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Short Communication

Immunocytochemical Evidence That the β-Protein Precursor Is an Integral Component of Neurofibrillary Tangles of Alzheimer's Disease

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Amyloid β (Aβ) immunoreactivity has been demonstrated in all extracellular neurofibrillary tangles (E-NFT) and most intraneuronal neurofibrillary tangles (I-NFT). We undertook this immunocytochemical study to understand the relationship between Aβ immunoreactivity localized in NFT and β-protein precursor (βPP). We found epitopes of amino-, mid-, and carboxyl-terminal domains of βPP in I-NFT and the majority of E-NFT. NFT retained βPP after ionic detergent extraction, demonstrating that βPP is an integral component of NFT. Finding βPP in regions of Aβ immunoreactivity raises the possibility that βPP or its fragments associate with amyloid, and that the stability of Aβ is responsible for its dominance in amyloid deposits. (Am J Pathol 1993, 143:1586–1593)

Immunocytochemical studies demonstrate that the primary component of senile plaques, amyloid β (Aβ), is also associated with neurofibrillary tangles (NFT).[1–8] Aβ was reported in only a low percentage of NFT in formalin-fixed, formic acid-denatured samples. However, in tissue not fixed by aldehyde and treated with low concentrations of formic acid, all extracellular NFT (E-NFT), and many intraneuronal NFT (I-NFT) were Aβ immunoreactive.[8] These previous studies were limited to antibodies to Aβ epitopes, which cannot distinguish between Aβ or a β-protein precursor (βPP) fragment containing Aβ. The association of βPP fragments with NFT is supported by the presence of βPP epitopes in NFT, which lie outside the Aβ domain.[9–11] Yet, βPP was only localized to the filaments of I-NFT[10,11] and not to E-NFT.[11] The absence of βPP from E-NFT[11] would suggest that βPP is removed, along with the neuronal cytoplasm, after neuronal death. This interpretation is consistent with the generalized cytoplasmic localization of βPP immunoreactivity in neurons.[9] The peripheral association of βPP with NFT is also consistent with ultrastructural localization of Aβ to the amorphous covering of E-NFT filaments[3] and a report that some βPP epitopes can be removed from NFT by detergent.[11]

The aim of this study was to determine quantitatively whether βPP-epitopes (amino-, mid-, and carboxyl-terminal domains) are integral or peripheral elements of NFT. The goal was to determine whether the failure to detect βPP epitopes in E-NFT and detergent-extracted NFT was related to poor epitope preservation or exposure. In contrast with previous reports, we show that βPP is specifically associated with the majority of NFT; I-NFT and E-NFT. Furthermore, βPP is, like τ, retained after ionic detergent extraction. Therefore, we suggest that the βPP in NFT may provide a source for the Aβ deposition on NFT. Further, this study suggests that the metabolism of some βPP molecules may occur extracellularly in amyloid deposits.[12,13]

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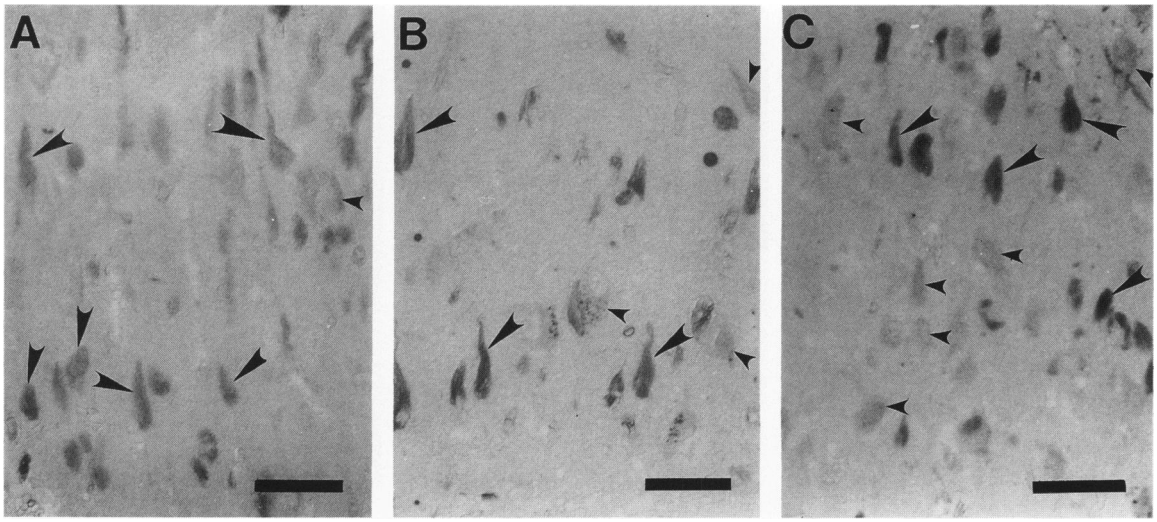

Figure 1. *Immunostaining of NFT with anti-βPP$_{45-62}$* (A) *anti-βPP$_{553-570}$* (B), *or anti-βPP$_{648-689}$* (C) *shows that numerous NFT are recognized by antibodies directed to amino-, mid-, and carboxyl-terminal domains of βPP, respectively. βPP antibodies recognized NFT* (*some indicated by* large arrowheads) *and neuronal perikarya* (*some indicated by* small arrowheads). *Scale bar = 50 μm.*

Materials and Methods

Tissue

The hippocampus and adjacent temporal cortex of five cases of Alzheimer's disease were studied (ages 71, 77, 85, 87, and 90 years). Clinical and pathological diagnoses were based on established criteria. Qualitatively identical results were obtained from all cases. Tissue taken at autopsy was fixed in methacarn (methanol-chloroform-acetic acid, 6:3:1) for 16 hours before paraffin embedding. Six-micron sections were cut.

Homogenates of hippocampal tissue were prepared by gently disrupting subiculum/Somer's sector gray matter (1:10, tissue:buffer ratio) in 50 mmol/L Tris-HCl, pH 7.6, with a Dounce homogenizer (Kontes Glass, Vineland, NJ). Aliquots of the homogenate (10 μl) were placed on slides, dried for 16 hours at 37 C, and treated with Tris buffer (50 mmol/L Tris-HCl, pH 7.6), Tris buffer-1% Triton X-100, or Tris buffer-1% sodium dodecyl sulfate (SDS) for 20 minutes at room temperature. The samples were then immunostained.

Immunological and Biochemical Reagents

Rabbit antisera raised to synthetic peptides homologous to three domains of βPP were used at a dilution of 1:100: 1) anti-βPP$_{45-62}$: amino-terminal, sequences 45–62 of βPP$_{695}$; 2) anti-βPP$_{553-570}$: mid-region, sequences 553–570 of βPP$_{695}$; and 3) anti-βPP$_{648-689}$: carboxyl-terminal, sequences 648–689 of βPP$_{695}$. None of the βPP antibodies recognized τ or neurofilament heavy subunit on immunoblots when used at the same concentration used for immunostaining but, as expected, recognized βPP on immunodots. In preliminary experiments, we found that formaldehyde fixation modified βPP epitopes such that they were not immunostained, and pretreatment with 50% formic acid for 5 minutes at room temperature was optimal for exposing βPP epitopes.

Immunoabsorption consisted of incubating the antiserum with 1 mg/ml of the appropriate synthetic peptide for βPP$_{553-570}$ and βPP$_{648-689}$ at 4 C for 16 hours before immunostaining. Immunoabsorption of anti-βPP$_{45-62}$ was performed by passage through a column containing βPP$_{45-62}$.

Heparan sulfate treatment consisted of incubating the sections for 1 hour at 37 C with 1 mg/ml heparan sulfate (molecular weight, approximately 8 kd; Sigma, St. Louis, MO) in 50 mmol/L Tris-HCl, pH 7.6. Heparan sulfate was eluted by treating with 2 mol/L NaCl for 16 hours at room temperature. Heparinase treatment involved incubating sections with heparinase 1 or heparinase 3 (1 U/ml; Sigma) in 10 mmol/L Tris-HCl, pH 7.0, and 20 mmol/L $CaCl_2$ for 16 hours at room temperature.

Standard markers to quantitatively assess NFT staining were basic fibroblast growth factor (bFGF) binding,[14] a probe for a form of heparan sulfate proteoglycans specific to E-NFT, and Alz-50, a monoclonal antibody that only recognizes I-NFT.[14] The peroxidase anti-peroxidase method using 3,3′-diaminobenzidine as chromogen was used to visualize immunoreactivity. Endogenous peroxidase

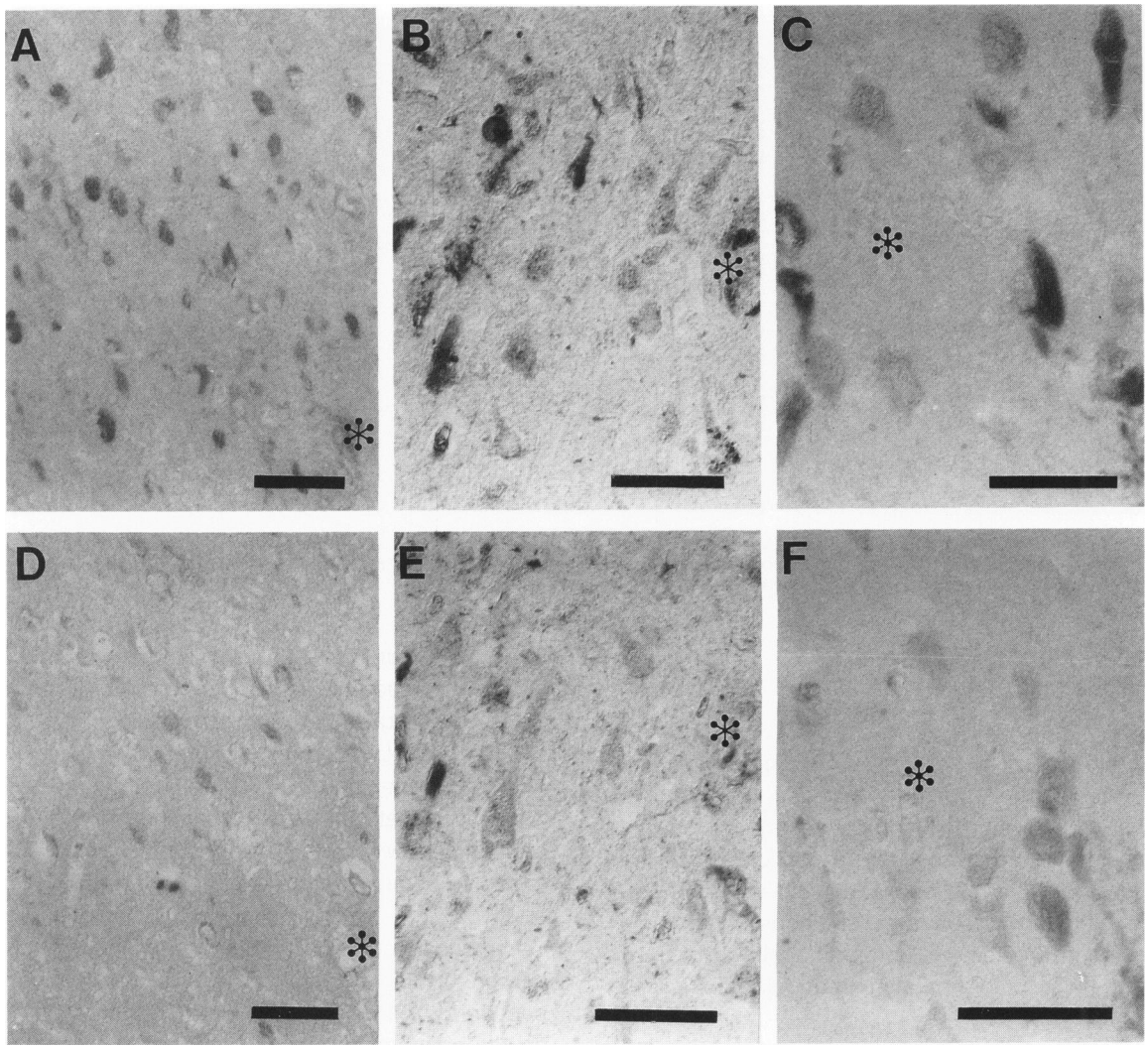

Figure 2. *Specificity of antibody binding was determined by absorption with the immunogen. Immunostaining of hippocampus with anti-βPP_{45-62}* (**A**), *anti-$\beta PP_{553-570}$* (**B**), *and anti-$\beta PP_{648-689}$* (**C**) *is reduced by preincubation with the respective immunogenic peptide: βPP_{45-62}* (**D**), *$\beta PP_{553-570}$* (**E**), *and $\beta PP_{648-689}$* (**F**). **A** *and* **D**, **B** *and* **E**, *and* **C** *and* **F** *are adjacent sections; a landmark is indicated* (•) *in each set. Scale bars = 50 μm.*

activity was quenched by treatment with 3% H_2O_2 in methanol for 30 minutes.

Quantitation of Stained Structures

The number of E-NFT and I-NFT recognized by the various probes in adjacent sections was determined by counting five fields, 0.2 mm^2 each, of the ×20 planapochromat objective of an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). Landmarks such as blood vessels were used to locate the same five fields of the subiculum within each group of adjacent sections. The fields evaluated were chosen in regions of maximum density for NFT. Immunostained NFT were defined by the intense staining of flame-shaped bundles of filaments; structures not displaying a filamentous structure were not counted as NFT. Congo red failed to recognize NFT after formic acid and, therefore, could not be used to augment our criteria to identify NFT. Differential interference contrast (Nomarski) was used to identify the cytoplasm and nucleus surrounding filaments of I-NFT and dispersed filaments of E-NFT.

Results

The initial goal of our study was immunocytochemical identification of specific βPP domains in NFT. Antibodies to the amino-terminal βPP_{45-62} (anti-βPP_{45-62}), mid-region $\beta PP_{553-570}$ (anti-$\beta PP_{553-570}$), and carboxyl-terminal $\beta PP_{648-689}$ (anti-$\beta PP_{648-689}$)

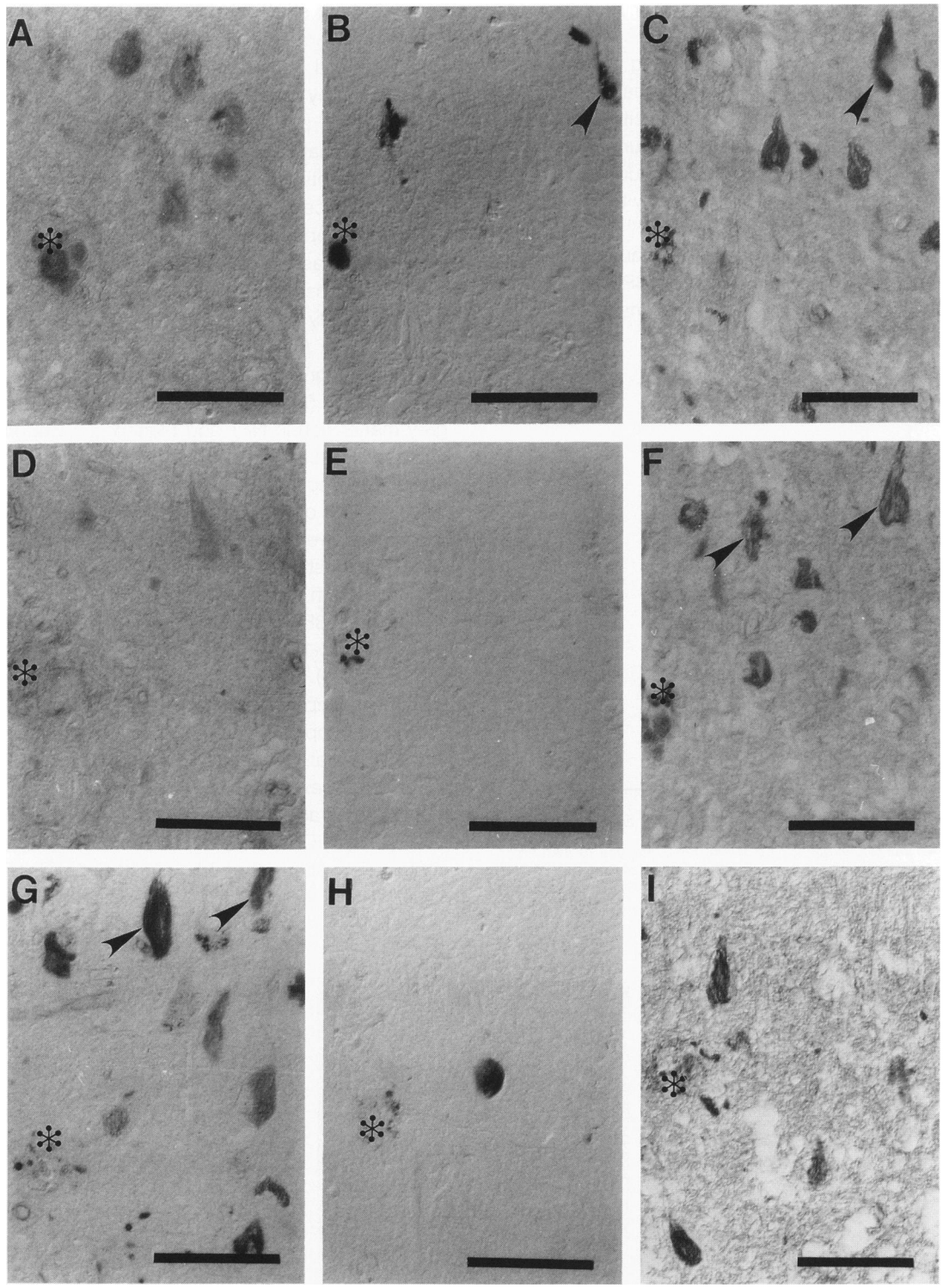

Figure 3. *NFT detected by antisera to three βPP domains compared to standards for E-NFT (bFGF binding) and I-NFT (Alz-50; see Figure 4). Adjacent serial sections are shown in order and defined by a senile plaque* (*). A: *Anti-βPP_{45-62};* D: *anti-$\beta PP_{553-570}$;* G: *anti-$\beta PP_{648-689}$;* B, E, H: *Alz-50;* C, F, I: *bFGF binding. Although individual NFT cannot always be followed in consecutive sections, the density and type of NFT recognized by these markers can be directly compared.* Arrowheads *indicate those cases where NFT recognized by different markers are seen in adjacent sections. Scale bars = 50 μm.*

all recognized NFT (Figure 1). Further, it was readily apparent that βPP is specifically associated with the filaments of NFT in addition to being diffusely distributed throughout neuronal cytoplasm (Figure 1) as previously reported.[11] The specificity of each antibody to βPP was verified by immunoabsorption, which reduced immunoreactivity (Figure 2).

The numbers of NFT recognized by the three βPP antisera were compared to Alz-50[14] (I-NFT) and bFGF[14] binding (E-NFT; Figure 3). A quantitative assessment in Figure 4 shows that βPP antibodies recognizes a majority of E-NFT and many I-NFT.

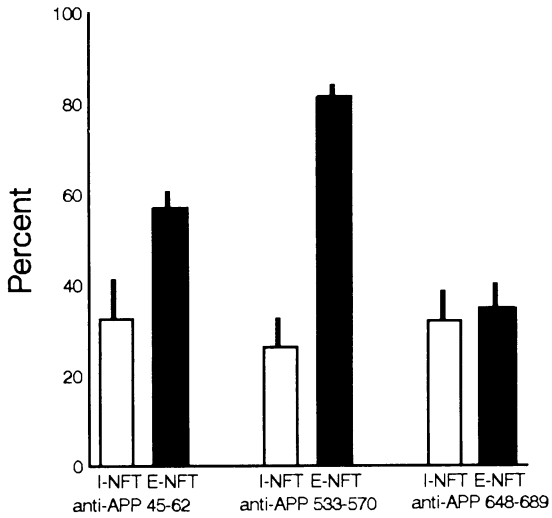

Figure 4. *The percentage of NFT containing βPP domains was determined from an area of 1 sq mm compared with the number of NFT stained by bFGF binding (E-NFT) and Alz-50 (I-NFT) in the immediately adjacent section defined by the same landmarks. Values are the mean ± SE for three cases.*

In a recent study, we showed that the heparan-binding protein cholinesterase, which is associated with NFT and senile plaques, can be specifically dissociated by competition with heparan sulfate.[15] As βPP has a heparan-binding domain,[16,17] we speculated that similar treatment might release βPP or its fragments. Indeed, pretreatment with heparan sulfate reduces immunoreactivity. However, we found that heparan sulfate blocks, rather than removes, βPP, as a high salt treatment after heparan sulfate reexposes βPP epitopes (Figure 5).

The inability to elute βPP with heparan sulfate suggests that βPP has an integral association with NFT. To demonstrate this aspect, we prepared homogenates in 50 mmol/L Tris-HCl from hippocampal grey matter rich in NFT and placed aliquots on microscope slides. The spots were dried and subsequently treated with Tris buffer alone or containing Triton X-100 or SDS. After detergent extraction, which dissolved most of the tissue, the sections were immunostained with the antisera to βPP, a preimmune serum, or an antiserum to τ.[18]

The three βPP domains were retained after nonionic (Triton X-100) or ionic (SDS) detergent extraction (Figure 6). Quantitative evaluations were made by direct comparison with the number of NFT containing τ epitopes. Comparison with τ immunostaining is essential, because NFT can be removed from the slide by extraction, and τ is retained by NFT after SDS extraction.[19] All three domains of βPP are retained by NFT morphologically identifical to those containing τ after either Triton X-100 or SDS (Table 1), indicating that βPP, like τ, is an integral component of NFT.[19]

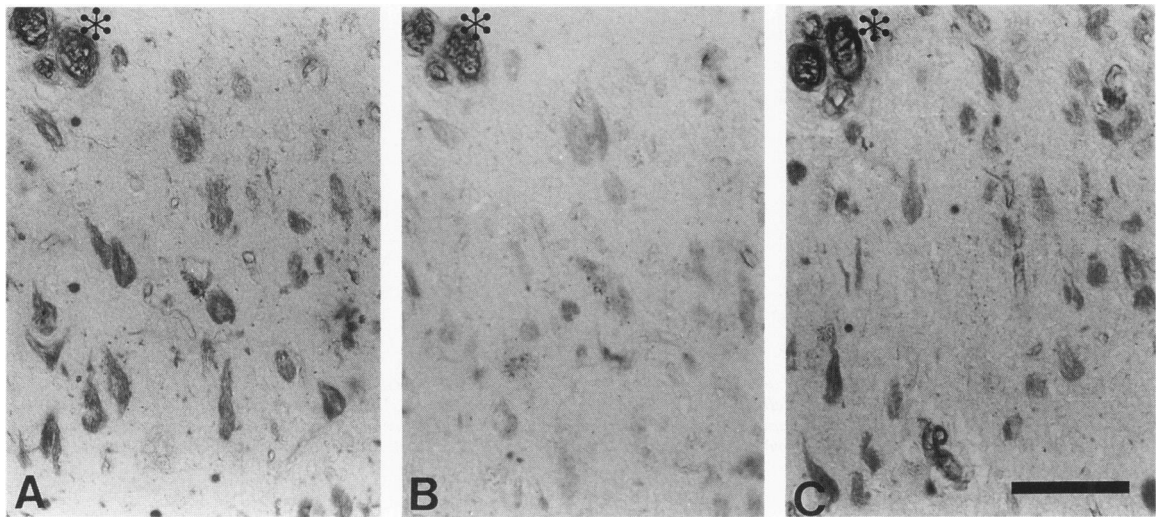

Figure 5. *Immunostaining of NFT by anti-βPP$_{533-570}$ (A) was reduced after incubation with 1 mg/ml heparan sulfate (B), but could be restored by treatment with 2 mol/L NaCl (C). Adjacent sections with the same blood vessel (*) found in each section are indicated. Scale bar = 50 μm.*

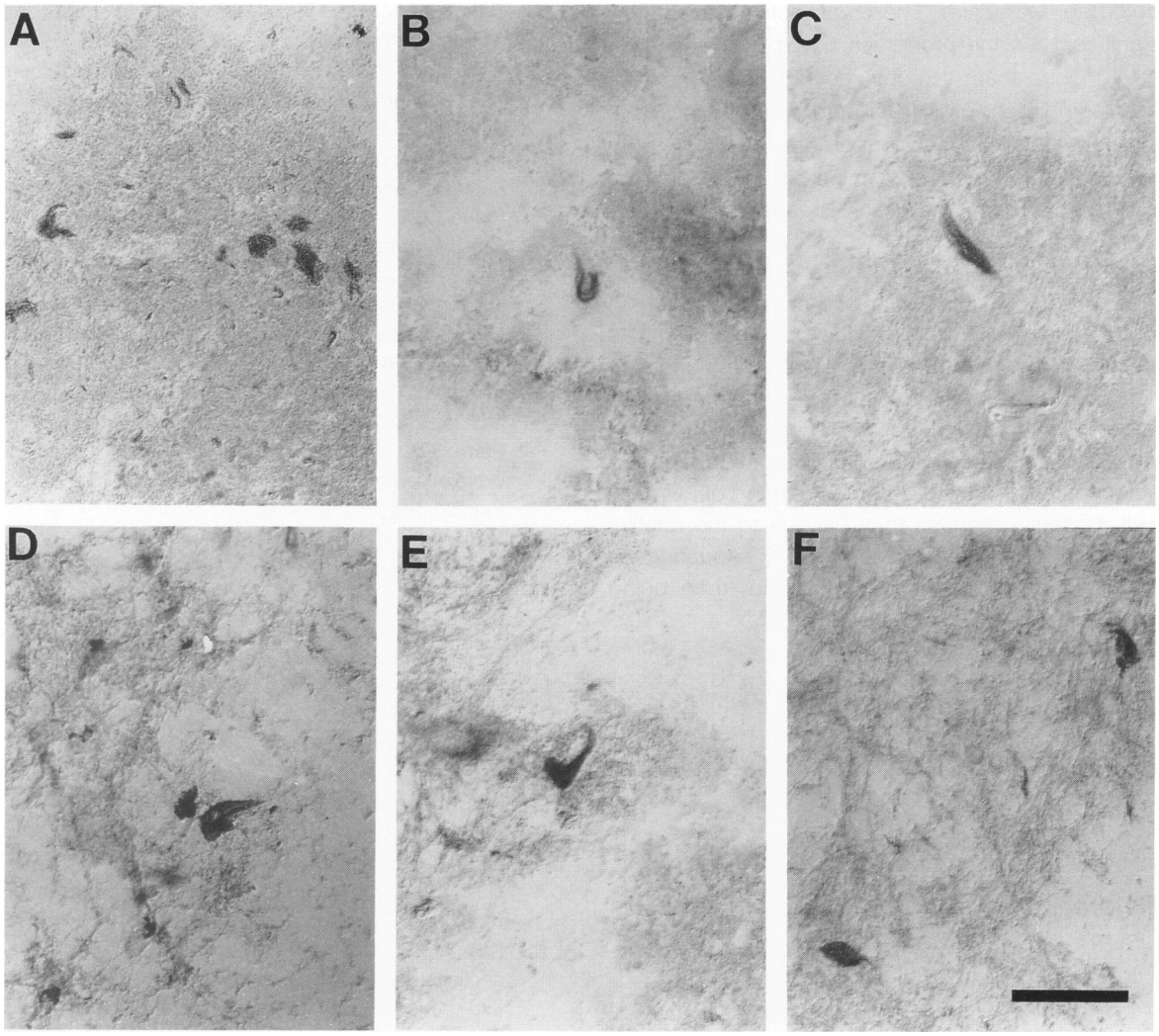

Figure 6. *βPP epitopes defined by anti-βPP_{45-62}* (**A**), *anti-$\beta PP_{553-570}$* (**B**), *and anti-$\beta PP_{648-689}$* (**C**) *were maintained after treatment with Tris buffer as well as after Tris buffer-1% SDS: anti-βPP_{45-62}* (**D**), *anti-$\beta PP_{553-570}$* (**E**), *and anti-$\beta PP_{648-689}$* (**F**). *Scale bar = 50 μm.*

Table 1. *βPP Epitopes Are Retained by NFT after either Nonionic (Triton X-100) or Ionic (SDS) Detergent Extraction to Approximately the Same Extent as τ Epitopes*

	Anti-βPP_{45-62} (n = 6)	Anti-$\beta PP_{553-570}$ (n = 2)	Anti-$\beta PP_{648-689}$ (n = 2)	Anti-τ (n = 6)	Preimmune (n = 2)
Tris	126.2 ± 6.9	14.5 ± 2.1	27	49 ± 7.5	2 ± 1.4
Triton	152 ± 13.6	34 ± 24	22.5 ± 6.4	54.8 ± 13.7	2.5 ± 0.7
SDS	46.8 ± 7.9	7 ± 8.5	30 ± 25.5	13.3 ± 4.4	0

Although in some cases there is considerable variability, all data indicate that βPP is retained by NFT. The lower number of NFT after SDS reflects removal of NFT from the slide. Data are expressed as the number of NFT stained in 10-μl aliquots of a homogenate (1:10, tissue:buffer ratio). Values are the mean ± SE.

Discussion

Our results demonstrate that three non-contiguous βPP epitopes are found in the majority of NFT. We also show that although heparan sulfate proteoglycans may be responsible for initial βPP incorporation,[16,17,20] it is unlikely that they play a significant role in retaining βPP in NFT, as βPP was not eluted by heparan sulfate. We speculate that once associated, βPP is retained in NFT by other interactions, such as those described between τ and βPP.[21,22]

Our findings differ from those of Yamaguchi and co-workers.[11] We readily detect three distinct βPP domains associated with NFT filaments and show βPP in E-NFT and retention of βPP after ionic

detergent extraction. These distinctions are highly significant, as the properties noted by Yamaguichi et al.[11] lead them to conclude βPP is an insignificant component of NFT, an interpretation inconsistent with the findings reported here.

A provocative aspect of this study is that βPP sequences flanking the Aβ domain are apparently integral components in the majority of E-NFT. This study raises the possibility that βPP or its fragments may directly associate with NFT in addition to amyloid filaments[9,23] in the extracellular space. This is consistent with the results of several immunocytochemical studies that identified βPP epitopes in Aβ deposits[9,23] and with a more recent study identifying βPP epitopes within highly purified amyloid plaque cores.[24] The presence of Aβ in both NFT as well as senile plaques may be due to the relative stability of Aβ compared to other βPP sequences to catabolic degradation,[12,13,25,26] rather than being the result of specific proteolytic events.

Acknowledgments

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