Characterization of the Precursor Protein of the Non-β Component of Senile Plaques (NACP) in the Human Central Nervous System

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Abstract. A novel and highly conserved presynaptic protein has been independently described in rodents (synuclein/SYN-1), songbirds (synelfin), and humans (the precursor protein of the non-β component of senile plaques, NACP); a fragment of the latter has been detected in senile plaques in Alzheimer’s disease (AD). We characterized the expression of NACP in human AD and non-AD brain. A subcellular fractionation study demonstrated that NACP was mainly localized to cytosolic fractions of human temporal cortex. NACP was also detectable in various membrane and vesicular fractions, suggesting that the protein was associated with membrane structures including synaptic vesicles. Pericellular immunostaining of the neuropil was observed in neocortical and limbic regions, supporting a synaptic localization. Senile plaques in AD brains were not immunoreactive, and confocal microscopy suggested a loss of NACP immunoreactivity in cores of plaques. No difference was found in the amount of protein in AD and control frontal cortex, as measured by immunoblotting. PCR analysis showed that the full-length mRNA product was the major splice form in both AD and control human brains. Thus, despite the association of a hydrophobic fragment of NACP with senile plaques, our data suggest that the precursor itself is not a significant component of plaques and NACP synthesis is not substantially altered in AD. Nevertheless, the protein is an abundant component of synaptic regions prone to degeneration in AD, and may have a role in the expression or advancement of the disease.

Key Words: Alzheimer’s disease; Amyloid; NACP; Synaptophysin; Synelfin; Synuclein.

INTRODUCTION

The principal component of Alzheimer’s disease (AD) amyloid plaques is the β peptide; however, a second intrinsic peptide was recently purified and termed the “non-β component” (NAC) (1). The full-length precursor to NAC, Non-β component of senile plaques precursor (NACP), is a highly conserved presynaptic protein of unknown function (2). Virtually identical sequences have been independently described in rat (synuclein/SYN1) (3, 4) and canary (synelfin) (5). Alternative splice forms of the mRNA were reported in the rat (3) and human (6, 7), and a closely related sequence, apparently derived from a second gene, has been identified in cows, rats (phosphoneuroprotein 14) (4, 8, 9), and humans (β-synuclein) (10). A striking feature of the conserved sequence of these proteins is an organization around an 11-residue repeating motif, predicting an α-helical secondary structure similar to the lipid-binding domains of exchangeable apolipoproteins, including apolipoprotein E (5). Studies in songbirds, where the NACP homologue was termed synelfin to reflect its synaptic localization and conserved 11-residue periodicity, have implicated the protein in the developmental regulation of plasticity in the circuit responsible for juvenile song learning (5).

In both songbirds and rats, the mRNA for this protein is neuron-specific and highly enriched in those portions of the cortex and basal forebrain which, in humans, are primary sites of AD-associated neuropathology (2, 3, 5, 10, 11). These findings raise the possibility that the human NACP protein could have a causal role in the formation of AD plaques or other aspects of AD pathology (1, 2, 5). Further support for this has come from studies showing that both the NAC peptide and NACP will nucleate the formation of fibrils of β in vitro (12, 13). Mapping of the human NACP gene to chromosome 4q21.3-q22 did not reveal mutations in early-onset familial AD or linkage of a TaqI RFLP to AD (7, 14).

The goal of the present study was to address several questions about the localization of the NACP protein in the human brain, in order to clarify its potential relevance to AD. First, although the NAC peptide has been found in AD plaques (1), little information is available about the distribution of the full-length precursor protein in the human brain. Is the precursor also expressed in brain regions where senile plaques typically occur, and is it detected as a soluble component of synaptosomal extracts, as in other species? Second, is the precursor itself a significant component of AD plaques? Finally, are there any detectable differences in the amount of protein or the splicing of its mRNA in AD brains versus controls?
MATERIALS AND METHODS

Materials

Monoclonal antibody (H3C) was raised in mice by the University of Illinois Hybridoma facility (5) against the 15 C-terminal amino acid residues of canary synclinin, 13 of which are identical in human NACP and the homologue in rat (synclinin/SYN1). This antibody does not react with the central portion of NACP that gives rise to the NAC peptide. In both songbirds and rodents, the H3C antibody has been shown to detect a single dominant protein migrating at approximately 19 kDa and localized to presynaptic terminals (5).

Monoclonal mouse antibody to synaptophysin SVP-38 was obtained from Sigma (St Louis, MO). Monoclonal mouse IODS antibody against Aβ was obtained from Athena Neurosciences (South San Francisco, CA) and polyclonal rabbit R1282 antibody to Aβ was a generous gift from D. Selkoe (Boston, MA).

Postmortem adult human brain was received at autopsy from the Alzheimer Disease Research Center. All AD cases met Khatchaturian (15) and CERAD (16) diagnostic criteria for AD based on examination of Bielschowsky-stained paraffin sections of multiple cortical and subcortical areas. Tissue for homogenates was rapidly frozen in liquid nitrogen. Tissue for immunostaining was fixed in paraformaldehyde lysine metaperiodate for 24 to 48 hours (h) before sectioning.

Subcellular Fractionation

Subcellular fractionation of human temporal cortex from 3 non-AD and 1 AD brains was performed as described (17–20) with modifications. Human brain tissue in 5 volumes (w/v) of solution A (0.32 M sucrose, 1 mM MgCl₂, 0.5 mM CaCl₂, 1 mM NaHCO₃) with protease inhibitors (1 µg/ml leupeptin, 20 µg/ml phenylmethyl-sulfonyl fluoride, PMSF, 1 µg/ml pepstatin, and 1 µg/ml aprotinin) was homogenized with a motor-operated Teflon-glass homogenizer at 900 rpm with 12 up- and downstrokes. The resulting total tissue homogenate (TH) was centrifuged at 1,475 × g. The pellet was resuspended in solution A, homogenized, and pelleted again at 710 × g. The supernatants from the above two centrifugation steps were pooled as the postnuclear homogenate (S1). High speed centrifugation of the S1 fraction at 13,800 × g yielded the crude synaptosomes (P2) containing myelin, medium-sized vesicles, synaptosomes, and mitochondria, and the supernatant (S2). The S2 fraction was further separated by ultracentrifugation (250,000 × g) to obtain the microsomal pellet (P3) and the supernatant cytosolic fraction (C). The P2 fraction was resuspended in solution B (0.32 M sucrose, 1 mM NaHCO₃) and lysed in 20 volumes of ice-cold 6 mM Tris-HCl, pH 8.1, by gentle stirring at 4°C for 1 h. The latter was centrifuged (16,000 × g) to yield the lysed supernatant of crude synaptosomes (LS1) and lysate pellet of crude synaptosomes (LP1). The LS1 fraction was subjected to ultracentrifugation (300,000 × g) to obtain the crude synaptic vesicle fraction (LP2), and the soluble fraction of crude synaptosomes (LS2). The LS2 fraction was concentrated by microfiltration. The LP1 fraction was resuspended in solution B and subjected to discontinuous sucrose density-gradient centrifugation with a 0.8 M, 1.0 M, 1.325 M sucrose solution at 82,500 × g for 1 h. The synaptic plasma membrane fraction (SPM) contained in the layer between 1.0 M and 1.325 M sucrose was collected and washed with solution B.

Immunoblotting of Brain Homogenates

Specific brain regions from human postmortem tissue were dissected and homogenized with a microtip sonicator in 1 mM ethylenediaminetetraacetic acid, 30 mM tris, 2% sodium dodecyl sulfate, 1 mM benzamidine, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, and 1 mM PMES. Protein was quantitated using the BioRad DC Protein Assay. Homogenates from brains with a postmortem interval of greater than 12 h were excluded from the dot blot study.

Proteins for western blotting (25 to 100 µg) were separated by SDS-PAGE under denaturing, reducing conditions on 10 to 20% tricine or 12% tris-glycine gels (Novex, San Diego, CA), and transferred to Immobilon-P PVDF membranes. Proteins (4 to 8 µg) were also immunoblotted onto an Immobilon-P PVDF membrane in a 96-well MINIFOLD I microsample filtration manifold (Schleicher & Schuell, Keene, NH) according to manufacturers instructions. The amount of protein blotted was chosen so that sample immunoreactivity occurred within the linear range of a standard curve of 1, 2, 4, 8, 16, and 32 µg of a control brain homogenate (21). Membranes were blocked with 3% nonfat dry milk in tris-buffered saline, pH 7.4, (TBS) and sequentially probed with primary (1:3,500 H3C or 1:5,500 SVP-38 in 3% milk in TBS) and secondary (horseradish peroxidase linked goat anti-mouse IgG, 1:3,000, Jackson, West Grove, PA) antibodies, visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, England) on X ray film (Fujifilm) and quantitated by a Bio-Rad GS-700 Imaging Densitometer as arbitrary units (pixel intensity multiplied by volume of blot adjusted for background) per µg of protein.

Immunocytochemistry

50 µm coronal temporal lobe sections were treated with 1% Triton X-100 in 3% hydrogen peroxide for 20 minutes, blocked with 3% nonfat dry milk in TBS, and sequentially probed with primary antibody (1:5,000 to 1:10,000 H3C, 1:200 SVP-38, or 1ODS 1:350 in 1.5% normal goat serum) and secondary antibody (horseradish peroxidase, Cy3, or Cy5 linked goat anti-mouse IgG, 1:200, Jackson). Adjacent sections from 7 non-AD control brains and 13 AD brains were processed for H3C and synaptophysin immunoreactivity. Double staining for Aβ was accomplished by reblocking, probing with R1282 1:500, and utilizing BODIPY conjugated anti-rabbit Ig 1:200 (Molecular Probes) as the secondary antibody. Control sections were incubated with no primary, H3C preincubated with immunizing peptide, or H3C preincubated with nonabsorbing control peptide. The chromagen for the peroxidase reaction was diaminobenzidine. Confocal images were obtained using the MRC-1024 Bio-Rad laser confocal imaging system at an excitation filter wavelength of 568 nm and an emission wavelength of 605 nm for Cy3, 647 nm and 680 nm for Cy5, and 488 nm and 522 nm for BODIPY.

Polymerase Chain Reaction

Primers flanking the coding region of synclinin/NACP were synthesized on an Applied Biosciences DNA synthesizer. Using the numbering scheme from Ueda, et al [1], the sense primer
was synthesized from bases 22 to 41 (5'-CGACAGTGTTGGTATTAGGA-3') and the antisense primer from complementary bases 506 to 487 (5'-GACTTCAAGAAACTGGGAGC-3') with a predicted full-length product of 485 base pairs. cDNA extracted from human brain was amplified in the presence of 25 pmol sense primer, 25 pmol anti-sense primer, 200 μM dNTP, 2.5 U Taq polymerase (Boehringer Mannheim, Indianapolis, IN), and 1.5 μM MgCl₂ (30 cycles of 1 cycle at 95°C, 1 cycle at 55°C, and 2 cycles at 72°C). For radioactive PCR, α-P-32 ATP was added to the reaction mixture. Nonradioactive DNA was separated on 1.5% and 4% agarose in TBE gel and visualized with 1 μg/ml ethidium bromide; radioactive reaction product was separated on 8% acrylamide in TBE and visualized by exposure to X-ray film (Fujifilm).

RESULTS

Subcellular Distribution of H3C Immunoreactivity

We examined the distribution of H3C immunoreactivity in subcellular fractions prepared from human temporal cortex of 3 non-AD and 1 AD cases (postmortem intervals less than 12 h) by Western blot analysis using antibody H3C (Fig. 1). H3C recognized a doublet with apparent molecular weight of 19 to 20 kDa. Immunoreactivity was most prominent in the cytosolic fraction (C), but was also detectable in both the heavy membrane/crude synaptosome fraction (P2) and the microsomal fraction (P3). From these data it appears that human NACP is distributed mainly in the cytosol but is also present in membranous subcellular compartments.

Among subsynaptic fractions examined, including the crude synaptic vesicle fraction (LP2), the soluble fraction of crude synaptosomes (LS2) and the synaptic plasma membrane fraction (SPM), H3C immunoreactivity was highest in LS2 followed by LP2. In SPM, the 19-kDa protein signal was significantly reduced. These results suggest that NACP is present in the synaptic compartment both free in the cytosol and associated with small vesicles. The pattern of immunoreactivity was identical among the four cases studied.

Fig. 1. Subcellular distribution of NACP in human temporal cortex. Western blot analysis of H3C immunoreactivity in total tissue homogenate (TH), post-nuclear homogenate (S1), crude synaptosomes (P2), cytosolic fraction (C), microsomal fraction (P3), and subsynaptic fractions including fractions enriched in heavy membranes (LP1), synaptic vesicles (LP2), soluble proteins (LS2), and synaptic plasma membranes (SPM). Fractions are defined as described in Methods. Equal amounts of protein (20 μg) from each of the indicated fractions were loaded in each lane of 7.5% to 15% gel (left panel) or 3.5 to 15% gel (right panel). H3C specifically detected a doublet comigrating with the 19.4 kDa molecular weight standard.

Fig. 2. Regional distribution of NACP in human brain. Western blot analysis of H3C immunoreactivity in homogenates of entorhinal cortex (ec), putamen (put), thalamus (thal), cerebellum (cbl), Brodmann area 17 (17), Brodmann area 18 (18), motor-sensory cortex (m/s), and frontal cortex (f ctx). Equal amounts of protein (25 μg) were loaded in each lane of a 12% gel.

Western Blot and Dot Blot Analysis

Western blot analysis using antibody H3C demonstrated a single band or doublet at approximately 19 to 20 kDa. These bands were not seen if the primary antibodies were preabsorbed with the immunizing peptide. Western blot of regional brain homogenates from 5 cortical and 3 subcortical areas in 4 brains showed the 19 to 20 kDa band in all regions studied (Fig. 2). Dot-blot analysis of 11 AD (age 72 ± 11, postmortem interval 7 ± 4) and 5 control (age 63 ± 20, postmortem interval 7 ± 3) brains revealed a broad distribution in the amount of H3C and synaptophysin immunoreactivity among different brains with no significant difference between the amount of proteins in AD and control (Fig. 3). There was no correlation between H3C signal intensity and synaptophysin signal intensity.

H3C Immunostaining

Comparison of Alzheimer's disease hippocampal formation with control hippocampal formation revealed a similar distribution of staining for both H3C and synaptophysin immunoreactivity (Fig. 4). There was pericellular H3C neuropil immunostaining outlining the granule cells of the dentate gyrus and the pyramidal cells of the
Figs. 3. Quantification of NACP and synaptophysin in brain homogenates. Densitometric analysis of H3C and synaptophysin immunoreactivity in 5 control and 11 AD frontal cortex homogenates in Arbitrary Units per µg of tissue protein, as defined in Methods. (Error bars depict standard error).

Fig. 4. Distribution of NACP and synaptophysin in the hippocampal formation. Control brain hippocampal formation immunostained with (A) H3C and (B) SVP-38. AD brain hippocampal formation immunostained with (C) H3C and (D) SVP-38.

Hippocampal subfields. The dentate gyrus molecular layer showed strong granular staining, and the stratum radiatum revealed streaks of staining outlining dendritic shafts (Figs. 4A, C, 5). Synaptophysin immunostaining was similar; however, laminarity of staining was more prominent with the synaptophysin antibody (Fig. 4B, D). Senile plaques were not H3C immunoreactive, suggesting that full-length NACP protein is not associated with senile plaques. Confocal scanning microscopy confirmed the impression that H3C immunoreactivity was confined to the neuropil, outlining the pyramidal neurons in the hippocampal formation (Fig. 6); double staining with H3C and anti-Ab R1282 showed decreased H3C staining within compact amyloid plaques compared to the adjacent neuropil without significant double staining (Fig. 7), although punctate staining could be observed on the periphery of plaques.

Fig. 5. NACP immunostaining in the (A) dentate gyrus and (B) CA1 region of the hippocampal formation demonstrating pericellular staining of the dentate gyrus granule cell layer (dg) and the CA1 pyramidal cell layer (py), with granular staining of the dentate gyrus molecular layer (ml) and linear staining of the apical dendrites in the CA1 stratum radiatum (image size A 890 µm x 700 µm, B 1270 µm x 1000 µm).
Splice Form Analysis

Comparison on GenBank of the NACP sequence (GenBank L08850) and clonal NACP sequences L36674 and L36675 suggested the possibility of human splice forms lacking NACP bases 172 to 213 in L36674 and NACP bases 357 to 440 in L36675. We used polymerase chain reaction to look for alternatively spliced mRNAs in 11 cDNAs prepared from 7 AD and 4 control brains. In all cases, a major band of 485 base pairs was seen after PCR amplification, suggesting that in mature brain, the significant splice form is the full-length 140 amino acid form. No smaller bands were reliably detected by either ethidium bromide or radioactive PCR.

DISCUSSION

Despite the diversity of names, the synelfin/synuclein/NACP protein has a sequence that has been highly conserved among birds, fish, and mammals (2, 5, 10). Previous evidence has suggested that this protein represents a novel and relatively abundant component of neurons in the vertebrate forebrain, with an unusual subcellular distribution: although found mostly as a soluble protein, it nevertheless comes to be enriched at presynaptic terminals (2, 3, 10). Most of the descriptive work on this protein has been performed in nonhuman species. Because a fragment of this protein was recently shown to be a component of amyloid plaques in AD, we studied the distribution of the full-length protein in human brain.

All of the evidence presented here indicates that the H3C antibody specifically recognizes the NACP protein in the human brain, and that the distribution of this protein in humans is consistent with previous observations in other species. H3C detects a tight 19–20 kDa doublet on western blots of human brain homogenates (Figs. 1, 2), similar to the pattern previously described using this antibody in songbirds and rats (5); 19 kDa bands have also been observed independently with antibodies raised against human NACP (2) and α-synuclein (10). It is not yet known why the protein migrates slowly on SDS gels relative to its peptide length (~140 amino acids) or why two distinct bands appear under some conditions; similar electrophoretic patterns have been seen using recombinant protein purified from E. coli (10).

The subcellular distribution of the protein after fractionation of human brain homogenate is very similar to the distribution previously described in songbirds: the protein is abundant in the cytosol, yet a portion co-fractionates with synaptosomal membranes through several steps of enrichment. A small disparity in the two studies is the finding here of some immunoreactivity associated with a crude synaptic vesicle fraction, whereas no significant synaptosomal association was detected in the previous songbird study (5). The pattern of NACP distribution in synaptosomal fractions suggests that the protein distributes between a soluble pool and vesicle-bound pool, similar to ribSec1, β-NAP, dynamin, and p145 (22–24). The pattern is distinct from that of proteins tightly associated with synaptic vesicles, such as synaptophysin, syntaxin,

Fig. 7. Confocal image of (A) BODIPY-labeled R1282 (anti-AB) double stained with (B) Cy3-labeled H3C in the temporal cortex of an AD case. H3C staining is decreased in the core of the plaque (scale bar 25 μm).
SNAP-25, synaptotagmin, and synaptobrevin II (25, 26). Given the predicted structural similarity to apolipoproteins (5) and the protein's capacity to serve as a kinase substrate (1), NACP may form reversible, and possibly regulated, associations with membranes.

Consistent with previous observations in rodents (2, 3), H3C immunostaining of human brain sections and western blots revealed strong expression in the hippocampal formation and cortex. Throughout all brain regions, the immunocytochemical pattern was suggestive of a localization of the protein to synapses, as assessed by comparison to synaptophysin immunostaining. The cerebral cortex displayed punctate pericellular granular labeling, with absence of staining in white matter tracts. Within the hippocampal formation, strong staining was seen within the molecular layer of the dentate gyrus with sparing of the dentate gyrus granule cell bodies (Fig. 5A). Similarly, the apical dendrites of CA1 and CA3 pyramidal cells were decorated, although neuronal cell bodies did not show immunoreactivity (Fig. 5B). These latter observations specifically replicate the findings of Jakes, et al. (10) who showed synaptic staining in human entorhinal cortex and in CA3 in the hippocampal formation using a different antibody (PER2).

An internal 35 amino acid hydrophobic fragment of the NACP protein has been detected in amyloid plaque cores, as shown both by biochemical purification and by immunostaining (1). Our studies here used an antibody reactive with a different epitope in the full-length precursor protein, and the resulting immunostaining was specifically excluded from the core of plaques as visualized by confocal microscopy. Punctate NACP staining distinct from Aβ immunoreactivity present in the periphery and within plaques likely represents neuritic staining consistent with the immunolabeling results of Masliyah (27). As the precursor protein does not appear to possess a signal sequence for export from the cell (1), these observations strongly suggest that the NACP precursor itself is an intracellular protein and is not a significant component of extracellular plaques.

We did not observe significant differences in NACP expression in AD brains versus controls, by the following two criteria. First, PCR analysis suggests that the largest splice form is the major mRNA species in human brain cDNA, and this is not altered in AD. Second, no significant change in the amount of H3C immunoreactivity was seen in AD frontal cortex homogenates compared to control brain homogenates, nor were there qualitative differences in the pattern of immunohistochemistry in the temporal lobe. An average increase of H3C immunoreactivity and decrease of synaptophysin immunoreactivity in AD compared to controls by immunoblot in this study did not reach statistical significance. Our methods would not necessarily detect a quantitative change in the amount of H3C immunoreactivity in the synaptic compartment of a subset of neurons. Masliyah et al recently reported no change in the amount of NACP, but a significant increase in the ratio of NACP to synaptophysin by radiolabeled immunoblots of cytosolic fractions of 25 AD cases (27). Moreover, this group reported relative enhanced intensity of NACP immunoreactivity and decreased intensity of synaptophysin immunoreactivity in synaptic structures in AD. Taken together, these results suggest that synaptic components are differentially affected by AD, with a relative preservation of NACP. Whether this relative preservation of NACP contributes to the deposition of NAC in plaques is unknown.

The contribution of this protein to AD pathology hence remains unclear. A causal role in plaque formation was originally suggested by the tight physical association of NAC with plaque amyloid (1), and by the ability of NAC and NACP to nucleate amyloid aggregation in vitro (12, 13). Furthermore, the gene is expressed only in neurons, and the mRNA is most abundant in neurons in telencephalic structures especially vulnerable to AD pathology (10). Yet here we found no evidence of any gross alteration in amount, distribution, or splicing of NACP in AD versus normal brains. These observations suggest that the presence of NACP may be necessary for the advancement of AD pathology, without itself being a primary cause. Alternatively, the presence of NAC in plaques may be entirely a secondary consequence of synaptic degeneration in AD. In any case, NACP appears to be an abundant constituent of synaptic elements vulnerable to degeneration in AD. An understanding of the protein's normal function in the brain may provide insight into the functional disruptions of AD.

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