American Journal of Pathology, Vol. 152, No. 2, February 1998

Short Communication

Abnormal Accumulation of NACP/α-Synuclein in Neurodegenerative Disorders

Ayako Takeda,*† Margaret Mallory,* Mary Sundsmo,* William Honer,‡ Lawrence Hansen,*§ and Eliezer Masliah*§

From the Departments of Neurosciences and Pathology§ University of California, San Diego, School of Medicine La Jolla, California; the Department of Psychiatry,‡ University of British Columbia, Vancouver, Canada; and the Department of Psychiatry,† Yokohama City University, School of Medicine, Kanazawa-ku, Yokohama, Japan*

The precursor of the non-Aβ component of Alzheimer's disease amyloid (NACP) (also known as α-synuclein) is a presynaptic terminal molecule that accumulates in the plaques of Alzheimer's disease. Recent studies have shown that a mutation in NACP is associated with familial Parkinson's disease, and that Lewy bodies are immunoreactive with antibodies against this molecule. To clarify the patterns of accumulation and differences in abnormal compartmentalization, we studied NACP immunoreactivity using double immunolabeling and laser scanning confocal microscopy in the cortex of patients with various neurodegenerative disorders. In Lewy body variant of Alzheimer's disease, diffuse Lewy body disease, and Parkinson's disease, NACP was found to immunolabel cortical Lewy bodies, abnormal neurites, and dystrophic neurites in the plaques. Double-labeling studies showed that all three of these neuropathological structures also contained ubiquitin, synaptophysin, and neurofilament (but not tau) immunoreactivity. In contrast, neurofibrillary tangles, neuropil threads, Pick bodies, ballooned neurons, and glial tangles (most of which were tau positive) were NACP negative. These results support the view that NACP specifically accumulates in diseases related to Lewy bodies such as Lewy body variant of Alzheimer's disease, diffuse Lewy body disease, and Parkinson's disease and suggests a role for this synaptic protein in the pathogenesis of neurodegeneration. *(Am J Pathol 1998, 152:367–372)*

Neurodegenerative disorders are characterized by damage to selective neuronal populations[1] that could be followed or preceded by synaptic injury.[2] The mechanisms triggering cell death and synaptic damage in these disorders might be related to the gain of a toxic property and/or loss of neuroprotective capacity of a specific neuronal cell protein.[3] Many of these neuronal molecules play an important role in the maintenance and functioning of the synaptic apparatus.[4–6] Therefore, specific mutations and other alterations of synaptic proteins might result in particular neurodegenerative diseases. Remarkably and in support of this concept, a recent paper by Polymeropoulos et al[7] showed that a mutation in the α-synuclein gene is associated with familial Parkinson's disease. Synuclein was originally cloned and identified as a synaptic protein in torpedo[8] and later on the bovine (PRP-14),[9] rat,[10] human (precursor of the non-Aβ component of Alzheimer's disease (AD) amyloid (NACP)),[11] and song bird (synelfin).[12] Subsequent studies showed that there are two types of synucleins (α and β)[13] and that NACP is homologous to α-synuclein.[14] NACP was originally found in human brains from patients with AD as a 19-kd protein precursor of non-Aβ component of AD amyloid, a 35-amino acid product found in amyloid plaques in AD.[11,15] Furthermore, NACP has been recently found to abnormally accumulate in synaptic terminals and axons in AD plaques[15] as well as in the Lewy bodies (LBs) in Parkinson's disease.[16] This suggests that accumulation of NACP and/or other synaptic proteins with amyloidogenic potential might play a role in neurodegeneration. To better understand the patterns of NACP accumulation in neurodegenerative disorders, we studied (by confocal microscopy and double-labeling techniques) the differences in abnormal compartmentalization of NACP in Lewy body variant of AD (LBV), diffuse Lewy body disease, Parkinson's disease, AD, Pick's disease, corticobasal degeneration, and progressive supranuclear palsy.

Supported in part by NIH Grants AG5131 and AG10689 (E. Masliah) and by the Medical Research Council of Canada (W. Honer).

Accepted for publication November 15, 1997.

Address reprint requests to Dr. E. Masliah, Department of Neurosciences, University of California, San Diego, La Jolla, CA 92093-0624. E-mail: emasliah@ucsd.edu.

Table 1. Summary of Clinical and Pathological Features

Diagnosis	Number of cases	Age (yrs ± SEM)	Disease duration (yrs ± SEM)	Brain weight (grams ± SEM)	History of dementia
Control	3	76.3 ± 3.3	0 ± 0	1195 ± 79	no
Parkinson's disease	3	80.7 ± 4.5	4.7 ± 1.5	1284 ± 26	no
Diffuse Lewy body disease	3	80.3 ± 3.5	11.7 ± 2.0	1213 ± 98	yes
Lewy body variant of Alzheimer's disease	6	76.2 ± 5.1	8.2 ± 2.0	1156 ± 81	yes
Alzheimer's disease	3	76.3 ± 3.7	14.3 ± 1.7	940 ± 94	yes
Progressive supranuclear palsy	3	75.0 ± 5.7	4.0 ± 1.0	1070 ± 44	yes
Pick's disease	3	73.7 ± 4.4	10.0 ± 0.6	1027 ± 26	yes
Corticobasal degeneration	2	62.0 ± 8.0	9.0 ± 1.0	972 ± 52	yes

Materials and Methods

Samples

Twenty-six autopsy cases from the Alzheimer's Disease Research Center at the University of California, San Diego, were included for this study. Briefly, as previously described,[17] LBV cases (n = 6) were confirmed by the presence of abundant plaques, occasional neurofibrillary tangles, and cortical LBs; clinically they presented with dementia and subsequently developed parkinsonism. Diffuse Lewy body disease cases (n = 3) had cortical and subcortical LBs but did not have either plaques or neurofibrillary tangles; clinically they presented with mild dementia and parkinsonian features. Parkinson's disease cases (n = 3) had subcortical LBs and few cortical LBs but did not have either plaques or neurofibrillary tangles; clinically they presented with parkinsonism but without dementia. Alzheimer's disease cases (n = 3) had numerous plaques and neurofibrillary tangles and presented clinically with dementia but no parkinsonian features. Pick's disease cases (n = 3) had lobar frontotemporal atrophy with numerous Pick bodies. Progressive supranuclear palsy cases (n = 3) were characterized by nigral degeneration and the presence of neurofibrillary tangles in the basal ganglia and brain stem and had no or few ballooned neurons. Cases of corticobasal degeneration (n = 2) had lateralized cortical and basal ganglial involvement with many ballooned neurons in the cerebral cortex. The remaining three cases were clinically and histopathologically free of neurological diseases and were considered controls (Table 1). For this study, tissues from superior temporal cortex, middle frontal cortex, and hippocampus were fixed in 2% buffered-paraformaldehyde for 72 hours at 4°C and sectioned at 40 μm with the vibratome (Leica Inc, Deerfield, IL). Paraffin sections from cortical and subcortical regions of all 24 cases were stained with hematoxylin and eosin, thioflavine S, and cresyl violet for routine histopathological examination.

Antibodies

Immunofluorescent studies were done with the affinity-purified rabbit polyclonal antibodies against the C-terminal NACP (aa131–140) prepared by inoculating rabbits with a synthetic peptide as previously described.[14] In addition, mouse monoclonal antibodies against ubiquitin (MAB1510, Chemicon, Temecula, CA), synaptophysin (SP15),[18,19] phosphorylated tau (AT8, BioSource International, Camarillo, CA), and neurofilament (SMI312, Sternberger Monoclonals, Baltimore, MD)[20] were used in combination with anti-NACP for double-immunolabeling studies.

Double Immunolabeling and Laser Scanning Confocal Microscopy

Briefly and as described previously,[15] 40-μm thick vibratome sections were double-immunolabeled with the following combinations of monoclonal/polyclonal antibodies: 1) ubiquitin (MAB1510) (1:200)/NACP (1:20); 2) synaptophysin (SP15) (1: 50)/NACP (1:20); and 3) tau (AT8) (1:100)/NACP (1:20), neurofilament (SMI312) (1: 2000)/NACP (1:20). After overnight incubation at 4°C, sections were incubated with Texas red-conjugated horse anti-mouse antibody (1:75, Vector Laboratories, Burlingame, CA) and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:50, Vector Laboratories). The double-immunolabeled sections were mounted on SuperFrost Plus slides (Fisher Scientific, Temecula, CA) and analyzed with the Bio-Rad (Richmond, CA) MRC1024 laser scanning confocal microscope.[15]

Results

Patterns of NACP Immunoreactivity in Disorders with LBs

Consistent with previous studies,[14,15] NACP strongly immunostained presynaptic terminals in the neuropil of the cortex in all LBV, diffuse Lewy body disease, and Parkinson's disease cases (Figure 1A). In LBV as well as in diffuse Lewy body disease and Parkinson's disease, NACP strongly immunolabeled LBs in the neurons of the deeper layers of the cortex (Figures 1, A, D, and G and 2A). Furthermore, the C-terminal NACP antibody strongly immunoreacted with virtually all of the LBs in substantia nigra in Parkinson's disease, diffuse Lewy body disease, and LBV cases (Figure 2B). Although the cortical LBs showed a diffuse NACP labeling, the subcortical LBs displayed stronger NACP immunoreactivity in the periphery than in their central portion (Figure 2). Double immunolabeling studies confirmed that virtually all of the ubiquitin-positive LBs were also NACP-positive (Figure 1A). Furthermore, NACP-positive LBs were also synaptophysin- (Figure 1D) and neurofilament-positive

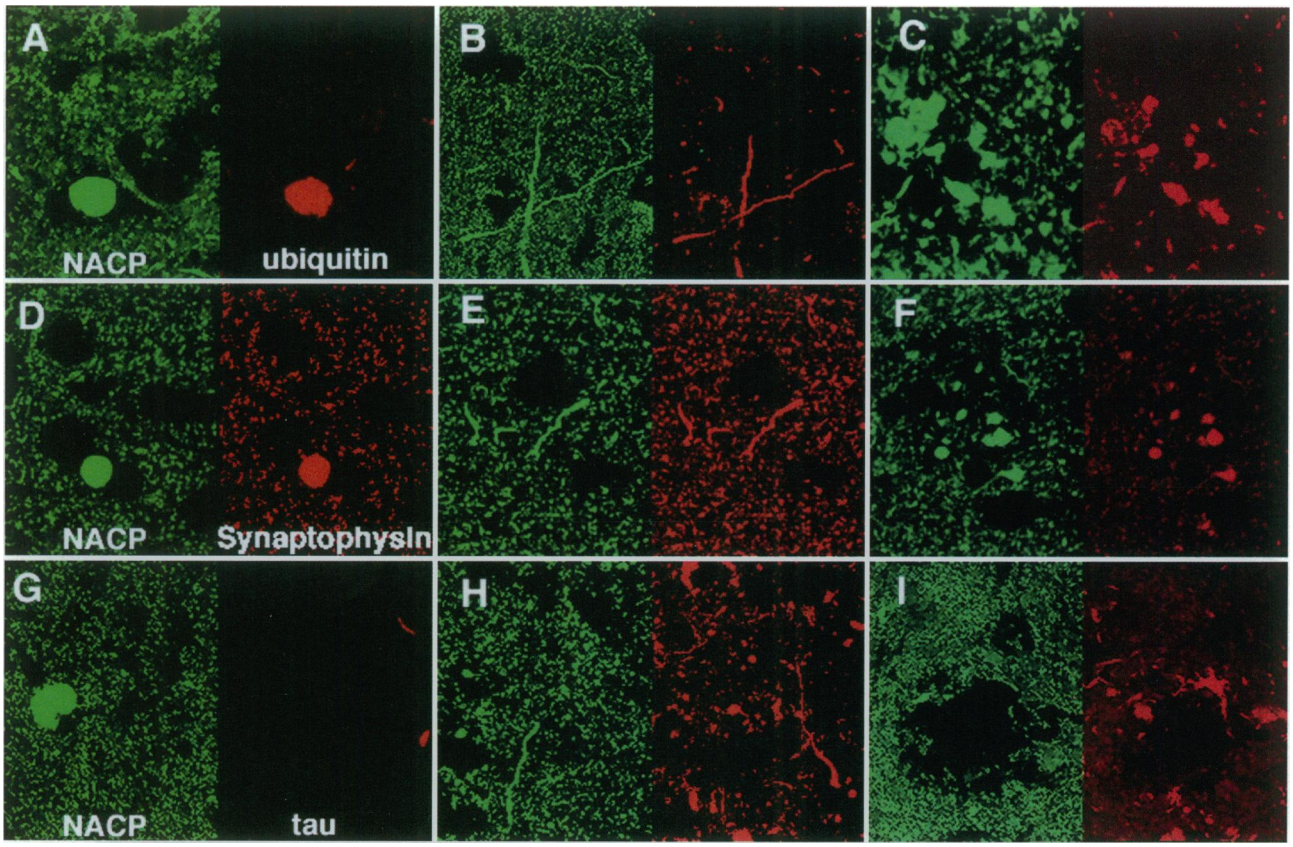

Figure 1. Patterns of NACP immunoreactivity in diseases with LBs. Sections were double immunolabeled and imaged with the laser confocal microscope. For each panel, the image to the left corresponds to NACP detected in the fluorescein isothiocyanate channel (**green**), and to the right, ubiquitin, synaptophysin, or tau detected in the Texas red channel (**red**). NACP-immunoreactive LBs were ubiquitin (**A**) and synaptophysin (**D**) positive but tau negative (**G**). NACP immunoreactive neurites were also ubiquitin (**B**) and synaptophysin (**E**) positive but tau negative (**H**). Furthermore, NACP-immunoreactive dystrophic neurites in the plaques were ubiquitin (**C**) and synaptophysin (**F**) positive but tau negative (**I**).

(not shown). Interestingly, in addition to LBs, NACP also strongly immunostained fine and tortuous neurites in the superficial and deeper cortical layers in cases with LBs (Figure 1, B, E, and H). Double immunolabeling studies showed that these NACP-positive neurites were also ubiquitin- (Figure 1B) and synaptophysin-positive (Figure 1E), but tau negative (Figure 1H). NACP, ubiquitin, and synaptophysin were also colocalized in dystrophic neuritic plaques in LBV cases (Figure 1, C and F).

Patterns of NACP Immunoreactivity in Other Neurodegenerative Diseases

In Pick's disease, tau strongly immunolabeled Pick bodies both in hippocampus (Figure 3A) and in superior temporal cortex (Figure 3B). Double immunolabeling studies showed that τ-positive Pick bodies and Pick cells were NACP negative (Figure 3, A and B) except for occasional Pick bodies in the hippocampus, which were faintly immunostained (not shown) in one of three cases analyzed. No NACP positive neurites were observed in Pick's disease (Figure 3B). In AD cases, NACP immunostained presynaptic terminals in the neuropil as well as some dystrophic neurites in the plaques; however, the neurofibrillary tangles were negative (Figure 3, C and D). Furthermore, τ-positive neuropil threads were also NACP negative (Figure 3C). Ballooned neurons in corticobasal degeneration (Figure 3E) and neurofibrillary tangles in progressive supranuclear palsy (Figure 3G) were also NACP negative. Glial tangles such as astrocytic plaques (Figure 3F), tuft-shaped astrocytes (Figure 3H), thorn-shaped astrocytes (not shown), glial coiled bodies (not shown), and argyrophilic threads were as well tau positive but NACP negative (not shown). In all of these cases, NACP strongly immunolabeled the synaptic terminals in the neuropil (Figure 3).

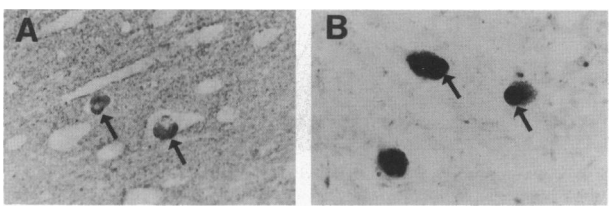

Figure 2. NACP immunoreactivity in LBs. **A:** In the temporal cortex of the case with diffuse Lewy body disease, NACP immunoreactivity was diffusely distributed in the LB. In contrast, in the substantia nigra (**B**), NACP strongly labeled the periphery of the LB, and it was less intense in the center.

Discussion

The present study showed that NACP consistently immunolabeled the cortical LBs in LB-related diseases such as

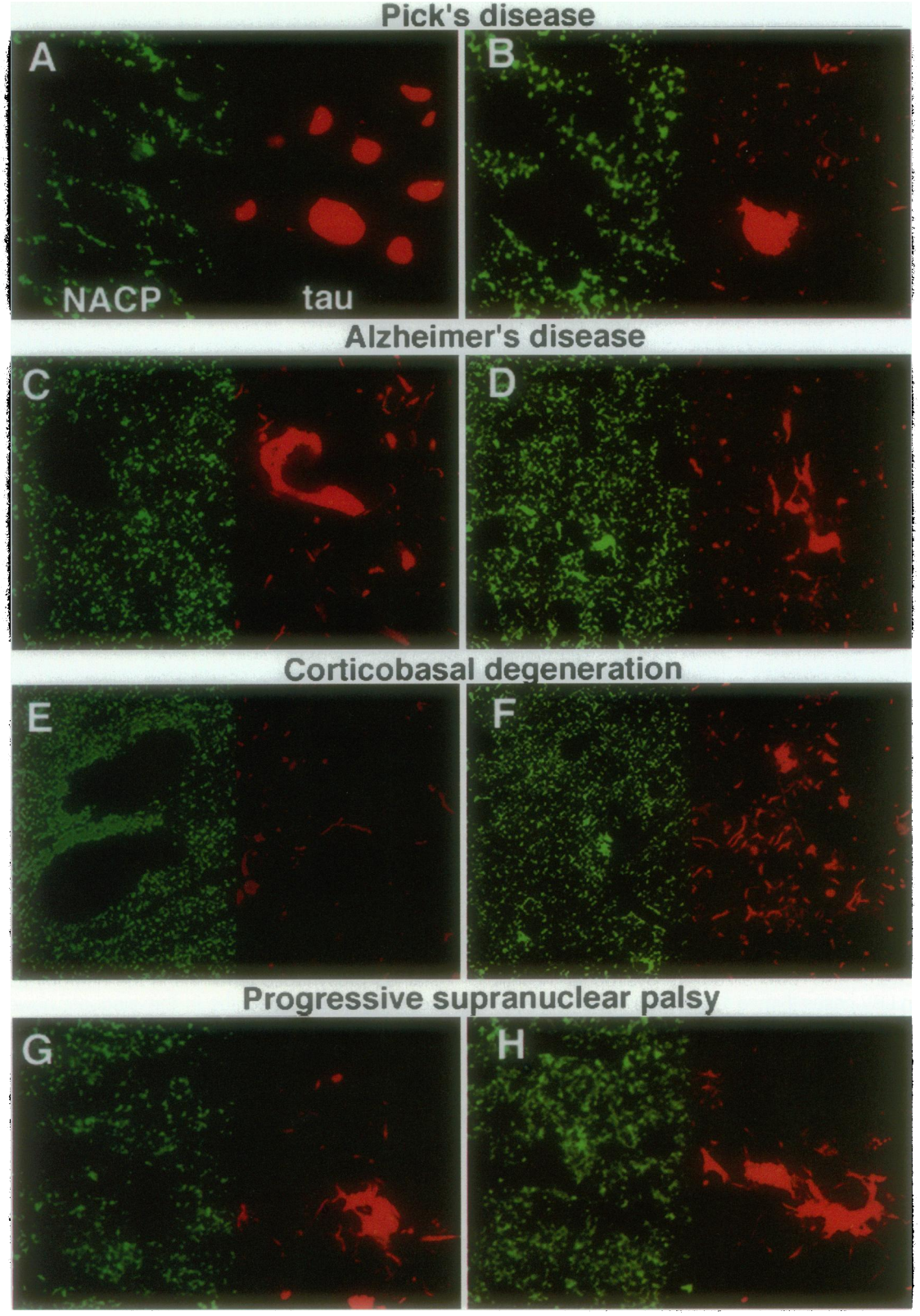

Figure 3. Patterns of NACP immunoreactivity in other neurodegenerative diseases. Sections were double immunolabeled and imaged with the laser confocal microscope. For each panel, the image to the left corresponds to NACP detected in the fluorescein isothiocyanate channel (**green**), and to the right, tau was detected in the Texas red channel (**red**). τ-immunoreactive Pick bodies in the hippocampus (**A**) and temporal cortex (**B**) were NACP negative. τ-immunoreactive neurofibrillary tangles (**C**) and dystrophic neurites in the plaque were also NACP negative in AD. τ-negative-ballooned neurons (**E**) were NACP negative, and a τ-positive astrocytic plaque (**F**) was NACP negative in corticobasal degeneration. Furthermore, τ-positive neurofibrillary tangles and tufts-shaped astrocytes were NACP negative in progressive supranuclear palsy (**G**, **H**).

LBV, diffuse Lewy body disease and Parkinson's disease but did not label the pathological intracellular inclusions in other neurodegenerative disorders. In fact, most of the ubiquitin positive LBs were NACP positive, indicating that this synaptic protein is widely involved in the process of LB formation. These findings are consistent with a recent report that showed strong labeling of cortical and subcortical LBs in Parkinson's disease and diffuse Lewy body disease with antibodies against the N- and C-terminal regions of α-synuclein (referred as NACP in this manuscript) but not with antibodies against β-synuclein.[16] The precise role of NACP in the LB formation or in the neurodegenerative process in disorders with LBs is unclear. Possible explanations that might be considered are: 1) NACP actively accumulates and might even form a center where the LBs are formed and 2) NACP accumulation in LBs might be related to abnormal transport of synaptic proteins in LB-bearing neurons and to entrapment of synaptic vesicles in them. Supporting the first possibility, previous studies have shown that NACP is a natively unfolded protein[21,22] with highly hydrophobic regions[11] that aggregate and form amyloid-like fibrills.[23,24] Furthermore, NACP binds amyloid β-protein and stimulates β-amyloid aggregation.[25,26] This suggests that, in the LBs, NACP might self-aggregate or form complexes with other components of the LB, including amyloid precursor protein, which has been also shown to be present in the LBs.[27] As to the second possibility, the present study showed that NACP is colocalized with other synaptic proteins (including synaptophysin) in LBs. In fact, previous studies have shown that LBs not only contain synaptophysin but also chromogranin A, which is a major soluble protein in dense core secretary vesicles.[28] Furthermore, detailed ultrastructural examination has confirmed that synaptic vesicles are present in LBs.[28] Future biochemical and immunoelectron microscopic studies will be necessary to better understand the subcellular distribution of NACP in the LB-bearing neurons.

This study also showed that NACP as well as other synaptic proteins are not only abnormally compartmentalized in the LBs but also in ubiquitin-positive neurites. These NACP-immunoreactive abnormal neuritic processes were exclusively present in LBV, diffuse Lewy body disease, and Parkinson's disease but not in other neurodegenerative disorders. NACP-positive neurites had the same immunocytochemical properties as LBs, which were synaptophysin- and neurofilament-positive and τ-negative. Ubiquitin-positive neurites were first noted in the CA2/3 region in diffuse Lewy body disease to distinguish this disorder from AD with τ-positive neurites.[29] Furthermore, ubiquitin-positive neurites in the hippocampus have been shown to coexist with cortical LBs[30] and have been found in the brainstem in Parkinson's disease.[31] Thus, the formation of ubiquitin-positive neurites might be closely associated with LBs, and therefore, they might share a similar pathogenic substrate.

Other τ-positive neuronal structures such as neurofibrillary tangles, neuropil threads, ballooned neurons in Pick's disease, corticobasal degeneration, and glial tangles were not detected with NACP except for a few Pick bodies. Occasional Pick bodies in hippocampus but not neocortex were faintly positive with NACP. Pick bodies mainly contain hyperphosphorylated τ, but previous studies have shown that they are also immunoreactive with antibodies against Chromogranin A and clathrin,[32] which are proteins that concentrate in synaptic terminals. Therefore, abnormal accumulation of NACP may be indicative of extensive axonal pathology. Supporting this possibility, the present study as well as previous studies showed that NACP abnormally accumulates in synaptic terminals[15,33] in AD and in the round dystrophic neurites in the plaques of AD and LBV.[15,34] Taken together, these findings suggest that both abnormal transport of synaptic proteins and abnormal compartmentalization of NACP might play a role during the progression of LBV.

Acknowledgments

The authors wish to thank Drs. Leon Thal and Robert Katzman for their scientific feedback.

References

1. Hof PR, Morrison JH: The cellular basis of cortical disconnection in Alzheimer disease and related dementing conditions. Alzheimer Disease. Edited by RD Terry, R Katzman, KL Bick. New York, Raven Press, 1994, pp 197–230
2. Masliah E, Mallory M, Hansen L, DeTeresa R, Alford M, Terry R: Synaptic and neuritic alterations during the progression of Alzheimer's disease. Neurosci Lett 1994, 174:67–72
3. Sisodia SS, Price DL: Role of the β-amyloid protein in Alzheimer's disease. FASEB J 1995, 9:366–370
4. Masliah E, Terry R: The role of synaptic proteins in the pathogenesis of disorders of the central nervous system. Brain Pathol 1993, 3:77–85
5. Masliah E, Mallory M, Alford M, DeTeresa R, Iwai A, Saitoh T: Molecular mechanisms of synaptic disconnection in Alzheimer's disease. Connections, Cognition and Alzheimer's Disease. Edited by BT Hyman, C Duyckaerts, Y Christen. Berlin, Springer-Verlag, 1997, pp 121–140
6. Masliah E: Mechanisms of synaptic dysfunction in Alzheimer's disease. Histol Histopathol 1995, 10:509–519
7. Polymeropoulos MH, Lavedant C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL: Mutation in the α-synuclein gene identified in families with Parkinson's disease. Science 1997, 276:2045–2047
8. Maroteaux L, Campanelli JT, Scheller RH: Synuclein: A neuron-specific protein localized to the nucleus and presynaptic nerve terminal. J Neurosci 1988, 8:2804–2815
9. Nakajo S, Tsukada K, Omata K, Nakamura Y, Nakaya K: A new brain-specific 14-kd protein is a phosphoprotein: its complete amino acid sequence and evidence for phosphorylation. Eur J Biochem 1993, 217:1057–1063
10. Maroteaux L, Scheller RH: The rat brain synucleins; family of proteins transiently associated with neuronal membrane. Mol Brain Res 1991, 11:335–343
11. Ueda K, Fukushima H, Masliah E, Xia Y, Iwai A, Otero D, Kondo J, Ihara Y, Saitoh T: Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer's disease. Proc Natl Acad Sci USA 1993, 90:11282–11286
12. George JM, Jin H, Woods WS, Clayton DF: Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. Neuron 1995, 15:361–372
13. Jakes R, Spillantini MG, Goedert M: Identification of two distinct synucleins from human brain. FEBS Lett 1994, 345:27–32

14. Iwai A, Masliah E, Yoshimoto M, De Silva R, Ge N, Kittel A, Saitoh T: The precursor protein of non-Aβ component of Alzheimer's disease amyloid (NACP) is a presynaptic protein of the central nervous system. Neuron 1994, 14:467–475
15. Masliah E, Iwai A, Mallory M, Ueda K, Saitoh T: Altered presynaptic protein NACP is associated with plaque formation and neurodegeneration in Alzheimer's disease. Am J Pathol 1996, 148:201–210
16. Spillantini MG, Schmidt ML, Lee VM-Y, Trojanowski JQ, Jakes R, Goedert M: α-Synuclein in Lewy bodies. Nature 1997, 388:839–840
17. Wakabayashi K, Hansen LA, Vincent I, Mallory M, Masliah E: Neurofibrillary tangles in the dentate granule cells of patients with Alzheimer's disease, Lewy body disease and progressive supranuclear palsy. Acta Neuropathol 1997, 93:7–12
18. Honer WG, Falkai P, Young C, Wang T, Xie J, Bonner J, Hu L, Boulianne GL, Lu Z, Trimble WS: Cingulate cortex synaptic terminal proteins and neural cell adhesion molecule in schizophrenia. Neuroscience 1997, 78:99–110
19. Wakabayashi K, Honer WG, Masliah E: Synapse alterations in the hippocampal-entorhinal formation in Alzheimer's disease with and without Lewy body disease. Brain Res 1994, 667:24–32
20. Masliah E, Mallory M, Hansen L, Alford M, DeTeresa R, Terry R: An antibody against phosphorylated neurofilaments identifies a subset of damaged association axons in Alzheimer's disease. Am J Pathol 1993, 142:871–882
21. Kim J: Evidence that the precursor protein of non-Aβ component of Alzheimer's disease amyloid (NACP) has an extended structure primarily composed of random-coil. Mol Cells 1997, 7:78–83
22. Weinreb PH, Zhen W, Poon AW, Conway KA, Lansbury PTJr: NACP, a protein implicated in Alzheimer's disease, and learning, is natively unfolded. Biochemistry 1996, 35:13709–13715
23. Iwai A, Yoshimoto M, Masliah E, Saitoh T: Non-Aβ component of Alzheimer's disease amyloid (NAC) is amyloidogenic. Biochemistry 1995, 34:10139–10145
24. Han H, Weinreb PH, Lansbury PT Jr: The core Alzheimer's peptide NAC forms amyloid fibrils which seed and are seeded by β-amyloid: is NAC a common trigger or target in neurodegenerative disease? Chem Biol 1995, 2:163–169
25. Jensen PH, Hojrup P, Hager H, Nielsen MS, Jacobsen L, Olesen OF, Gliemann J, Jakes R: Binding of Aβ to α- and β-synucleins: identification of segments in α-synuclein/NAC precursor that bind Aβ and NAC. Biochem J 1997, 323:539–546
26. Yoshimoto M, Iwai A, Kang D, Otero DAC, Xia Y, Saitoh T: NACP, the precursor protein of non-amyloid β/A4 protein (Aβ) component of Alzheimer disease amyloid, binds Aβ, and stimulates Aβ aggregation. Proc Natl Acad Sci USA 1995, 92:9141–9145
27. Arai H, Lee VM-Y, Hill WD, Greenberg BD, Trojanowski JO: Lewy bodies contain β-amyloid precursor proteins of Alzheimer's disease. Brain Res 1992, 585:386–390
28. Nishimura M, Tomimoto H, Suenaga T, Nakamura S, Namba Y, Ikeda K, Akiguchi I, Kimura J: Synaptophysin and chromogranin A immunoreactivities of Lewy bodies in Parkinson's disease brains. Brain Res 1994, 634:339–344
29. Dickson DW, Ruan D, Crystal H, Mark MH, Davies P, Kress Y, Yen S-H: 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 1991, 41:1402–1409
30. Kim H, Gearing M, Mirra SS: Ubiqutin-positive CVA2/3 neurites in hippocampus coexist with cortical Lewy bodies. Neurology 1995, 45:1768–1770
31. Gai WP, Blessing WW, Blumberg PC: Ubiqutin-positive degenerating neurites in the brainstem in Parkinson's disease. Brain 1995, 118: 1447–1459
32. Yasuhara O, Matsuo A, Tooyama I, Kimura H, McGeer EG, McGeer PL: Pick's disease immunohistochemistry: new alteration and Alzheimer's disease comparisons. Acta Neuropathol 1995, 89:322–330
33. Iwai A, Masliah E, Sundsmo MP, DeTeresa R, Mallory M, Salmon DP, Saitoh T: The synaptic protein NACP is abnormally expressed during the progression of Alzheimer's disease. Brain Res 1996, 720:230–234
34. Irizarry MC, Kim TW, McNamara M, Tanzi RE, George JM, Clayton DF, Hyman BT: Characterization of the precursor protein of the non-A β component of senile plaques (NACP) in the human central nervous system. J Neuropathol Exp Neurol 1996, 55:889–895