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Altered Presynaptic Protein NACP Is Associated with Plaque Formation and Neurodegeneration in Alzheimer's Disease

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We have recently identified, in the brain tissue of patients afflicted with Alzheimer's disease (AD), the non-Aβ component of AD amyloid (NAC) as a new constituent of amyloid. NAC is derived from a larger precursor, NACP, a presynaptic protein. To better understand the role of NACP/NAC in the pathogenesis of AD, we used semiquantitative immunoblotting and combined double-immunocytochemistry/laser scanning confocal microscopy to study the concentration and distribution of NACP/NAC in human brain, and compared them to the concentration and distribution of the presynaptic marker synaptophysin and the amyloid marker Aβ. The semiquantitative immunoblotting demonstrated that the NACP concentration is slightly increased in the AD frontal cortex without statistical significance, whereas synaptophysin was reduced in its levels in AD. Consequently, the proportion of NACP/synaptophysin was more than double in the AD frontal cortex as compared with controls. In the AD neocortex, NACP was colocalized with ~80% of the synaptophysin-immunoreactive structures (presumably the presynaptic terminals) and with the dystrophic neuritic component of the plaques. Computer-aided analysis showed that numbers of NACP-immunoreactive structures along synaptophysin-immunoreactive structures were significantly diminished (30 to 40%) in AD. Although the overall numbers of NACP-positive structures were decreased, there was a significant increase in the intensity of NACP-immunoreactivity per structure in AD. This increased intensity of NACP immunoreactivity per structure in AD was not observed with anti-synaptophysin, consistent with immunoblotting-based quantification. Antibodies against NAC immunoreacted with amyloid in 35% of the diffuse plaques and 55% of the mature plaques. Normal aged control brains containing small groups of diffuse plaques were negative with anti-NAC. Double-immunolabeling studies with Aβ antibodies showed that NAC immunoreactivity is more abundant in the center portion of amyloid rather than in the periphery. These studies suggest that there is a connection between metabolism of presynaptic proteins and amyloid formation, and that NAC might follow diffuse Aβ accumulation resulting in the formation of compact amyloid and mature plaques. (Am J Pathol 1996, 148:201–210)

Although several different molecular components have been identified to be associated with Alzheimer's disease (AD) amyloid,[1–7] it is still not clear by which mechanism amyloid deposits are formed. We have recently identified a novel non-Aβ component of AD amyloid (NAC) and cloned the cDNA corresponding to its precursor, NACP.[8] NAC is a 35-amino acid peptide derived from a larger precursor protein, NACP, composed of 140 amino acids.[8] Secondary structure analysis has shown that NAC has a strong tendency to form β-pleated structure, and immunocytochemical analysis has shown that antibodies raised against synthetic peptides corresponding to the NAC sequence recognize Aβ fibrils

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in plaques.[8] On the other hand, recent double-immunocytochemical and immunoelectron microscopic studies have shown that the precursor protein NACP is actually a presynaptic protein loosely associated with synaptic vesicles.[9] The NACP sequence showed 95% homology with the rat synuclein 1[9,10] and some homology with bovine phosphoneuroprotein 14.[9,11] Given that AD is characterized by extensive synapse loss in the association cortex and limbic system[12–16] in addition to the plaque and tangle formation,[17] and that synaptic loss is strongly correlated with cognitive dysfunction,[18,19] alterations in NACP metabolism might be involved in synaptic pathology and in the process of amyloid formation in AD. To better understand the role of NACP/NAC in the pathogenesis of AD, we compared levels and patterns of distribution of NAC and NACP in normal aging and AD brain.

Materials and Methods

Samples

Seventy-five autopsy cases from the Alzheimer Disease Research Center at the University of California, San Diego were included for the present study. Sixty-eight of the cases had clinical histories of AD, confirmed at autopsy. The average age of the AD patients was 80 ± 2 years, with a postmortem delay of 5 ± 2 hours. The other seven cases were clinically and histopathologically free of neurological disease. The average age of this control group was 75 ± 3 years with a postmortem delay of 8 ± 4 hours. For immunocytochemical analysis blocks from the frontal cortex of six AD and five control cases were fixed in 2% buffered paraformaldehyde for 72 hours at 4°C and serially sectioned at 40 μm with the Vibratome 2000.[20] The average age of these selected AD and control cases was 79 ± 2 years with a postmortem delay of 5 ± 2 hours and 77 ± 2 years with a postmortem delay of 9 ± 3 hours, respectively. Paraffin sections from cortical and subcortical regions of all 75 cases were stained with hematoxylin and eosin, thioflavine-S, and cresyl violet for routine histopathological examination and morphometric analysis, as previously described.[21] Homogenates from all 75 cases were prepared from the frontal cortex, fractionated into particulate and soluble fractions, as previously described[22] and used for the dot blot-based quantification of synaptophysin (SYN) and NACP. In the current study, SYN determination was done on only 41 AD cases with the average age of 79 ± 1 year and a postmortem delay of 5 ± 1 hour.

Antibodies

Immunocytochemical and immunochemical studies were done with the affinity-purified rabbit polyclonal antibodies against N-terminal and C-terminal NACP prepared by inoculating rabbits with synthetic peptide, as previously described.[9] Polyclonal antibodies against NAC were prepared with synthetic peptides, as previously described.[8] Further corroboration of the patterns of synaptic immunoreactivity was done with a mouse monoclonal antibody against SYN (SY38, Boehringer Mannheim, Indianapolis, IN).[12,23] In addition, mouse monoclonal antibodies against Aβ (clone 4G8, courtesy of Dr. H. Wisniewski),[24] ubiquitin (Chemicon International, Temecula, CA), neurofilament SMI312 (Sternberger Monoclonals, Baltimore, MD), amyloid precursor protein (APP, clone 22C11, Boehringer Mannheim)[25] and A68 (Alz50, courtesy of Dr. P. Davies)[26] were used in combination with anti-NAC and anti-NACP for double-immunocytochemical analyses.

Immunocytochemistry, Morphometry, and Dot Blot Analysis

As previously described,[8,20,22] vibratome sections from control and AD cases were first washed in phosphate-buffered saline (PBS) (pH 7.4), blocked with 10% normal serum and incubated overnight at 4°C with anti-NAC or NACP (1:100). Serial antibody dilutions (1:10, 1:50, 1:100, 1:500) were used to find the optimal antibody concentration. The free-floating sections were then washed in PBS and incubated with a secondary biotinylated antibody (goat anti-rabbit or horse anti-mouse IgG), followed by avidin D-HRP (ABC Elite, Vector Laboratories, Burlingame, CA) and reacted with diaminobenzidine (0.2 mg/ml) in 50 mmol/L Tris buffer (pH 7.4) with 0.001% hydrogen peroxide. Additional frontal cortex sections, immunostained with anti-NAC, were counterstained with thioflavine-S.[8] To verify the staining specificity, sections were immunostained with NAC antibodies adsorbed with NAC synthetic peptides at a 20-fold concentration.[8]

The NAC-immunoreactive plaques were counted in 10 consecutive fields (0.1 mm^2 each) along the side of the gyrus using a 40× objective and a gridded 10× eyepiece lens.[27] Additional sections were reacted with the anti-SYN antibody, followed by fluorescein isothiocyanate (FITC)-coupled secondary antibody as previously described,[20,28] utilizing for this purpose, combined image analysis/laser scanning confocal microscopy (LSCM).

Further quantification of the immunoreactivity (IR) of anti-NACP and SYN in the human brain was done by dot blot analysis.[29] Homogenates from control and AD frontal cortex were separated into particulate and cytosolic fractions, as previously described.[29] Aliquots assayed by the Lowry method were blotted (2 μg/dot) onto nitrocellulose paper. Blots were incubated at an antibody dilution of 1:1000, followed by ^{125}I protein A. Finally, blots were exposed to PhosphorImager screens and analyzed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Double Immunolabeling and Laser Confocal Imaging

Forty-μm thick vibratome sections from control and AD cortex were double-immunolabeled[20,28] with the following combinations of monoclonal/polyclonal antibodies: 1) SY38/NACP, 2) SMI312/NACP, 3) ubiquitin/NACP, 4) APP/NACP, 5) Alz50/NACP, and 6) Aβ (4G8)/NAC. Sections were then incubated with the goat anti-rabbit biotinylated antibody (1:100, Vector Laboratories) followed by a mixture of FITC-conjugated horse anti-mouse IgG (1:75, Vector Laboratories) and Avidin Cy5 D (1:150) (Jackson ImmunoResearch Labs, West Grove, PA). Cy5 D was used because, as we have shown in previous studies,[30] this fluorochrome emits in the infrared channel. This prevents the artifactual detection of fluorescein bleeding into the red channel as well as lipofuscin autofluorescence.[31] The double-immunolabeled sections were transferred to SuperFrost slides (Fisher Scientific, Tustin, CA) and mounted under glass coverslips with antifading media (Vector Laboratories). The sections were studied with the Bio-Rad (Richmond, CA) MRC-600 LSCM[20] mounted on an Axiovert Zeiss microscope. This system permits the simultaneous analysis of double-immunolabeled samples in the same optical plane. The aperture, contrast, and gain level were initially adjusted manually to obtain images with a pixel intensity within a linear range.[20]

Statistical Analyses

Statistical analyses of the results were performed using the STAT VIEW II software package (Abacus Concepts) running on a Macintosh personal computer. Statistical comparisons among the different groups of the control and AD cases were done with the unpaired, two-tailed, Student's *t*-test (values expressed as mean ± SEM).

Results

The ratio of two presynaptic proteins (NACP to SYN) doubles in AD. Western blot analysis showed that, consistent with previous studies,[9] in the cytosolic fraction of both normal aged and AD cases, the antibody against NACP detected a single band at an apparent molecular weight of 19 kd. Preincubation of NACP antibody with purified NACP specifically abolished the 19 kd band (data not shown). No statistically significant differences were observed between the two groups ($P = 0.185$) in the levels of NACP-IR determined by dot blot analysis, although AD cases showed a trend toward higher levels of NACP-IR (Figure 1A). PhosphorImager analysis of a dot blot immunoreacted with anti-SYN showed a significant decrease in levels of SYN-IR in AD cases (Figure 1B; $P < 0.005$). Consequently ratio of NACP to SYN doubled in AD compared with controls (Figure 1C; $P < 0.05$).

Intensity of NACP (but Not of SYN) IR Is Increased in Individually Immunolabeled Structures

To understand the morphological basis of the imbalance between NACP and SYN, we carried out immunohistochemical experiments. NACP antibodies immunostained the neuropil throughout the human brain with a punctuate pattern similar to that of SYN (Figure 2). Cell bodies were excluded from immunostaining by both antibodies. Preadsorption of anti-NACP antibodies with purified NACP or NACP peptides specifically blocked the immunostaining. Computer-aided analysis of images generated with the LSCM from sections double-immunolabeled with a polyclonal antibody against NACP and a monoclonal antibody against SYN showed that NACP was colocalized with SYN in ~80% of the structures. Consistent with this observation, comparison of immunostaining in 11 AD cases demonstrated that the numbers of SYN-immunoreactive structures were strongly correlated with the numbers of NACP-immunoreactive structures ($r = 0.93$, $P < 0.001$, $n = 11$). Alzheimer's disease cases showed a 40% decrease in the numbers of SYN- and NACP-immunoreactive structures (Figures 2B and 3; $P < 0.001$), although no effect of AD was detected in the size of the immunostained structures by anti-NACP of anti-SYN (data not shown). Analysis of double-immunolabeled neuritic plaques (Figure 2C) showed that ~52% of the dystrophic neurites contained both NACP- and SYN-IR, whereas 27% were only SYN-positive and 21% were only NACP-positive. NACP-IR in the neu-

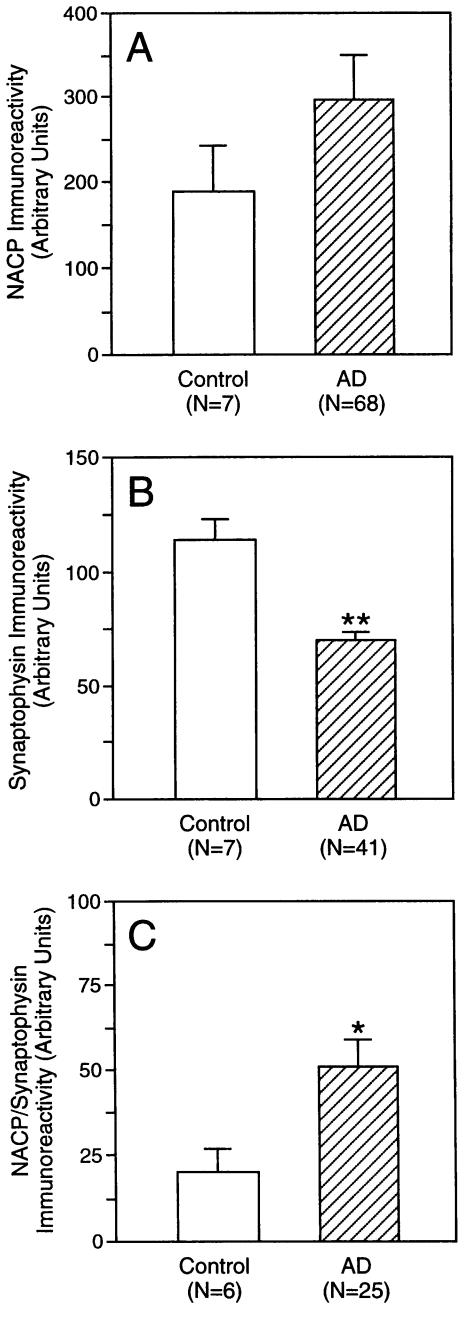

Figure 1. *Immunoquantification of NACP and SYN in brain homogenates.* (**A**) *PhosphorImager analysis of anti-NACP-immunolabeled dot blot showed a trend toward an increase in AD cases compared with controls* ($P = 0.185$, $t = 1.33$) ($n = 68$ *AD and* $n = 7$ *control cases*). (**B**) *PhosphorImager analysis of anti-SYN-immunolabeled dot blot showed a significant decrease in AD cases compared with controls* (**$P < 0.005$, $t = 4.3$) ($n = 41$ *AD and* $n = 7$ *control cases*). (**C**) *Ratio of NACP/SYN is doubled in AD* (*$P < 0.05$, $t = 2.07$) ($n = 25$ *AD and* $n = 6$ *control cases*).

ritic component of plaques was also colocalized with ubiquitin, neurofilament, and APP in the spherical dystrophic neurites, but was not colocalized with Alz50 in the fusiform neurites (data not shown). Although the numbers of NACP-immunoreactive structures per unit area were reduced in AD, there was a significant increase in NACP-IR per structure, compared with normal aged controls (Figure 4A; $P < 0.005$). On the other hand, no significant differences were observed in the intensity of SYN-IR per structure between AD and controls (Figure 4B).

Immunohistochemistry Differentiates NAC from Aβ in Amyloid Deposits

Whereas antibodies against NACP recognized the neuritic component of plaques, antibodies against NAC immunoreacted with the amyloid component of the plaques (Figure 5). Anti-NAC immunostained amyloid in diffuse plaques (Figure 5, A (left) and E), primitive immature plaques (Figure 5, B (left) and E), and mature plaques (Figure 5, C and D, left). Interestingly, anti-NAC immunostained the center portion of compact amyloid more densely than its periphery, whereas anti-Aβ immunostained the amyloid evenly (Figure 5, C and D; compare left and right). Noncompact amyloid was immunostained lightly but evenly by anti-NAC (Figure 5, A and B (compare left and right); and E). Immunohistochemical analysis of normal aged controls with antibodies against NAC showed only occasional IR in the neuronal cell bodies, but no amyloid deposits were detected. Anti-NAC-immunostained and thioflavine-S-counterstained sections from normal aged cases showed that although occasional thioflavine-S-positive diffuse plaques were detected in the neocortex, these structures were anti-NAC-negative (Figure 6A and B). In contrast, AD sections immunoreacted with anti-NAC and counterstained with thioflavine-S showed that many diffuse and mature plaques and amyloid angiopathy were anti-NAC positive (Figure 6, C–F). Computer-aided analysis of images generated with the LSCM from sections double-immunolabeled with a polyclonal antibody against NAC and a monoclonal antibody against Aβ showed that 35% of the diffuse plaques detected by the Aβ antibody contained NAC-IR, whereas 55% of the Aβ-immunoreactive mature plaques contained NAC-IR. Preadsorption of anti-NAC antibodies with NAC synthetic peptide blocked plaque immunostaining.

Discussion

Previous light and electron microscopic studies have demonstrated that NACP is a presynaptic protein homologous to synuclein 1 and PNP-14, associated with the presynaptic terminals.[9–11] Although the physiological role of NACP is still unclear, the syn-

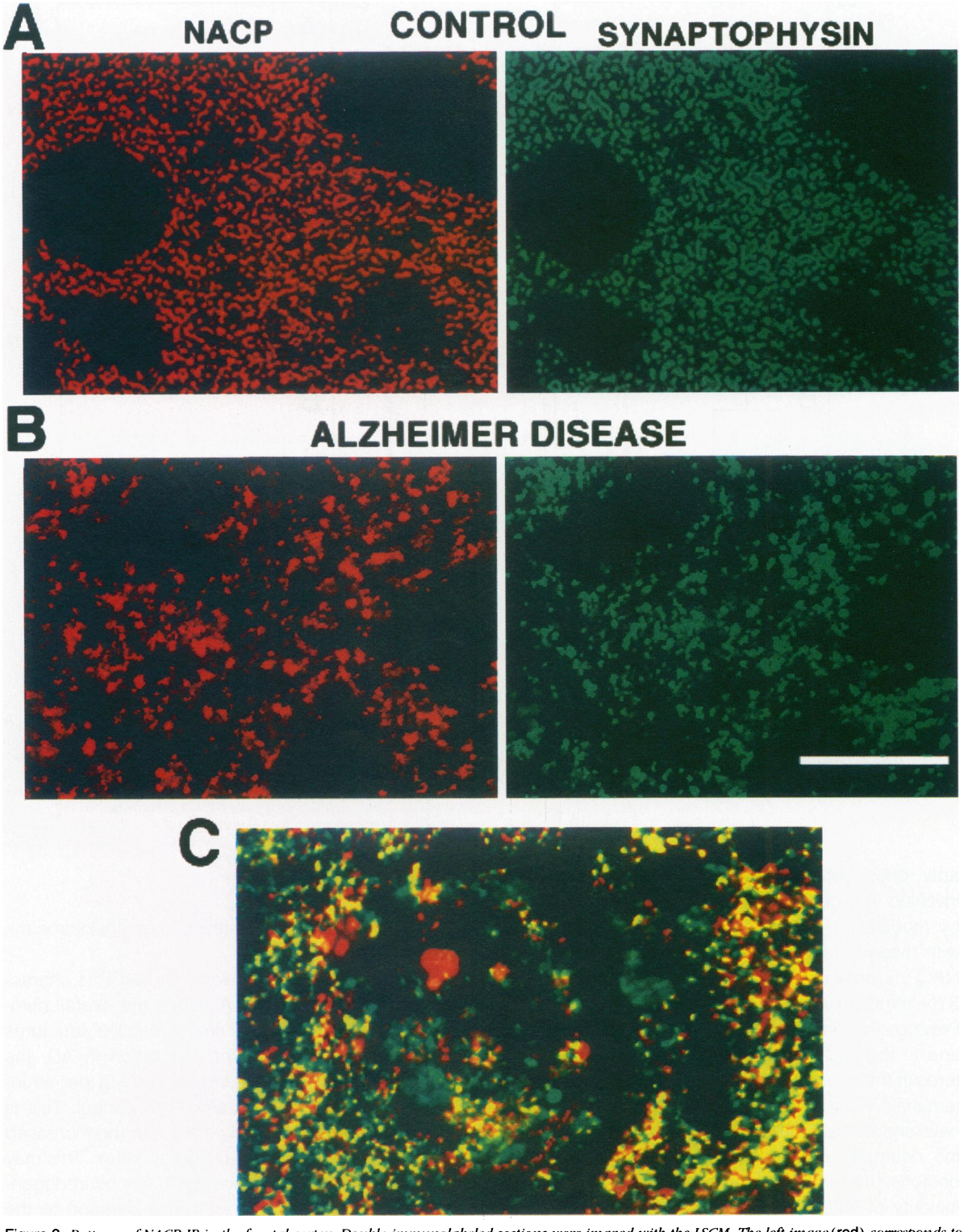

Figure 2. *Patterns of NACP-IR in the frontal cortex. Double-immunolabeled sections were imaged with the LSCM. The left image* (red) *corresponds to punctate structures immunolabeled with anti-NACP, and the image on the right* (green) *corresponds to structures immunolabeled with anti-SYN that presumably correspond to presynaptic boutons. Spaces in black correspond to neuronal cell bodies. In both control* (A) *and AD* (B) *cases, the SYN-immunoreactive structures are colocalized with NACP-immunoreactive elements. Note that NACP- and SYN-immunoreactive structures are more sparse in AD compared with controls.* (C) *Merged image corresponding to a neuritic plaque double-immunolabeled with anti-NACP and anti-SYN. Structures in yellow correspond to dystrophic neurites that contain both SYN and NACP. Neurites in red are uniquely NACP-immunoreactive and neurites in green are uniquely SYN-immunoreactive. Bar = 15 μm. The magnification is the same for all panels.*

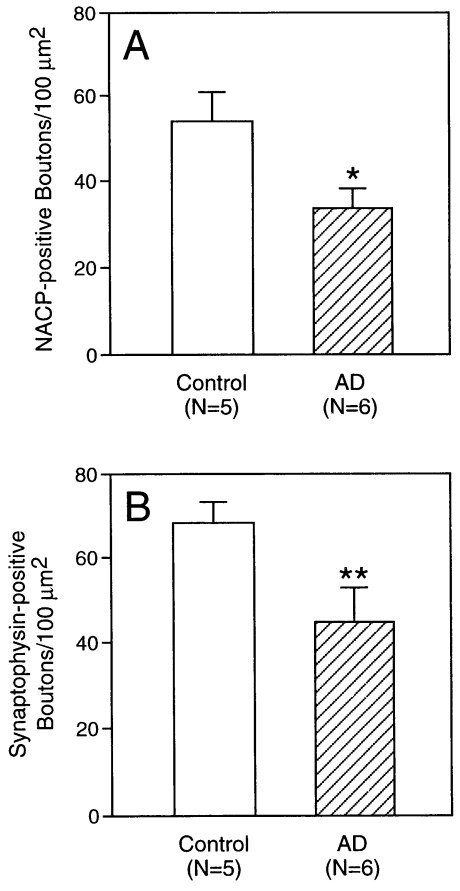

Figure 3. *Quantification of the number of NACP- and SYN-immunoreactive structures in AD and control frontal cortex. Both markers detected a decrease of ~40% in the numbers of immunolabeled structures.* (*P < *0.001*; t = *5.19*; **P < *0.0005*, t = *5.77*) (*n* = *6 AD and n* = *5 control cases*).

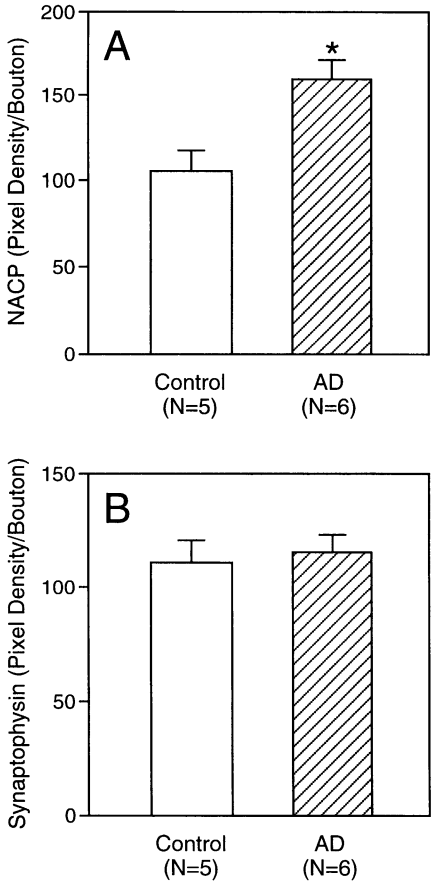

Figure 4. *Semiquantitative assessment of immunostaining intensity of SYN- and NACP-IR per individual structure in AD and control frontal cortex. Anti-NACP-IR per structure was increased in AD* (*P < *0.005*, t = *4.59*), *whereas anti-SYN-IR per structure was not different* (*n* = *6 AD and n* = *5 control cases*). *Note that the pixel intensity is scaled from 0 to 255. Thus, neither NACP- nor SYN-IR is saturated in the current quantification, although the intensity of anti-NACP-stained structures appears to be saturated in Figure 2 because of the sensitivity of photographic reproduction of the image.*

aptic phosphoprotein in the NACP family might be involved in regulation of synaptic function[9] possibly by modulation of signal transduction.[32] Consistent with these studies, the present study showed that NACP is colocalized with the great majority of the SYN-immunoreactive structures in the human brain. Previous immunoelectron microscopic studies in AD and in rodents have shown that the SYN-IR structures in the neuropil are associated with presynaptic terminals.[33] Similarly, immunogold studies in rodents have shown that NACP-immunoreactive structures in the neuropil are associated with the presynaptic boutons.[9] Therefore it is possible that in AD the great majority of NACP-IR structures in the neuropil are associated with presynaptic terminals. However, given that in pathological conditions such as AD there might be significant alterations in axonal transport[34] and neuritic regeneration,[29] these synaptic proteins might be abnormally compartmentalized. Then, the exact subcellular localization of NACP in AD awaits further more detailed immunoelectron microscopic studies.

The present study showed that NACP is characteristically altered in AD. Although the overall numbers of NACP- and SYN-immunoreactive structures were reduced in the same proportion in AD, the intensity of the NACP-IR and not SYN-IR per structure was increased compared with control. This is reflected, at the biochemical level, in the increased ratio of NACP to SYN in AD frontal cortex. This may have relevance to AD, because in this neurodegenerative disorder there is extensive damage to the synaptic site,[12–14,16,20,33], which appears to be present at very early stages of the disease.[35] Furthermore, the extent of damage to the synaptic site in AD is well correlated to the severity of the characteristic cognitive alterations.[18,19] Moreover, some of the dystrophic neurites in the plaques contained strong

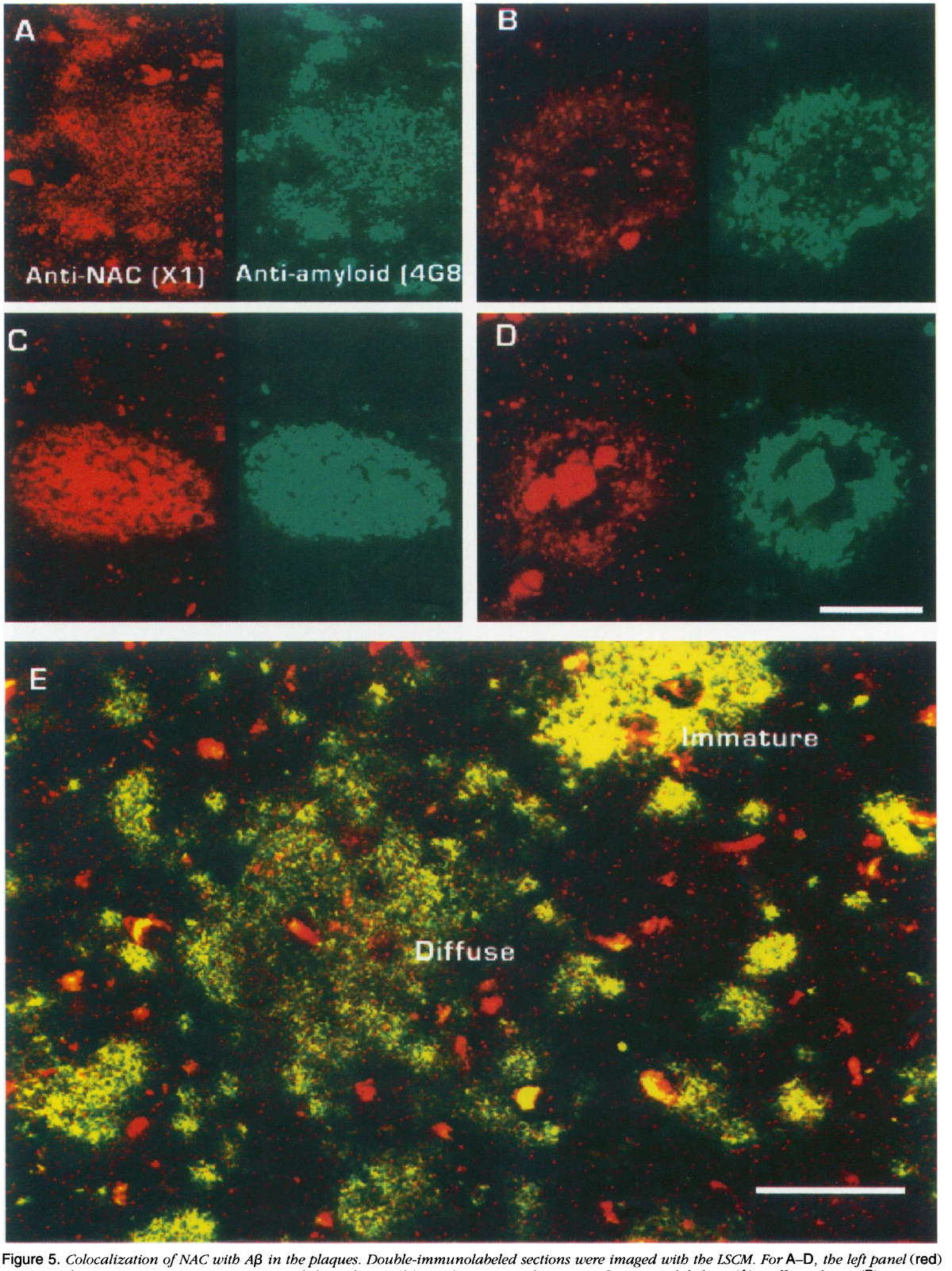

Figure 5. *Colocalization of NAC with Aβ in the plaques. Double-immunolabeled sections were imaged with the LSCM. For* A–D, *the left panel* (red) *corresponds to anti-NAC immunostaining and the right panel* (green) *corresponds to anti-Aβ immunolabeling.* (A) *Diffuse plaque* (B) *primitive immature plaque, and* (C *and* D) *mature plaques. Note that in the mature plaque the center portion of amyloid is strongly immunolabeled with anti-NAC, whereas anti-Aβ stained amyloid evenly.* (E) *Colocalization* (yellow) *of NAC and Aβ. Anti-NAC immunostaining* (red) *is colocalized with Aβ-IR* (green) *in diffuse and immature plaques. Bars = 20 μm. The magnification for panels* A–D *was identical.*

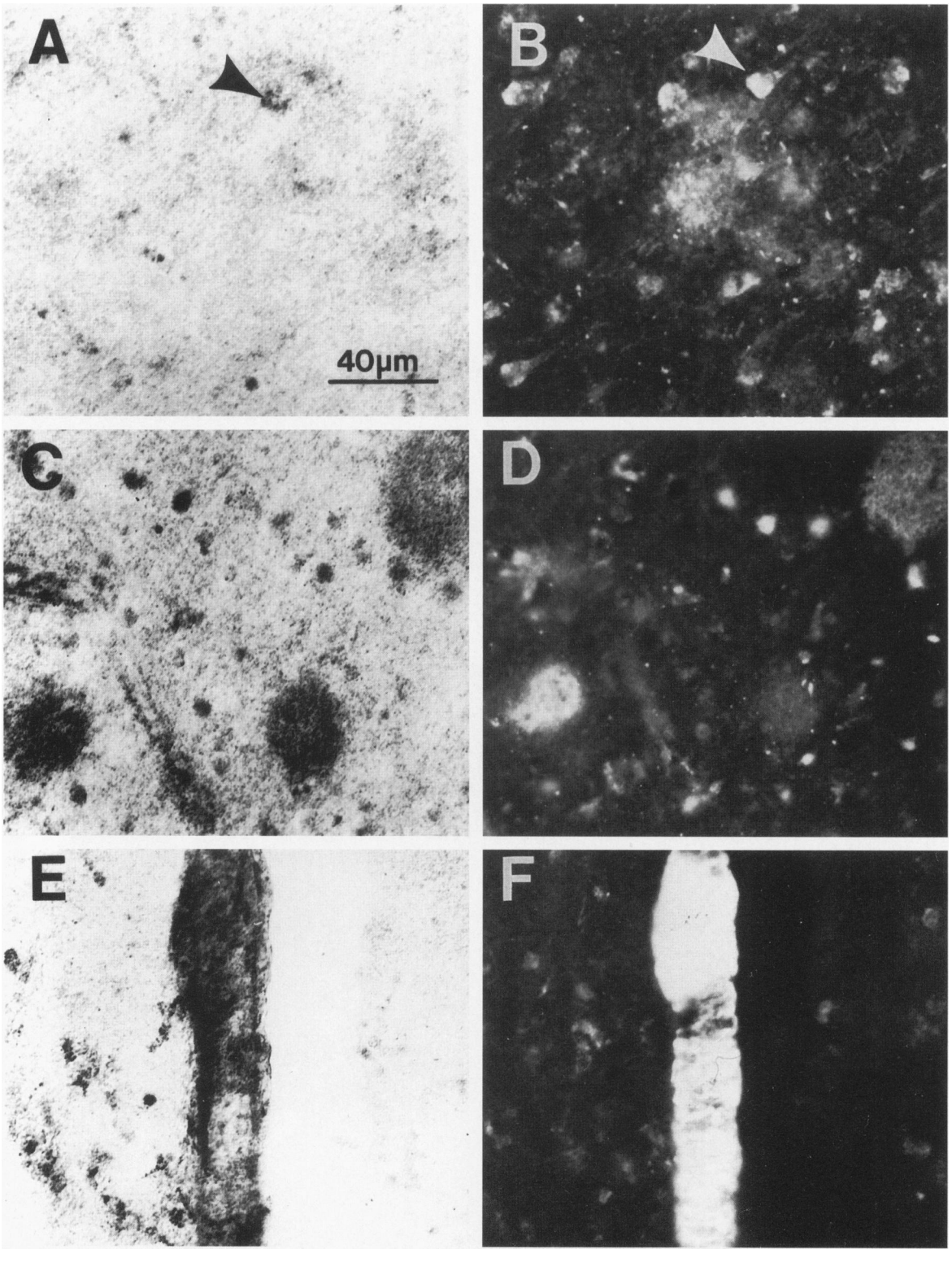

Figure 6. *Immunolocalization of NAC to amyloid in AD.* **A**, **C**, *and* **E** *are immunostained with the anti-NAC antibody;* **B**, **D**, *and* **F** *correspond to thioflavine-S staining.* (**A** *and* **B**) *In normal aged controls, thioflavine-S-stained diffuse plaques were anti-NAC negative.* (**Arrowheads** *indicate the autofluorescent lipofuscin).* (**C** *and* **D**) *In AD, thioflavine-S-stained diffuse and mature plaques were strongly immunolabeled by anti-NAC.* (**E** *and* **F**) *Amyloid angiopathy in large vessels was also immunostained by anti-NAC. For all panels final magnification was* ×*290.*

NACP-IR without SYN-IR. This local imbalance of NACP in relation to other synaptic proteins such as SYN at the boutons and in the neuritic plaques might be critically important in understanding the pathophysiology of AD. The mechanisms by which NACP accumulates locally at the boutons and abnormal neurites in AD are not clear. It is possible that this local increase in NACP-IR could be related to abnormal transport and accumulation of synaptic proteins and/or to abnormal processing of NACP. It is noteworthy that, in this brain region of healthy adult rat,[9] a majority of NACP-positive boutons are also SYN-positive, and only a small percentage of boutons are NACP-immunopositive without SYN-IR.[9] On the other hand, as much as 21% of boutons in plaques were NACP-immunopositive, but SYN-negative. It is tempting to speculate that this excess of NACP in the boutons and neuritic plaques might be involved in some aspect of AD pathogenesis. It will be critically important to identify the NACP-positive and SYN-negative structures in neuritic plaques in the future. Also, the ultrastructural analysis of boutons in AD that have higher levels of NACP will be required to understand the precise nature of the synaptic changes in AD. Although our previous electron microscopic study localized NACP to synaptic vesicles,[9] it is possible that fractions of NACP might be found in a free form. Further, it is interesting to note that, in diffuse plaques of clinically healthy individuals where no neuritic components are associated, amyloid is not stained with anti-NAC. On the other hand, many of the diffuse amyloid plaques become anti-NAC-positive when they are accompanied by neuritic components that are positive with NACP, APP, neurofilament, and ubiquitin. These results suggest that NAC or NACP might play a role in promoting and enhancing the Aβ aggregation into compact amyloid in the plaque. Consistent with this possibility, recent studies have shown that NAC and NACP bind Aβ and that NAC can form birefringent amyloid fibrils.[36–38] We suggest that abnormal processing of synaptic proteins, including APP and NACP, is involved in the pathogenesis of AD, particularly in plaque and amyloid formation, leading eventually to neurodegeneration and dementia.

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