing a clear laminar predilection. The latter inclusions were occasionally visible on H&E-stained sections and were consistent with cortical Lewy bodies. Cortical Lewy bodies were most numerous in limbic and paralimbic cortices, including the parahippocampal cortex, insular cortex and the cingulate gyrus. The number and distribution of cortical Lewy bodies were consistent with neocortical stage of DLB (see Table 2 for distribution).

The hippocampus had extensive neuronal loss and gliosis in CA2/3 and extending into the proximal part of CA1 (Fig. 4A). On the other hand, neurons in the CA1 and subiculum were spared (Fig. 4B). There were no hippocampal neurofibrillary tangles, senile plaques or granulovacuolar degeneration, but many Hirano bodies were present (Fig. 4B). Some of the Hirano bodies were much larger than usual and visible with thioflavin fluorescence

Table 2 Lewy body density and distribution with α-synuclein immunostaining. Multiple fields were scanned at low power to identify regions with the greatest lesion density and then counts were taken manually of the number of Lewy bodies in a × 200 field. Representative low- and high-density fields were recorded to give a semiquantitative estimate of lesion density in specific neuroanatomic regions

Cortical region	Lewy bodies (range at × 200)
Mid-frontal	8–10
Superior temporal	9–11
Inferior parietal	2–5
Cingulate gyrus	10–12
Parahippocampal gyrus	5–7

microscopy. The α-synuclein immunostain revealed extensive and unprecedented neuritic pathology in the endplate of the hippocampus (Fig. 4C). Surprisingly, there were also a few α-synuclein-immunoreactive inclusions in the granular neurons of the dentate fascia (Fig. 4D). Other regions of the brain with comparable degrees of α-synuclein-immunoreactive neuritic pathology were the amygdala (Fig. 4E) and the basal ganglia (Fig. 4F). In addition to thread-like staining, there were round dot-like structures and some perikaryal neuronal inclusions. The amygdala had numerous Lewy neurites and cortical Lewy bodies. The Lewy bodies in the amygdala were often pleomorphic. The ventrolateral amygdala had dense fibrillary gliosis.

The basal nucleus of Meynert had neuronal depopulation with gliosis and many Lewy bodies and Lewy neurites, but no neurofibrillary tangles. Some of the Lewy

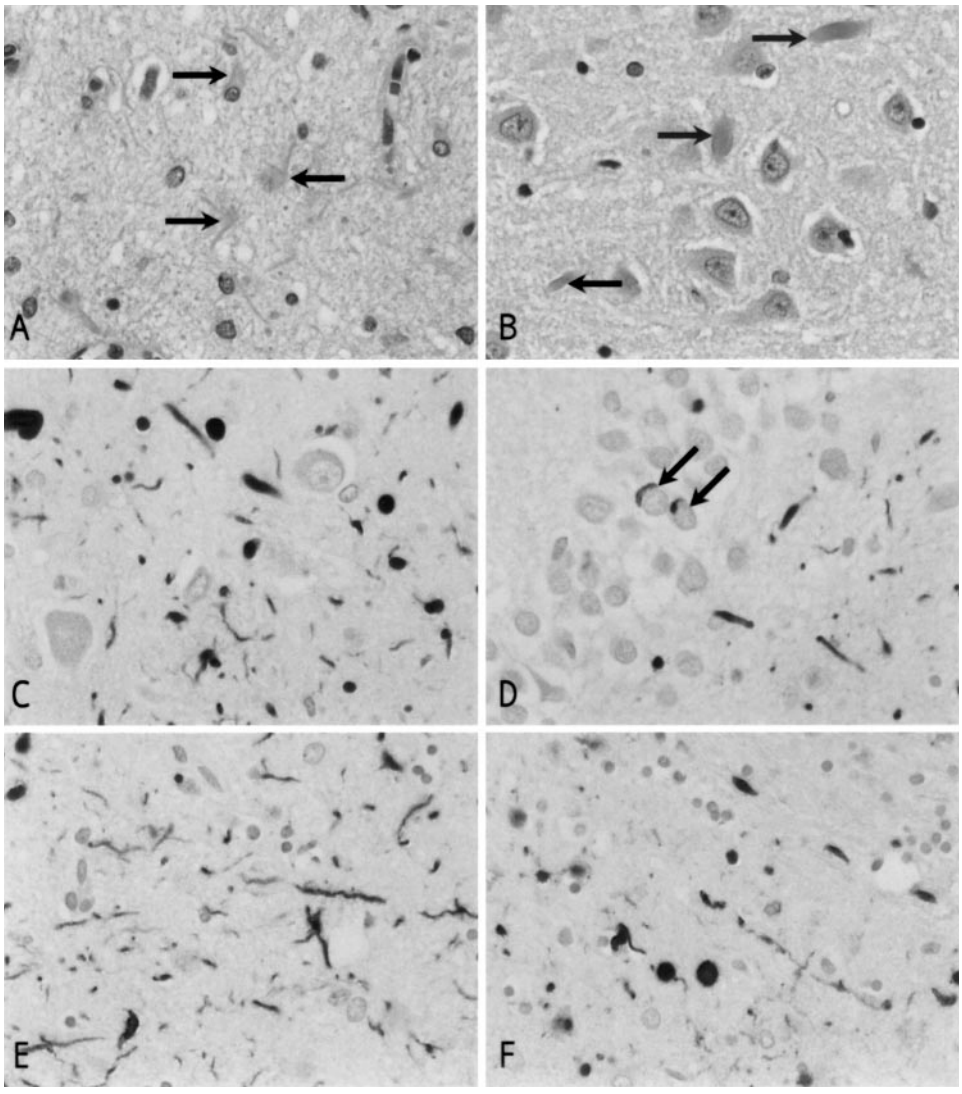

Fig. 4 A–F Limbic pathology. **A** The CA2/3 region of the hippocampus has almost complete loss of neurons with mild spongiosis and prominent reactive astrocytes (*arrows*), H&E staining. **B** In contrast, the CA1 region has preserved neuronal population, but many Hirano bodies (*arrows*), H&E staining. **C–F** Synuclein immunostaining reveals numerous lesions in the limbic lobe. **C** The hippocampal endplate has many dystrophic neuronal processes. **D** Synuclein-immunoreactive inclusions are also present in neurons in the dentate fascia (*arrows*). **E** The amygdala has marked neuritic pathology and a few cortical Lewy bodies. **F** The basal ganglia, including the nucleus accumbens and the medial putamen, have synuclein inclusions in neurons as well as immunoreactive lesions in fiber tracts. **A–F** × 400

bodies were bizarre and pleomorphic (Fig. 3 A). The hypothalamus also had many Lewy bodies and Lewy neurites (Fig. 5 B). There was widespread involvement of the hypothalamus from anterior to posterior extent, with striking sparing of the mammillary body. The basal ganglia showed extensive neuronal loss and gliosis in the medial putamen and the nucleus accumbens. These regions also had many α-synuclein-immunoreactive neuronal inclusions, Lewy neurites and a few glial inclusions (Figs. 3 B, C; 4 F). The globus pallidus was less affected with respect to neuronal inclusions, but had scattered granular dystrophic neurites and glial inclusions. There was brown granular (hemosiderin-like) pigment in the globus pallidus and pars reticularis of the substantia nigra.

The substantia nigra had severe neuronal loss with only a few residual neurons in the most medial part of the pars compacta. There were many Lewy neurites and a few Lewy bodies in the substantia nigra (Fig. 5 A). There were also a few glial inclusions, especially in the medial substantia nigra. The periaqueductal gray and the dorsal midbrain tegmentum were also affected, whereas the red nucleus was spared. Bizarre neuritic Lewy bodies were common in the raphe nuclei (Fig. 5 E, G). A few Lewy bodies were detected in the oculomotor nucleus. The locus ceruleus had marked neuronal loss and many α-synuclein-immunoreactive neuronal processes and Lewy bodies in the few residual neurons.

The lower brain stem was remarkable for sparse α-synuclein-immunoreactive dystrophic processes in the pontine base (Fig. 5 C) and neuritic Lewy bodies in the dorsal motor nucleus of the vagus and throughout the reticular formation of the medullary tegmentum (Fig. 5 F). A few α-synuclein-immunoreactive neurites were even detected in the inferior olive. The cerebellum had well-preserved Purkinje and internal granular cell layers. There were scattered glial inclusions in the cerebellar white matter that were somewhat similar to glial cytoplasmic inclusions in MSA (Fig. 3 D–F). The neuronal population of the dentate nucleus was preserved, but rare neurons had Lewy bodies in the dentate nucleus.

Ubiquitin immunostaining revealed similar pathology to α-synuclein, but had less sensitivity and specificity.

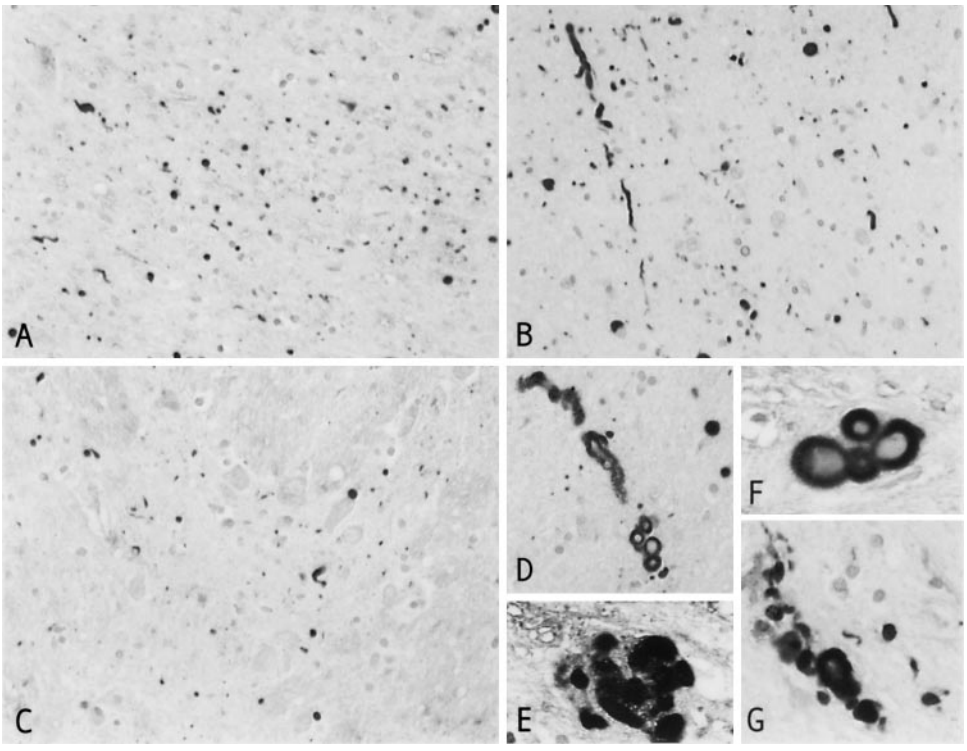

Fig. 5 A–G Deep gray matter pathology. **A** Dot-like synuclein-positive dystrophic neurites are present in the substantia nigra, which is almost completely devoid of neurons. **B** Synuclein-positive lesions in cross-section and longitudinal section are abundant in the hypothalamus. **C** Sparse dystrophic processes are present in the pontine base. **D–G** Pleomorphic Lewy bodies are present in the raphe nucleus (**D**, **G**) and the medullary tegmentum (**E**, **F**). **A–C** × 200, **D–G** × 400

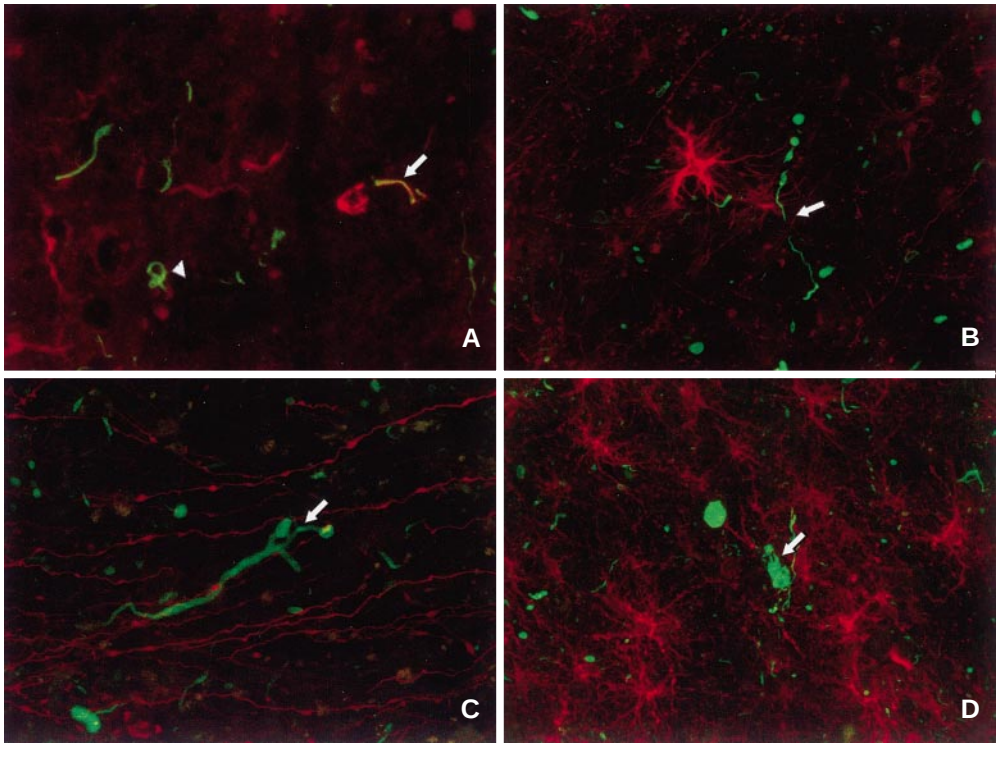

Fig. 6 A–D Laser confocal microscopy of amygdaloid pathology. **A** Double immunolabeling of glial cells with C4d (fluorescein) and synuclein (rhodamine). Note that some of the ring-like forms show colocalization of both antigens (*arrow*), whereas others are only positive for synuclein (*arrowhead*). **B–D** Sections double labeled with glial fibrillary acidic protein (rhodamine) and synuclein (fluorescein) show no colocalization of antigens. Synuclein-positive processes are segmented (*arrow*) (**B**) or branched (*arrow*) (**C**). **D** Pleomorphic (*arrow*) and round synuclein-positive inclusions are associated with gliosis

Surprisingly, the tau antibody, PHF-1, reacted with a few brain stem-type, hyaline Lewy bodies, as well as intraneuritic Lewy bodies, but not cortical-type Lewy bodies or Lewy neurites in limbic regions and the basal forebrain.

Double immunostaining for C4d and α-synuclein revealed that a few of the small cells that were positive for C4d occasionally had co-labeling for α-synuclein (Fig. 6 A). The C4d immunostain also labeled select myelin sheaths. The GFAP and α-synuclein double stain showed complete separation of the two antigens (Fig. 6 B–D). None of the α-synuclein-positive processes were within GFAP-positive astrocytes. Thus, the glial inclusions were probably mostly within oligodendroglia.

Western blotting

Homogenates from various neuroanatomical regions were probed with the affinity-purified rabbit antibody and a mouse monoclonal to α-synuclein (LB 509) (Fig. 7). Both antibodies reacted specifically with a band having an apparent molecular mass of 19 kDa, consistent with α-synuclein. In addition to the 19-kDa band, the antibodies detected additional bands. A band of approximately 26 kDa was consistently detected in the Iowa kindred brain with both α-synuclein antibodies, especially in frontal and temporal cortices and the amygdala, areas with many cortical Lewy bodies. It was absent from regions that lacked Lewy neurites, such as the cerebellum and occipital cortex. It was also not obvious in the substantia nigra, but this region had very severe pathology and only very few residual neurons. A 38-kDa band, which was detected with the polyclonal but only weakly recognized by the monoclonal antibody, may represent a dimer of α-synuclein. Its precise molecular identity remains uncertain, but it was also detected in control brains (Fig. 8). The 26-kDa band was absent in cell lines overexpressing α-synuclein (not shown) and not detected in control brain tissues, including normal brains and brains with other synucleinopathies, including MSA and Lewy body disease ranging from brain stem to diffuse cortical types (Fig. 8).

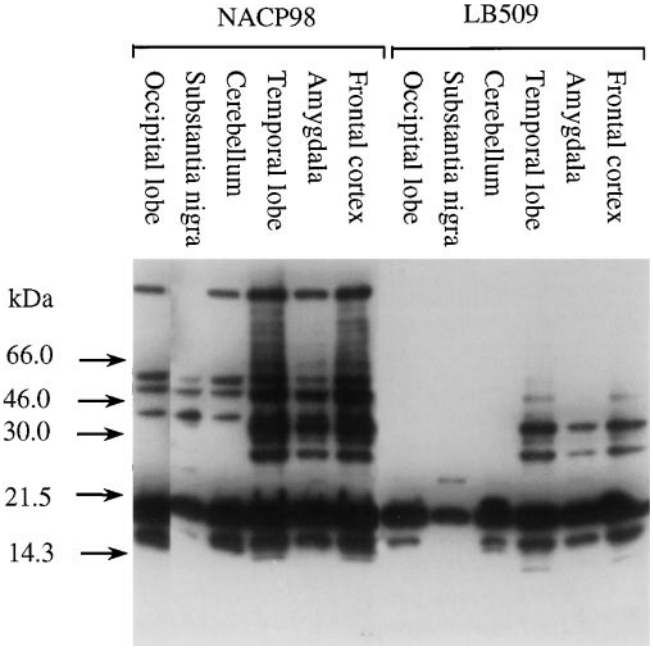

Fig. 7 A Western blot of extracts from several brain regions from the Iowa kindred are probed with a rabbit polyclonal antibody (NACP98) and a murine monoclonal antibody (LB509) generated to α-synuclein. Both antibodies recognize a 19-kDa band in all brain regions. Higher molecular mass bands are detected in areas with the most synuclein pathology, namely, frontal and temporal cortices and the amygdala. A 26-kDa band is recognized by both antibodies in these regions, while a 38-kDa band is recognized by NACP98 in all regions, but only weakly by LB509

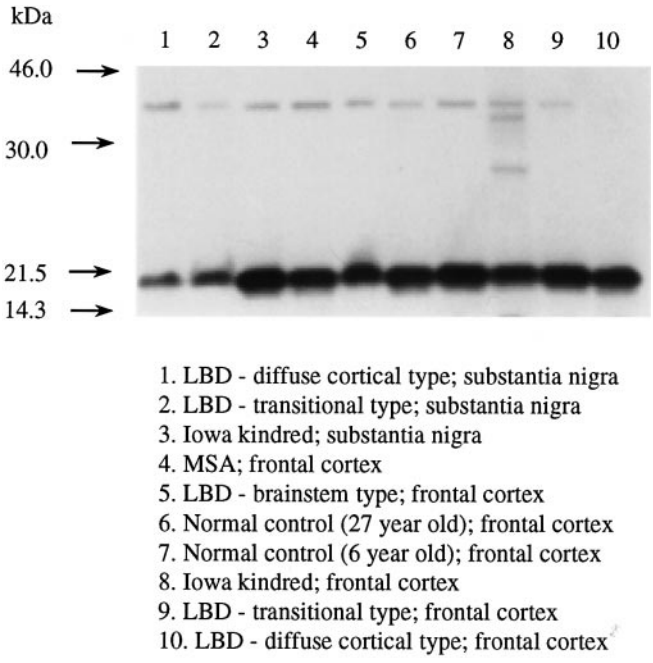

Fig. 8 Immunoblots are shown of brain homogenates using the polyclonal antibody (NACP98) of the Iowa kindred and normal controls and brains of other synucleinopathies. A 19-kDa band and a 38-kDa band are present in all cases. In contrast, a 26-kDa band and several other higher molecular weight species are only found in the affected cortex of the Iowa kindred. This 26-kDa band is not present in the substantia nigra in the Iowa kindred or the other synucleinopathies

Discussion

Clinicopathological correlations

From a clinicopathological perspective, the diagnosis in this case best fits DLB, since the patient had widespread cortical and brain stem Lewy bodies and he exhibited the cardinal clinical diagnostic features, as well as a number of supporting features [19]. In particular, he had dementia, visual hallucinations and spontaneous parkinsonism. The only essential feature missing was fluctuation of cognition; however, this clinical feature is also the least reproducible and reliable in a number of studies testing the sensitivity and specificity of the proposed DLB criteria [1, 20]. The criteria for DLB call for dementia to be present "within a year" of the onset of parkinsonism. In the present case dementia was clearly present 1–2 years into the disease process, and possibly, earlier. When dementia follows parkinsonism after 12 months or more, it is suggested that "parkinsonism with dementia" rather than DLB be used for the disorder. This essentially semantic issue may be resolved in careful comparative clinicopathological studies of DLB and parkinsonism with dementia in the future. One could argue that since clinical and pathological features are similar, but simply in a different order, that the distinction may not be biologically meaningful. In other members of this kindred dementia

was less prominent. Together with changing concepts in neurological diagnosis over the four generations for which they have been followed, this phenotypic variability has led to differing diagnoses being made for affected individuals of the Iowa kindred, with diagnoses ranging from PD to psychosis. Except for the early age of onset, some of the other members of the family fit consensus criteria for PD [14].

Among other notable clinical features in this man was a pervasive sleep disorder, which is increasingly recognized as a manifestation of DLB [5]. He had sleep walking and talking, suggestive of REM behavior disorder. He may have had sleep dysfunction even earlier in his course, according to an interview with his spouse. Other sleep abnormalities in DLB have been reported in less detail and are felt to be at least partially due to involvement of brain stem structures by Lewy bodies [4].

Autonomic dysfunction has also been described in DLB and probably correlates with the Lewy body involvement of central and peripheral neurons of the autonomic nervous system [23, 29]. In the present case autonomic dysfunction was termed "central" based upon laboratory tests, but cannot be more adequately localized. The gastrointestinal dysfunction may be due to abnormalities of autonomic nervous system at multiple levels of the neuraxis. In the setting of parkinsonism the presence of autonomic dysfunction raises the possibility of MSA. Indeed, both MSA and Lewy body disease are now recognized as synucleinopathies. While the inclusions in MSA are predominantly glial and those in Lewy body disease are predominantly neuronal, the converse is also true in both disorders – neuronal inclusions are detected in MSA and glial inclusions in Lewy body disease. Neuronal inclusions in MSA have a number of features in common with cortical-type Lewy bodies, including irregular shape, indistinct appearance of routine stains, cytoplasmic location and immunoreactivity with synuclein. Whereas glial inclusions are not widely recognized in Lewy body disease, they have been reported in previous studies using sensitive silver impregnation methods [28] and more recently immunocytochemistry for synuclein [6]. In the present case glial inclusions were widespread, although not as numerous as in MSA. They were mostly within cells that were consistent with oligodendroglia, since they were negative for GFAP and sometimes positive for C4d, which has been shown to label oligodendroglia in pathological conditions [32, 33].

Unusual neuropathological findings

Previously published cases in this kindred had documented gross pathological abnormalities similar to the present case. Notably, atrophy of the anterior temporal lobe, including medial temporal lobe structures as well as the superior temporal gyrus, was present in all cases studied [21]. This pattern of cortical atrophy is typical of DLB and corresponds to cortical regions that are vulnerable to Lewy body pathology. In the present case, additional neuritic pathology was prominent in these cortical areas and was associated with spongiosis and gliosis. Neuritic pathology has only been recognized as a significant feature of DLB with the advent of immunocytochemical methods of analysis; neuritic pathology in the hippocampus was first described in the early 1990s [7] when ubiquitin immunostaining was widely used to evaluate dementia disorders. While more than adequate for revealing the characteristic histopathology of Lewy body disease, ubiquitin staining lacks disease specificity. This problem has been surmounted with synuclein immunostaining. Synuclein has much greater specificity. In a survey of a host of degenerative diseases in our laboratory and others, only Lewy-related lesions and glial inclusions in MSA were positive for synuclein [8, 27]. Furthermore, novel pathology is revealed with synuclein immunostaining. Although neuritic lesions are often exposed with ubiquitin antibodies, unless double staining is done it is difficult to distinguish this from Alzheimer-type pathology, which is often present in DLB. Synuclein immunostaining does not recognize Alzheimer-type neuritic pathology. The neuritic pathology in the present case is certainly detected in sporadic cases of DLB, but this case had far more lesions than usual, even in the most severe cases of DLB.

In addition, synuclein revealed lesions that were heretofore unknown. In particular, synuclein-immunoreactive neuritic dystrophy in the hippocampal endplate and inclusion bodies in the dentate fascia were very unusual. Finally, neuronal inclusions were found in abundance in the nucleus accumbens, the globus pallidus and the internal segment of the putamen. These are observations that have yet to be confirmed in non-familial forms of DLB, even in its most marked pathological manifestation. A few neuronal inclusions were found in unusual locations, such as the inferior olive and the dentate nucleus of the cerebellum. These are neurons that are resistant to Lewy body pathology and suggest that the pathology in the present case is very advanced.

Immunochemical findings

Western blotting demonstrated abundant α-synuclein in this brain, as well as unusual immunoreactive higher molecular weight species. In addition to a 19-kDa band representing monomeric α-synuclein there was a 38-kDa band in the present case and in control brains that may represent a multimer of synuclein given its molecular mass and immunoreactivity with antibodies to α-synuclein. In addition, a band of about 26 kDa was detected in the Iowa kindred brain, but not in other synucleinopathies. This band was detected in brain regions that have the most synuclein-immunoreactive lesions and was absent in areas that either had no α-synuclein pathology (e.g., occipital lobe) or had end-stage neuronal loss (e.g., substantia nigra). This band was not observed in cell lines overexpressing α-synuclein. The 26-kDa protein was not detected in Lewy body cases with transitional or predominant brain stem pathology. This suggests that in DLB cases with se-

vere pathology, such as familial forms of DLB, additional immunoreactive species may be detected, which may represent polymerized or complexed synuclein. It is worth noting that higher molecular mass α-synuclein-immunoreactive proteins were also detected in the brains of the Contursi kindred, a form of familial Lewy body parkinsonism linked to mutations in α-synuclein [16]. Determining the nature of the high molecular mass protein may define other genetic determinants of parkinsonism. Of note is a recent report [10] of a synuclein-binding protein; the molecule, synphilin, has a molecular mass of about 90 kDa. However, complexing of α-synuclein with synphilin is unlikely to explain the observed 26-kDa band in the present study.

In summary, this individual had clinically overt disease of at least 17 years duration, and the pathology presumably reflects end-stage disease. He initially presented with decreased right arm swing and subsequently developed parkinsonism, consistent with involvement of the left "extrapyramidal" system, specifically substantia nigra. His symptoms were initially responsive to dopamine-replacement therapy. It is notable that at autopsy the substantia nigra pathology was end stage, with almost complete neuronal depopulation and numerous synuclein-positive dystrophic processes. He subsequently became depressed and demented. Although these features are difficult to localize, it is plausible that the disease extended to other subcortical gray matter and at a relatively early stage to limbic and isocortical areas. He became withdrawn, possibly due to subcortical abulia or frontal lobe dysfunction. One can envision the disease beginning in substantia nigra, with the next most vulnerable regions being the limbic system and finally the neocortex. The clinical and neuropathological findings in this kindred support the hypothesis that DLB is one end of a Lewy body disease spectrum with idiopathic PD at the other end.

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References

1. Ala TA, Yang K-H, Sung JH, Frey WH II (1997) Hallucinations and signs of parkinsonism help distinguish patients with dementia and cortical Lewy bodies from patients with Alzheimer's disease at presentation: a clinicopathological study. J Neurol Neurosurg Psychiatry 62: 16–21
2. Arima K, Ueda K, Sunohara N, Arakawa K, Hirai S, Nakamura M, Tonozuka-Uehara H, Kawai M (1998) NACP/α-synuclein immunoreactivity in fibrillary components of neuronal and oligodendroglial cytoplasmic inclusions in the pontine nuclei in multiple system atrophy. Acta Neuropathol 96: 439–444
3. Baba M, Nakajo S, Tu PH, Tomita T, Nakya K, Lee VM, Trojanowski JQ, Iwatsubo T (1998) Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. Am J Pathol 152: 879–884
4. Biggins CA, Boyd JL, Harrop FM, Madeley P, Mindham RH, Randall JI, Spokes EG (1992) A controlled, longitudinal study of dementia in Parkinson's disease. J Neurol Neurosurg Psychiatry 5: 566–571
5. Boeve BF, Silber MH, Ferman TJ, Kokmen E, Smith GE, Ivnik RJ, Parisi JE, Olson EJ, Petersen RC (1998) REM sleep behavior disorder and degenerative dementia: an association likely reflecting Lewy body disease. Neurology 51: 363–367
6. Dickson DW (1999) Tau and synuclein and their role in neuropathology. Brain Pathol 9: 657–661
7. Dickson DW, Ruan D, Crystal H, Mark MH, Davies P, Kress Y, Yen SH (1991) Hippocampal degeneration differentiates diffuse Lewy body disease (DLBD) from Alzheimer's disease: light and electron microscopic immunocytochemistry of CA2-3 neurites specific to DLBD. Neurology 41: 1402–1409
8. Dickson DW, Farrer MJ, Mehta ND, Perez-Tur J, Tiseo P, Yen S-H, Hardy J (1998) Antibodies to non-amyloid component of plaques (NACP) specifically label Lewy bodies and Lewy neurites, but not other inclusions in neurodegenerative diseases. J Neuropathol Exp Neurol 57: 516
9. Dickson DW, Liu W-K, Hardy J, Farrer M, Mehta N, Uitti R, Mark M, Zimmerman T, Golbe L, Sage J, Sima A, D'Amato C, Albin R, Gilman S, Yen S-H (1999) Widespread alterations of alpha-synuclein in multiple system atrophy. Am J Pathol 155: 1241–1251
10. Engelender S, Kaminsky Z, Guo X, Sharp AH, Amaravi RK, Kleiderlein JJ, Margolis RL, Troncoso JC, Lanahan AA, Worley PF, Dawson VL, Dawson TM, Ross CA (1999) Synphilin-1 associated with α-synuclein and promotes the formation of cytosolic inclusions. Nat Genet 22: 110–114
11. Farrer M, Wavrant-De Vrieze F, Crook R, Boles L, Perez-Tur J, Hardy J, Johnson WG, Steele J, Maraganore D, Gwinn K, Lynch T (1998) Low frequency of alpha-synuclein mutations in familial Parkinson's disease. Ann Neurol 43: 394–397
12. Farrer M, Gwinn-Hardy K, Muenter M, DeVrieze FW, Crook R, Perez-Tur J, Lincoln S, Maraganore D, Adler C, Newman S, MacElwee K, McCarthy P, Miller C, Waters C, Hardy J (1999) A chromosome 4p haplotype segregating with Parkinson's disease and postural tremor. Hum Mol Genet 8: 81–85
13. Green N, Alexander H, Olson A, Alexander S, Shinnick TM, Sutcliffe JG, Lerner RA (1982) Immunogenic structure of the influenza virus hemagglutinin. Cell 28: 477–487
14. Hughes AJ, Ben-Shlomo Y, Daniel SE, Lees AJ (1992) What features improve the accuracy of clinical diagnosis in Parkinson's disease: a clinicopathologic study. Neurology 42: 1142–1146
15. Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, Reiss O (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. Nat Genet 18: 106–108
16. Langston JW, Sastry S, Chan P, Forno LS, Bolin LM, Di Monte DA (1998) Novel α-synuclein-immunoreactive proteins in brain samples from the Contursi kindred, Parkinson's, and Alzheimer's disease. Exp Neurol 154: 684–690.
17. Lee S, Park YD, Yen SH, Ksiezak-Reding H, Goldman JE, Dickson DW (1989) Infantile motor neuron disease: a case report with electron microscopic and ubiquitin and neurofilament immunohistochemical studies. Neuropediatrics 20: 107–111
18. Lippa CF, Fujiwara H, Mann DM, Giasson B, Baba M, Schmidt ML, Nee LE, O'Connell B, Pollen DA, St George-Hyslop P, Ghetti B, Nochlin D, Bird TD, Cairns NJ, Lee VM, Iwatsubo T, Trojanowski JQ (1998) Lewy bodies contain altered alpha-synuclein in brains of many familial Alzheimer's disease patients with mutations in presenilin and amyloid precursor protein genes. Am J Pathol 153: 1365–1370

19. McKeith IG, Galasko D, Kosaka K, et al (1996) Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop. Neurology 47: 1113–1124
20. Mega MS, Masterman DL, Benson DF, Vinters HV, Tomiyasu U, Craig AH, Foti DJ, Kaufer D, Scharre DW, Fairbanks L, Cummings JL (1996) Dementia with Lewy bodies: reliability and validity of clinical and pathological criteria. Neurology 47: 1403–1409
21. Muenter MD, Forno LS, Hornykiewicz O, Kish SJ, Maraganore DM, Caselli RJ, Okazaki H, Howard FM Jr, Snow BJ, Calne DB (1998) Hereditary form of Parkinsonism-dementia. Ann Neurol 43: 768–781
22. Polymeropoulos MH, Lavedan C, Leroy E, et al (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276: 2045–2047
23. Schmidt ML, Murray J, Lee VM-Y, Hill WD, Wertkin A, Trojanowski JQ (1991) Epitope map of neurofilament protein domains in cortical and peripheral nervous system Lewy bodies. Am J Pathol 139: 53–65
24. Spellman GG (1962) Report of familial cases of Parkinsonism. Evidence of a dominant trait in a patient's family. JAMA 179: 160–162
25. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. Nature 388: 839–840
26. Spillantini MG, Crowther RA, Jakes R, Cairns NJ, Lantos PL, Goedert M (1998) Filamentous α-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. Neurosci Lett 251: 205–208
27. Takeda A, Mallory M, Sundsmo M, Honer W, Hansen L, Masliah E (1998) Abnormal accumulation of NACP/alpha-synuclein in neurodegenerative disorders. Am J Pathol 152: 367–372
28. Wakabayashi K, Takahashi H (1996) Gallyas-positive, tau-negative glial inclusions in Parkinson's disease midbrain. Neurosci Lett 217: 133–136
29. Wakabayashi K, Takahashi H, Ohama E, Ikuta F (1990) Parkinson's disease: an immunohistochemical study of Lewy body-containing neurons in the enteric nervous system. Acta Neuropathol 79: 581–583
30. Wakabayashi K, Matsumoto K, Takayama K, Yoshimoto M, Takahashi H (1997) NACP, a presynaptic protein, immunoreactivity in Lewy bodies in Parkinson's disease. Neurosci Lett 239: 45–48
31. Waters CH, Miller CL (1994). Autosomal dominant Lewy body parkinsonism in a four-generation family. Ann Neurol 35: 59–64
32. Yamada T, McGeer PL (1990) Oligodendroglial microtubular masses: an abnormality observed in some human neurodegenerative diseases. Neurosci Lett 120: 163–166
33. Yasuhara O, Matsuo A, Tooyama I, Kimura H, McGeer EG, McGeer P (1995) Pick's disease immunohistochemistry: new alterations and Alzheimer's disease comparisons. Acta Neuropathol 89: 322–330