α-Synuclein Inclusions in Amygdala in the Brains of Patients with the Parkinsonism-Dementia Complex of Guam

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Abstract. We investigated by immunohistochemistry the deposition of α-synuclein in the brains of deceased patients with the parkinsonism-dementia complex (PDC) of Guam. Five of 13 PDC brains showed numerous α-synuclein positive neuronal inclusions and abnormal neurites, chiefly in the amygdala. Similar α-synuclein positive lesions were observed, although to a lesser extent, in the entorhinal cortex and the dorsal vagal nucleus. No α-synuclein positive inclusions were observed in motor cortex or locus coeruleus, and only a small number of positive inclusions were found in the Sommer’s sector, temporal cortex, or substantia nigra. Some of the α-synuclein positive inclusions were reminiscent of cortical Lewy bodies (LB), but many of those in the amygdala coexisted with tau-positive pretangles and/or neurofibrillary tangles (NFT) within the same neurons. In these neurons, tau-positive shells encapsulated α-synuclein positive central cores or irregularly shaped α-synuclein-positive deposition intermingled with pretangles/NFT. Thus, the present study suggests that a common mechanism may govern aggregation of α-synuclein and tau in the amygdala, and that aggregation of α-synuclein may play some role in the neurodegenerative process of a tauopathy (i.e. PDC) in which Aβ deposition is virtually absent.

Key Words: α-synuclein; Amygdala; Guam; Parkinsonism-dementia complex; Tau.

INTRODUCTION

α-synuclein was originally found as the precursor of the non-Aβ component of Alzheimer disease (AD) amyloid in human brains (1, 2), and subsequently was recognized as a constituent of Lewy bodies (LB) (3–6) and glial cytoplasmic inclusions in multiple system atrophy (7, 8). However, it has recently been reported that numerous α-synuclein-positive structures, that is, LBs and Lewy-related neurites, were detected mainly in the amygdala of patients with familial Alzheimer disease (FAD) (9) and Down syndrome (DS) with AD (10).

The parkinsonism-dementia complex (PDC) of Guam is characterized by a marked loss of neurons and deposition of a large amount of neurofibrillary tangles (NFTs) in the neocortex, entorhinal cortex, hippocampus, amygdala, and brainstem (11–14). Despite the occurrence of NFTs morphologically (12) and biochemically (15) similar to those in AD, PDC has been definitively distinguished from AD by the virtual absence of senile plaques (11, 12, 14) and the presence of granular, hazy astrocytic inclusions (16).

These observations led us to examine whether α-synuclein aggregation may occur in the brains of PDC patients. Such brains had previously been considered to show tauopathy, only without senile plaques, but in this study we found that α-synuclein deposited in various forms is frequently observed in the amygdala in PDC.

MATERIALS AND METHODS

Cases

Brains from 13 patients with PDC of Guam (age at death: 44–75 years, mean: 60.7 years) autopsied by one of the authors (KO) and his colleagues at Guam Memorial Hospital between 1978 and 1982 were studied. All fulfilled the clinicopathological criteria for PDC (12, 14, 16, 17). Disease duration ranged from 2 to 13 years (mean: 7.4 years; Table). No LB was seen in hematoxylin and eosin (H&E)-stained sections of the brainstem of these PDC cases.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were cut at 6 μm from the amygdala (accessory, central and medial nucleus), entorhinal cortex, motor cortex, midbrain, pons (including the locus ceruleus) and medulla oblongata (including the dorsal vagal nucleus) and stained with H&E and the Klüver-Barrera and Bodian methods. Immunostaining was performed with polyclonal anti-ubiquitin antibody (DAKO, 1:500), anti-human Aβ monoclonal antibody (mAb) 6F/3D (DAKO, 1:100), anti-α-synuclein mAb LB509 (1:100) (5), anti-α-synuclein antisemur #17 (1:100) (18), anti-β-synuclein antisemur 253 (1:100) (18, 20–22) and anti-tau mAb AT8 (Innogenetics, 1:1000). LB509, raised against purified LBS (5), recognizes a human-specific epitope at the C terminus of α-synuclein (23). #17 was raised against a synthetic peptide corresponding to residues 1–10 of human α-synuclein. The specimens were pretreated by hydrated autoclaving (121°C, 15 min) (24). Primary antibody labeling was detected using the avidin-biotinylated horseradish peroxidase complex (ABC) system (Vector, Elite kit) and visualized...
### TABLE
Clinical Data and Pathological Findings of PDC Cases Examined

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<tr>
<th>Case</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Duration (y)</th>
<th>Brain Weight (g)</th>
<th>Neuronal Loss</th>
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Neuronal loss and NFT formation were rated on a scale of 4+. The number of α-synuclein-positive inclusions in each region on 1 section were arranged in grades 0–3: -; no α-synuclein-positive inclusions; +1; 1–5 α-synuclein-positive inclusions; +2; 5–20 α-synuclein-positive inclusions; +3; more than 20 α-synuclein-positive inclusions. Abbreviations: Age, age at death; Duration, duration of disease; M, male; F, female; Aβ(+)-SP, number of Aβ-positive senile plaques; Nigra, substantia nigra; LC, locus ceruleus, Medulla, medulla oblongata; MC, motor cortex; ERC, entorhinal cortex; T3, inferior temporal cortex; ne, not examined.
using diaminobenzidine (DAB) as a chromogen. The sections from 5 cases of AD and dementia with LB were similarly examined as positive controls. Negative control sections were processed in parallel with the omission of primary antibodies.

The number of α-synuclein-positive intraneuronal inclusions in each region in a single section was divided into 3 groups using the following semiquantitative criteria: -, no α-synuclein-positive inclusions; +1, 1–5 α-synuclein-positive inclusions; +2, 5–20 α-synuclein-positive inclusions; +3, more than 20 α-synuclein-positive inclusions per entire area of a given region. The neuronal loss and NFT in the amygdala of Guam PDC cases were rated 1+ to 4+ based on K-B staining and tau immunostained sections, respectively.

**Immunofluorescence Microscopy**

Sections were observed under a fluorescence microscope combined with a laser confocal system (TCS-SP, Leica, Heidelberg, Germany). For double immunostaining with anti-tau antibody (AT8) and anti-α-synuclein antiserum (#17), the specimens were blocked with nonimmune sera from horse and goat corresponding to the secondary antibodies used. Sections were first incubated with the mixture of the 2 primary antibodies and then followed by fluorescence-labeled secondary antibodies (i.e. Alexa 488 labeled anti-rabbit IgG (H+L) and Alexa 594 labeled anti-mouse IgG (H+L)).

**Immunoelectron Microscopy**

Paraffin-embedded, 6-μm sections from the amygdala of PDC cases, in which α-synuclein-positive inclusions were observed, were immunostained with LSB09, visualized by DAB as for light microscopic immunohistochemistry and then processed for immunoelectron microscopy. After being postfixed in 4% OsO4 for 15 min, the sections were dehydrated in a graded ethanol series, and embedded in Epon 812 and polymerized at 60°C for 24 h. Ultrathin sections were stained with 3% lead acetate for 2 min and viewed with an electron microscope (H-9000, Hitachi, Japan).

**RESULTS**

Numerous NFTs were observed in amygdala (Table) and entorhinal cortex. A small number of tau-positive neuropil threads were observed in amygdala in all cases. A few diffuse plaques were found in the amygdala in 1 case (case 13), and no Aβ-positive plaques were observed in the amygdala of 9 other cases (Table).

α-synuclein immunohistochemistry revealed many α-synuclein-positive intraneuronal inclusions in the amygdala in 5 of 13 PDC cases (Fig. 1A). A smaller number of α-synuclein-positive inclusions were found in the entorhinal cortex, substantia nigra, or dorsal vagal nucleus of the medulla in these cases. No α-synuclein-positive inclusions were detected in the motor cortex or locus coeruleus, and only a small number of α-σynuclein-positive inclusions were found in the Sommer’s sector, temporal cortex, or substantia nigra. In total, 7 of 13 PDC cases (54%) showed α-synuclein-positive inclusions in at least 1 brain region and 6 other cases (46%) exhibited no α-synuclein-positive structures in any regions examined in this study (Table). Re-examination by H&E or ubiquitin immunostaining showed only a few cortical LBs in 1 PDC case (case 6). The anti-β-synuclein antiserum 253 did not immunostain any of the α-σynuclein-positive inclusions.

For the PDC patients whose brains contained α-synuclein-positive inclusions, age, duration of disease, clinical manifestations, and brain weight were variable (Table). No significant difference was found in the degree of amygdala degeneration (i.e. neuronal loss, NFT formation, and Aβ deposition) between α-synuclein-positive and -negative cases (Table).

Notably, most of the α-synuclein-positive inclusions showed unique morphologies that were distinct from those of typical cortical LBs, which had globose figures and regular outlines, and some showed either ragged or curvilinear profiles (Fig. 1B). Only a small number of α-synuclein-positive inclusions showed globular and solid structures that closely resembled cortical or brainstem LBs (Fig. 1C, D). Many of these inclusions coexisted with tau-positive NFTs in the same neuronal perikarya in the amygdala or entorhinal cortex (Fig. 1E, F). Three major patterns of coexistence of α-synuclein positive inclusions with NFTs were observed: 1) α-synuclein-positive inclusions (green) intermingled with tau-positive NFTs (red) (Fig. 2A, B); 2) some NFT were encapsulated by an α-synuclein-positive shell (green) (Fig. 2C, D); and 3) α-synuclein-positive cores encapsulated by tau-positive shells (Fig. 2E, F).

The α-synuclein-positive covering in type (2) was so thin that it was only weakly immunopositive using the conventional ABC method (Fig. 1G). α-synuclein and tau deposits did not overlap and were always completely segregated from each other (Fig. 2A–F). In this study, however, all cases with α-synuclein-positive inclusions invariably exhibited tau-positive inclusions in amygdala and entorhinal cortex.

We also observed α-synuclein-positive threads similar to those designated Lewy-related neurites, though the number was very small and they were formed only in the amygdala and the dorsal vagal nucleus of the medulla. No α-synuclein-positive neurites were observed in any other regions, such as the CA2-3 regions of the hippocampus, where a number of Lewy-related neurites have been typically reported in cases of dementia with LBs (25).

Immunoelectron microscopic observation (Fig. 3B, C) showed that α-synuclein-positive intraneuronal inclusions resembling cortical LBs were composed of granulo-filamentous elements, and the diameter of the filamentous components decorated with the DAB was ~15–22 nm.

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Fig. 1. Immunostaining with antibodies against α-synuclein (LB 509) revealed α-synuclein-positive inclusions in PDC cases (A–G). The low magnification photomicrograph (A) shows the frequency and distribution of the α-synuclein-positive inclusions in the amygdala. Immunostaining pattern of intraneuronal α-synuclein inclusions was variable (B–G); most of the α-synuclein-positive inclusions were different from brainstem-type LBs, and were either amorphous or linear in shape (D). Some were globular with solid inclusions, thus closely resembling brainstem and cortical LBs (B, C). Many α-synuclein-positive inclusions coexisted with NFTs (arrowheads) in the same neuron (E, F). Some NFTs were encapsulated by α-synuclein-positive shells and they were weakly immunopositive for α-synuclein using conventional ABC method (G) Scale bars: A, 50 μm; B–G, 10 μm.

Fig. 2. Double immunofluorescence labeling with AT8 (Alexa 594: red) and #17 (Alexa 488: green) showed that many α-synuclein-positive inclusions coexisted with NFTs in the same neurons (A–F). The features of coexistence were of 3 types: 1) α-synuclein-positive inclusions were intermingled with NFTs (A, B); 2) α-synuclein-positive inclusions were covered by a thin, tau-positive layer (C, D); and 3) a small number of pretangles/NFTs were encapsulated by a α-synuclein-positive thin shell (E, F). Scale bars: A–F, 10 μm.

DISCUSSION

Hirano (26) and Oyanagi (14) reported that very few LBs, most of which coexisted with NFTs, were observed in the substantia nigra in approximately 10% of PDC cases. In the present study, we have clearly shown that α-synuclein-positive inclusions are detectable in neurons of the amygdala in approximately 40% of cases of Guam PDC, and that more than 50% of PDC cases harbored α-synuclein reactive inclusions in at least 1 region examined. α-synuclein-positive inclusions were not observed in amygdala in cases 5 and 10, but were detected in entorhinal cortex and medulla, respectively. Nonetheless, α-synuclein-positive inclusions were found chiefly in amygdala and the total number of α-synuclein-positive inclusions in amygdala was much larger than those in any other sites examined in most of the cases. Therefore, we considered that amygdala is the major site for α-synuclein accumulation in PDC.

Interestingly, the shape and size of α-synuclein positive-inclusions varied and they were often distinguishable
from cortical LBs. Typically, the inclusions were classified as 1) irregularly shaped, bundle-like inclusions intermingled with pretangles/NFTs (Fig. 1E, F); 2) a small number of α-synuclein-positive thin shells which encapsulated pretangles/NFTs; and 3) round inclusions that were wrapped up within a tau-positive shell, showing a reverse of the immunostaining pattern seen with type (2). Although it has previously been reported that α-synuclein-positive inclusions (LBs) and NFTs were intermingled within the same neurons in a similar fashion to type (1) in the amygdala of patients with sporadic or FAD and dementia with LB (DLB) (9, 10, 27), type (2) or (3) patterns of codeposition of α-synuclein and tau have never been documented. These findings may imply a closer relationship between aggregation of α-synuclein and tau, as well as a common mechanism underlying these biochemically distinct fibrous aggregations.

In this study, we did not have a case with α-synuclein-positive inclusions but lacking tau-positive inclusions, including NFTs and pretangles. All cases with α-synuclein-positive inclusions invariably contained tau-positive inclusions in the same sites. It is not known whether tau or α-synuclein accumulates first in the PDC brain. From the findings described above, it is conceivable that tau-positive inclusions including NFTs and pretangles precede the deposition of α-synuclein.

It has been reported that various types of α-synuclein-positive structures are found in PD and DLB, and some researchers have proposed that the diffuse cytoplasmic α-synuclein staining of an otherwise morphologically normal neuron in PD and DLB may represent an early stage of LB formation (28). In Guam PDC, the same diffuse cytoplasmic α-synuclein staining was also observed in neurons with or without tau-positive pretangles/NFTs, but there were few LBs. This finding may indicate that the diffuse cytoplasmic α-synuclein staining does not represent the process of LB formation. If so, all the diffuse cytoplasmic α-synuclein staining should not correspond to an early stage of LBs in PDC. Rather, the staining may represent a unique form of α-synuclein aggregation specific to this disorder. Similarly, in multiple system atrophy, α-synuclein invariably aggregates to form glial cytoplasmic inclusions (7, 8), but never develops LBs. Another possibility is that the diffuse cytoplasmic α-synuclein staining represents an early stage of LB formation in PDC as well. If this were the case, the speed of LB maturation would be much slower, and this may explain why the total number of LBs is smaller in PDC.

Lippa and coworkers reported that in about half of all cases of FAD and DS with AD, α-synuclein-positive LBs were detected mainly in the amygdala (9, 10). They emphasized that the frequency of LBs in DS with AD and FAD brains was higher than that in brains with sporadic AD and speculated that the development of α-synuclein-positive lesions could be influenced by genetic factors. However, in PDC of Guam, some recent epidemiological studies have indicated that genetic factors do not play a pivotal role in the pathogenesis (29–32). The present study suggests that some, as yet undefined, epigenetic factors may contribute to the aggregation of α-synuclein in PDC.

Deposition of both LBs and NFTs without Aβ accumulation has also been reported in a few cases of Hallervorden-Spatz syndrome of long duration (33, 34), in a young patient with dementia of unknown origin (35), and a case of Ewing’s sarcoma (36). Nevertheless, our observation expands the spectrum of well-defined neurodegenerative disorders that are characterized by widespread aggregation of α-synuclein.
This is the first report to indicate that α-synuclein can aggregate in the amygdala in a form of tauopathy that lacks Aβ deposition. Future studies should elucidate the common mechanism (e.g., hyperphosphorylation, failure of proteolysis, or promotion of protein precipitation) whereby α-synuclein and tau coaggregate and lead to neuronal death in these disorders.

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REFERENCES


