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EXPRESS COMMUNICATION

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NACP/α-synuclein immunoreactivity in fibrillary components of neuronal and oligodendroglial cytoplasmic inclusions in the pontine nuclei in multiple system atrophy

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Abstract We examined neuronal cytoplasmic inclusions (NCIs) and oligodendrocytic glial cytoplasmic inclusions (GCIs) in the pontine nuclei in multiple system atrophy (MSA) using antibodies against the non-amyloid β component of Alzheimer's disease amyloid precursor protein (NACP/α-synuclein). Our immunohistochemical study revealed that anti-NACP antibodies labeled both NCIs and GCIs. Immunoelectron microscopy showed that positive reaction products were localized on the 15- to 30-nm-thick filamentous components of NCIs and GCIs. The present study demonstrates that NACP is associated with cytoplasmic inclusions of MSA, and suggests a role of NACP in abnormal filament aggregation in neuronal degeneration.

Key words NACP · Synuclein · Multiple system atrophy · Neuronal cytoplasmic inclusions · Glial cytoplasmic inclusions

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Introduction

Non-amyloid β component (NAC), a 35-amino acid peptide, was originally identified as the second component of senile plaque amyloid in Alzheimer's disease brains [15]. The precursor protein of NAC, a 140-amino acid protein named NACP [15], was demonstrated to be a presynaptic protein [4] and to be localized in the dystrophic neuritic component of senile plaques [9]. Later, NACP was identified independently in human brain, and referred to as human α-synuclein [5], which is highly homologous to the synuclein of the *Torpedo* electric organ and of rat brain [7, 8]. Thus, NACP is a member of the the synuclein family [5, 7]. Shortly after the identification of a mutation in the NACP/α-synuclein gene in families with Parkinson's disease [13], Lewy bodies were reported to be immunolabeled by antibodies to NACP/α-synuclein protein [14]. Here we report for the first time that both neuronal cytoplasmic inclusions (NCIs) [1, 6] and glial cytoplasmic inclusions (GCIs) of oligodendrocytic origin [10–12] in the pontine nuclei in multiple system atrophy (MSA, [2]) express epitopes for NACP.

Materials and methods

Tissue source

This study was carried out on brain samples from five Japanese MSA patients. The samples were obtained from the archives of the Laboratory of Neuropathology, Department of Laboratory Medicine, National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP). They were from two males and three females whose clinical duration of the disease ranged from 3 to 11 years (mean 7.6 ± 3.4 years), age at death ranged from 51 to 65 years of age (mean 58.6 ± 5.2 years), brain weight ranged from 1075 to 1440 g (mean 1253 ± 155 g), and in whom the postmortem delay ranged from 1 to 9 h (mean 6.2±3.5 h). Nineteen brains from patients with various neurological or psychiatric disorders including Alzheimer's disease, sporadic Parkinson's disease, progressive supranuclear palsy, amyotrophic lateral sclerosis, Machado-Joseph's disease, multiple cerebral infarctions, myotonic muscular dystrophy, traumatic cerebral injury, epilepsy, schizophrenia, and depression, whose age of

death ranged from 28 to 98 years of age, were obtained from the same archives and used as controls. The pons was examined by routine histological staining including Bodian's impregnation method.

Primary antibodies and their specificities

The primary antibodies employed were anti-NACP antibody (MDV2, rabbit polyclonal antibody raised against a synthetic peptide corresponding to residues 1–15 of the N terminus of NACP), anti-NAC antibody (EQV1, rabbit polyclonal antibody raised against a peptide corresponding to residues 1–15 of the N terminus of NAC that also recognizes the NAC domain in NACP), and anti-β-synuclein antibody (REE1, rabbit polyclonal antibody raised against a peptide corresponding to residues 86–100 of human β-synuclein [5]). The characterization of MDV2 and EQV1 have been partially reported previously [3].

To further confirm the specificities of the MDV2, EQV1, and REE1 antibodies, both immunoblot analysis and immunohistochemical study were carried out. For the immunoblot analysis, a rat brain cytosolic fraction was separated through 10–20% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, Mass.), blocked, and then incubated with either of MDV2 (1:5000 dilution), EQV1 (1:5000), or REE1 (1:5000). After washing, the membrane was incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:10 000; Vector, Burlingame, Calif.), followed by the Western blot-chemiluminescence reagent (DuPont NEN, Boston, Mass.). The immunoreactivities on the membrane were detected using Hyperfilm-ECL (Amersham, Little Chalfont, UK).

Specificity of the staining was demonstrated by an absorption experiment that comprised preincubation of each antibody with 20 μg of the corresponding peptide per ml. Paraformaldehyde-fixed and paraffin-embedded hippocampal sections from a control human brain were stained with each antibody (1:5000 dilution) or pre-absorbed antibody using the immunoperoxidase method described below. Anti-ubiquitin antibody (1:150 dilution; DAKO, Glostrup, Denmark) was also examined.

Immunohistochemical study

Formalin-fixed, paraffin-embedded 6-μm-thick sections of the pons were examined. The deparaffinized sections were incubated with either of the primary antibodies (1:3000 dilution), and the streptavidin-biotin complex peroxidase method was carried out with 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the final chromogen. To demonstrate simultaneous immunohistochemical localization in the same inclusions, several sets of serial sections were alternately stained with the following three pairs of antibodies: MDV2 and anti-ubiquitin, EQV1 and anti-ubiquitin, and MDV2 and EQV1.

Pre-embedding immunoelectron microscopic study

A paraformaldehyde-fixed pontine base was sectioned at 50 μm with a vibratome. The sections were first incubated with MDV2, labeled with 1.4-nm-diameter gold immunoprobe solution (Nanogold, 1:200 dilution, Nanoprobes, Stony Brook, N.Y.), and then treated with a silver enhancement kit (HQ Silver, Nanoprobes). The sections were subsequently embedded in epoxy resin and sectioned for transmission electron microscopy. As a reference, tissue blocks of glutaraldehyde-fixed and osmium tetroxide-postfixed pontine base from the same case were examined by conventional electron microscopy.

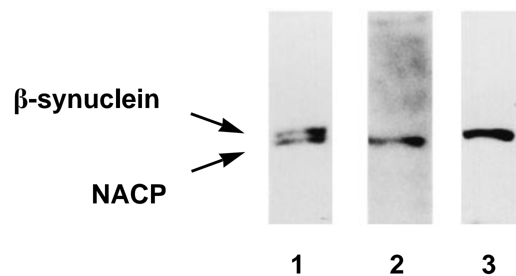

Fig. 1 Characterization of MDV2 (an antibody raised against the N-terminal sequence of the non-amyloid β component of Alzheimer's disease amyloid precursor protein, NACP), EQV1 (an antibody raised against the N-terminal sequence of the non-amyloid β component, NAC), and REE1 (an antibody raised against the residue 86–100 of β-synuclein) antibodies on immunoblots of the rat brain cytosolic fraction. EQV1 (*lane 2*) specifically reacts with NACP, migrating at ca. 19 kDa, and REE1 (*lane 3*) specifically recognizes β-synuclein, which ran with a slightly slower mobility. MDV2 (*lane 1*) reacts with both NACP and β-synuclein

Results

Specificities of the primary antibodies

In immunoblot analysis of the rat brain cytosolic fraction, EQV1 detected a band of approxomately 19 kDa (Fig. 1, lane 2), consistent with our previous observation [15]. REE1 detected β-synuclein that ran with a slightly slower mobility than NACP in accord with a previous report [5], but REE1 did not immunoreact with NACP (Fig. 1, lane 3). MDV2 reacted with both NACP and β-synuclein (Fig. 1, lane 1).

The specificities of the antibodies, MDV2, EQV1, and REE1 were tested on the control human hippocampal sections. Each of the antibodies immunolabeled fine or coarse granular structures only in the gray matter that is expected to includes synaptic terminals. Immunoreactivity was not present in axonal tracts such as the white matter or fornix. In the subiculum, intense granular immunolabeling was demonstrated in the neuropil, while neuronal somata, apical dendrites, and perivascular areas were unstained (Fig. 2A, C, E). Granules occasionally appeared in a row along the outer surface of neuronal somata or dendrites. These immunohistochemical profiles were consistent with the synaptic staining patterns. MDV2, EQV1, and REE1 produced essentially similar staining patterns in the hippocampus. These staining patterns were abolished on the sections in which the primary antibodies were pre-absorbed with their specific peptides (Fig. 2B, D, F). Immunoreaction was not detected when sections were incubated with each of pre-immune serum (not illustrated).

Histological and immunohistochemical studies

Bodian's silver impregnation preparations revealed both NCIs and GCIs in the pontine nuclei of all the cases of

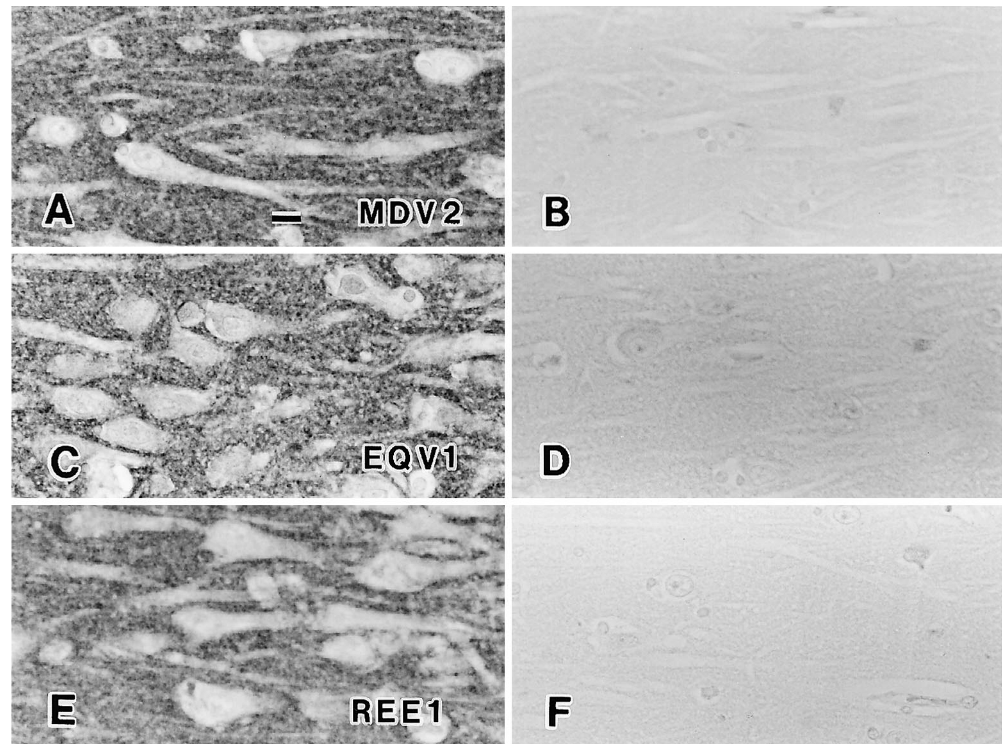

Fig. 2A–F Immunohistochemical detection and absorption experiment of MDV2, EQV1, and REE1 antibodies in the subiculum of the control human hippocampal sections. Peroxidase-DAB staining without counter staining. Each of MDV2 (**A**), EQV1 (**C**), and REE1 (**E**) stains fine or coarse granular structures only in the neuropil. Absorption with the corresponding peptide eliminates the staining in MDV2 (**B**), EQV1 (**D**), and REE1 (**F**) (*DAB* diaminobenzidine). *Bar* 10 µm

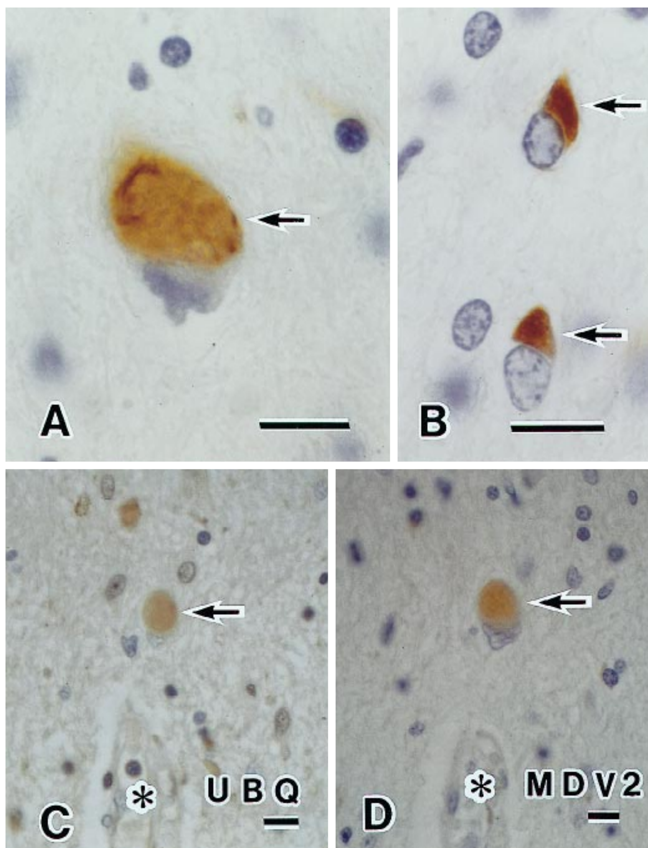

Fig. 3A–D Immunohistochemistry of NCIs and GCIs in the pontine nucleus in MSA. **A** The MDV2 antibody immunolabels the NCIs moderately (*arrow*). **B** The MDV2 antibody stains GCIs moderately to strongly (*arrows*). **C**, **D** Simultaneous immunohistochemical colocalization of anti-ubiquitin reactivity (**C**) and MDV2 reactivity (**D**) in the same NCI (*arrow*) in the vicinity of the arteriole (*asterisk*) is demonstrated by the serial section study (*NCI* neuronal cytoplasmic inclusion, *GCI* glial cytoplasmic inclusion, *MSA* multiple system atrophy). **A–D** Peroxidase-DAB/hematoxylin; *bar* 10 µm

MSA, but none in the pontine nuclei of the control brains. Lewy bodies in the locus coeruleus of Parkinson's disease brains reacted positively with anti-ubiquitin, MDV2, and EQV1, but negatively with REE1. In MSA, the anti-ubiquitin antibody stained NCIs and GCIs moderately and there was slight background staining. Similarly, the MDV2 and EQV1 antibodies immunostained NCIs moderately and GCIs moderately to strongly in all MSA brains without appreciable background staining. NCIs appeared as irregularly demarcated, unevenly stained spherical structures in the neuronal perikarya (Fig. 3A). The GCIs were identified as bundles of filaments having fusiform, crescent-shaped, or perinuclear structures in the perikaryal cytoplasm of small cells with round nuclei in the pontine base (Fig. 3B). The REE1 antibody did not immunostain either of the inclusions. Serial section analysis demonstrated that the ubiquitin-immunoreactive filamentous elements in the neuronal perikarya (NCIs, Fig. 3C) were immunoreactive for MDV2 (Fig. 3D) in adjacent sections. Simultaneous colocalization of epitopes in NCIs was demonstrated by anti-ubiquitin and EQV1, as well as MDV2 and EQV1. Likewise, colocalization of immunoreactivity for anti-ubiquitin, MDV2, and EQV1 was revealed in the GCIs.

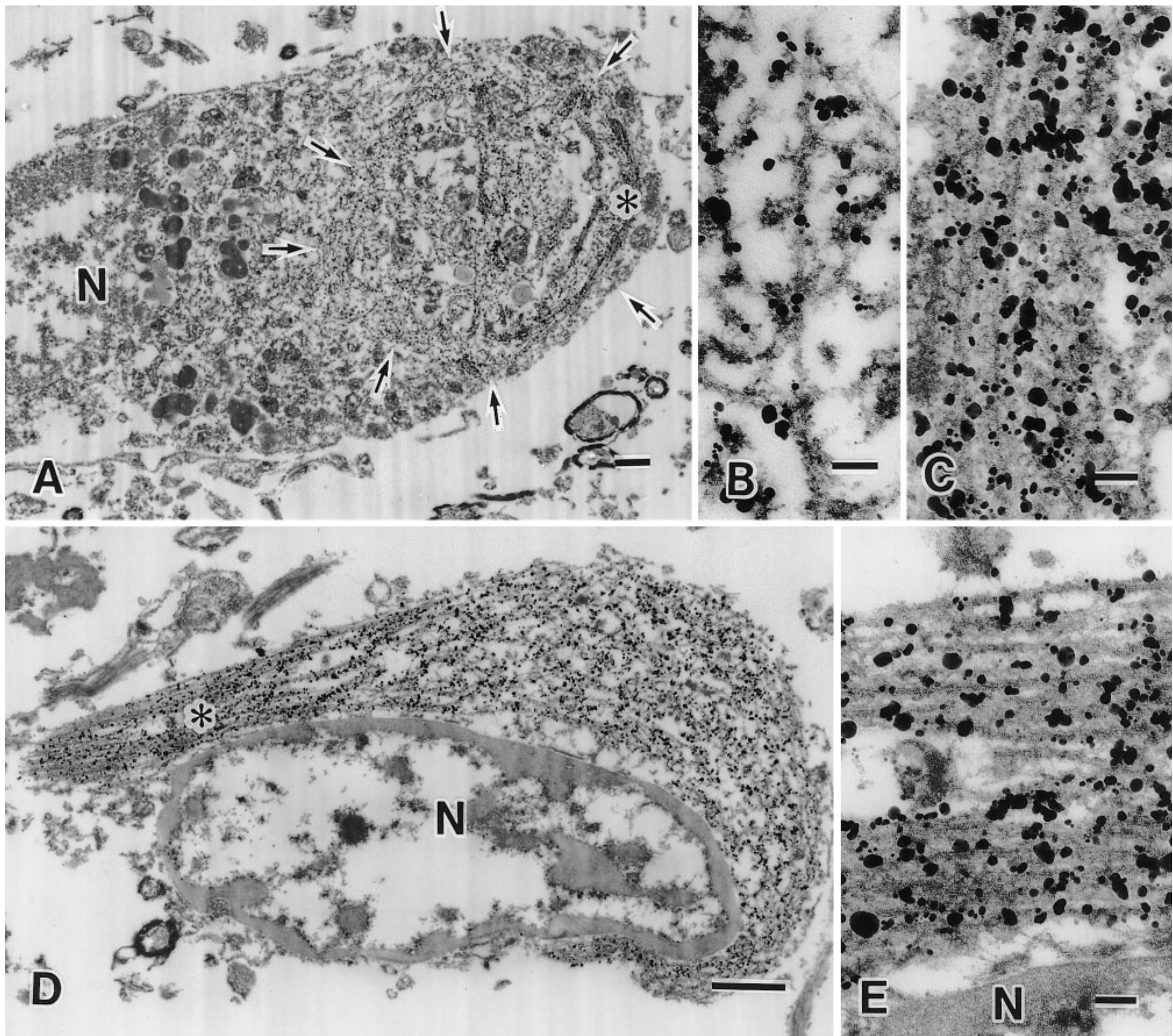

Fig. 4A–E Immunogold-labeled and silver-intensified pre-embedding immunoelectron micrographs using MDV2 antibody. The pontine nucleus in MSA. **A** Overview of an NCI. The inclusion is comprised of filaments that form small bundles or are arranged at random, as outlined by the *arrows*. Silver particles selectively label the filaments but not the cell organelles in the cytoplasm or the nucleus. **B** High-power view of an isolated filament of an NCI showing silver particle deposition along 15- to 30-nm-thick filaments with an irregular outer surface. **C** High-power view of the area indicated by the *asterisk* in **A**, showing dense silver particle deposition on a bundle of filaments. **D** Overview of a GCI showing selective labeling of filaments by silver particles. **E** High-power view of the area indicated by the *asterisk* in **D**, showing dense silver labeling on 15- to 30-nm-thick filaments with an irregular outer surface (*N* nucleus). *Bars* **A**, **D** 1 μm; **B**, **C**, **E** 100 nm

In control brains, no positive immunoreactions were detected in the perikarya of neurons or glial cells by MDV2, EQV1, or REE1, except in the Parkinson's disease brains.

Pre-embedding immunoelectron microscopic study

Immunocytochemistry on immunogold-labeled silver-intensified sections with MDV2 revealed that silver particles, the final immunoreaction products selectively precipitated on gold particles, laid on the 15- to 30-nm-thick filamentous components of NCIs (Fig. 4A–C). The filaments were irregular on their outer surface and were arranged at random (Fig. 4B) or occurred in small bundles (Fig. 4C) in the perikarya. The antibody did not immunolabel cell organellae, nuclei, 10-nm-thick neurofilaments, or the surrounding neuropil. In perikaryal GCIs, 15- to 30-nm-thick filaments with an irregular outer surface were coated with silver granules (Fig. 4D, E). The diameter of the filaments of both the NCIs and the GCIs measured 15–26 nm in conventional electron micrographs. Fairly good structural preservation in our immunocytochemical specimens allowed us to identify the immunolabeled cells as NCIs and GCIs, by comparison with those examined by conventional electron microscopy.

Discussion

In this study, we employed three antibodies, MDV2, EQV1, and REE1, against peptides corresponding to certain residues of NACP/α-synuclein, NAC, and β-synuclein, respectively. Since there is close homology in amino acid alignment in the N-terminal domain of synuclein family proteins, MDV2 cross-reacts with β-synuclein. Thus, MDV2 alone cannot distinguish NACP from other synucleins. EQV1 recognizes the N-terminal region of NAC and, therefore, also recognizes the NAC domain of NACP, which was demonstrated by our current blotting study and also reported previously [3]. EQV1 does not react with β-synuclein, since β-synuclein does not have the NAC sequence. We consider the positive immunoreaction with both MDV2 and EQV1 to be specific to NACP. We also used REE1 to detect β-synuclein. Taking the characteristics of each antibody into consideration, we will refer the MDV2-positive, EQV1-positive, REE1-negative pattern as "NACP immunoreactivity" in the following discussion. Lewy bodies in the control brains exhibited a similar immunohistochemical pattern, in good agreement with the original description by Spillantini et al. [14]. Lewy bodies and Lewy neurites are reported to be specifically stained with anti-α-synuclein sera (PER1 and PER2) but not with anti-β-synuclein serum (PER3) [14].

Our immunohistochemical study using serial sections revealed that the epitopes of both MDV2 and EQV1 were colocalized in the same inclusions and in a similar manner in NCIs and GCIs, corroborating the previous observation that EQV1 recognizes the NAC domain in NACP [3]. The negative immunoreaction of these pathological structures for REE1 indicates that β-synuclein is not associated with them. Since the overall immunohistochemical profile of MDV2-positive, EQV1-positive, REE1-negative pattern is observed in NCIs and GCIs, these MSA inclusions possess NACP immunoreactivity, an immunocytochemical profile similar to that of Lewy bodies.

So far as we are aware, the synuclein family members have only been reported to be associated with neuronal membranes in the brain [4, 7]. However, our previous Northern blot analyses revealed that expression of the NACP gene was not restricted to the brain, but was also found in significant amounts in several non-neuronal cells in certain extracerebral tissues [15, 16]. Thus, it is possible that oligodendroglial cells also produce NACP. It remains to be seen whether glial cells express NACP at undetectable levels physiologically or begin to express it under certain pathological conditions.

It is widely accepted that granule-associated filaments are the cardinal ultrastructural constituents of both NCIs [1, 6] and GCIs [10–12] in MSA, although the diameter of the filaments described has been inconsistent, presumably due to differences in tissue preservation and errors in measurement. In our previous reports, 18- to 28-nm-thick granule-associated filaments were the primary components of NCIs [1], and granule-associated filaments of about 25 nm in diameter were the predominate constituents of GCIs [10]. The present gold-based immunocytochemistry using MDV2 antibody showed that the antibody selectively labeled the granule-associated filaments in both NCIs and GCIs. It is noteworthy that certain epitopes of NACP are associated with abnormal filaments in MSA. We postulate that NACP may play a role in the filament-aggregating processes.

The common core protein of the filaments has not yet been identified in spite of previous immunohistochemical studies of MSA. We consider NACP to be a promising candidate protein in MSA. It is still unclear whether NACP is the primary damaged protein in MSA. Further investigation is urgently needed to clarify this issue.

In conclusion, MSA is the third pathological manifestation of NACP-associated neuronal degeneration after Alzheimer's disease and Parkinson's disease/dementia with Lewy bodies.

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