The Neurotoxic Carboxy-Terminal Fragment of the Alzheimer Amyloid Precursor Binds Specifically to a Neuronal Cell Surface Molecule: pH Dependence of the Neurotoxicity and the Binding

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One of the hallmarks of Alzheimer's disease neurodegeneration is the accumulation of deposits of amyloid in neuritic plaques and in the cerebral vasculature. Recent studies have implicated carboxy-terminal fragments of the Alzheimer amyloid precursor protein (βAPP) in the processes of amyloidogenesis and neurodegeneration. In particular, the carboxy-terminal 104 amino acids of βAPP (βAPP-C104) have been shown to cause amyloid-like fibrils when expressed in non-neuronal cells and to cause the degeneration of neuronal cells. These data suggest that it may play a role in the development of the progressive neuropathology of Alzheimer's disease. We hypothesized that βAPP-C104 may cause the degeneration of neurons by interacting with a cell surface receptor. In the present report, we show that βAPP-C104 synthesized *in vitro* binds specifically and with high affinity to the surface of NGF-treated PC12 cells. Both the cell surface binding and the neurotoxicity of βAPP-C104 are pH dependent and are not inhibited by tachykinins. Mutational analysis suggests that both the binding and the neurotoxicity are dependent at least in part on the presence of a tyrosine residue that is a potential site of phosphorylation at the carboxy terminus of the fragment.

The deposition of amyloid in neuritic plaques and along the walls of the cerebral vasculature is a key feature of Alzheimer's disease pathology (Terry et al., 1981; Glenner, 1983). The principal component of amyloid is a 4 kDa protein termed β/A4 (Glenner and Wong, 1984a,b; Masters et al., 1985) that is probably derived from one or more of four identified precursor proteins with predicted lengths of 695 (Kang et al., 1987), 751 (Ponte et al., 1988; Tanzi et al., 1988), 770 (Kitaguchi et al., 1988), and 714 amino acids (Golde et al., 1990), respectively.

DNA sequence (Kang et al., 1987) and biochemical (Dyrks et al., 1988; Selkoe et al., 1988) studies suggest that the amyloid

precursor protein (βAPP) is an integral membrane protein with the β/A4 peptide spanning the border between the extracellular domain and the transmembrane region. βAPP in cultured cells is rapidly processed from a membrane-bound form to a secreted form (Weidemann et al., 1989); pulse-chase studies show that the secretion of βAPP leaves behind a stable 11.5 kDa fragment consisting of the transmembrane region and the cytoplasmic domain (Oltersdorf et al., 1990). This normal processing of βAPP is a membrane-associated event that occurs within the extracellular domain of β/A4 (Esch et al., 1990; Sisodia et al., 1990). Hence, the generation of β/A4 in Alzheimer's disease appears to be the result of abnormal cleavage. However, the molecular mechanisms of amyloidogenesis and the role of amyloidogenesis in the development of Alzheimer's disease pathology remain unclear.

Recent evidence has implicated the 100–104 amino acid carboxy-terminal fragment of βAPP in the processes of amyloidogenesis and neurodegeneration. This fragment, which spans the β/A4 and cytoplasmic domains, has a tendency to self-aggregate (Dyrks et al., 1988). Moreover, the expression of this carboxy-terminal βAPP fragment in primate cells has been shown to lead to the production of a 16 kDa protein that aggregates and accumulates into deposit-like structures (Wolf et al., 1990) and that results in the formation of amyloid-like fibrils (Maruyama et al., 1990).

We have shown that this same carboxy-terminal βAPP fragment is neurotoxic (Yankner et al., 1989). PC12 cells transfected with a retroviral recombinant expressing the carboxy-terminal 104 amino acids of βAPP (formerly termed AB1, Yankner et al., 1989; now termed βAPP-C104) degenerate when induced to differentiate into neuronal cells with NGF. Moreover, conditioned medium from these cells is toxic to differentiated neuroblastoma cells (Slavc et al., 1989) and to neurons but not non-neuronal cells in primary rat hippocampal cultures (Yankner et al., 1989). The neurotoxicity can be removed from the medium by immunoabsorption with an antibody to βAPP-C104 (Yankner et al., 1989), suggesting that βAPP-C104 is secreted by the transfected cells and is neurotoxic. We have recently demonstrated that brain transplants of cells expressing βAPP-C104 cause specific neuropathology *in vivo* (Neve et al., in press). We have also shown that transgenic mice expressing βAPP-C104 in the brain manifest intracellular and neuropil deposition of β/A4 protein, as well as other abnormalities that resemble aspects of Alzheimer's disease pathology (Kammesheidt et al., 1991; and A. Kammesheidt and R. L. Neve, unpublished observations).
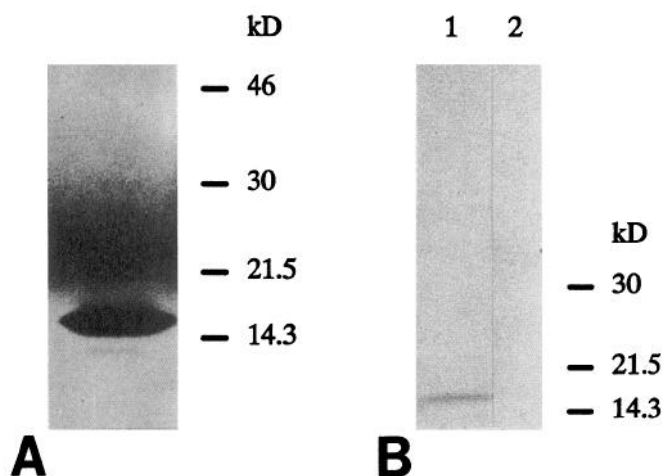
Figure 1. SDS-PAGE (*A*) and immunoblot (*B*) analysis of βAPP-C104 made by *in vitro* transcription-translation. In *B*, the *first lane* shows reactivity of antibody BX6 with βAPP-C104; the *second lane* is a control with no primary antibody.

The neurotoxicity of this carboxy-terminal βAPP fragment suggests that it may play a role not only in amyloidogenesis but also in the development of the progressive neuropathology of Alzheimer's disease. We have therefore sought to determine the mechanism by which βAPP-C104 causes the degeneration of neurons. We report here that βAPP-C104 synthesized *in vitro* binds specifically and with high affinity to the surface of NGF-treated PC12 cells. Both the cell surface binding and the neurotoxicity of βAPP-C104 are pH dependent. Mutational analysis suggests that both the binding and the neurotoxicity are dependent at least in part on the presence of a tyrosine residue that is a potential site of phosphorylation at the carboxy terminus of the fragment.

Materials and Methods

Cell culture. PC12 cells for the binding assays were grown in RPMI1640 (GIBCO) with the addition of 10% heat-inactivated horse serum (Hana Biological) and 5% fetal bovine serum (J.R. Scientific). T-175 flasks (Falcon) were seeded with 5×10^5 cells/ml in 60 ml of growth medium containing 10 ng/ml NGF. An additional 60 ml of this medium were added every 2 d until the cells were harvested (usually at 6 d). Where indicated, PC12 cells were cultured as above except in the absence of NGF. Cells were collected by centrifugation at $138 \times g$ for 8 min. The supernatant was discarded and the pellet resuspended in 5 ml of the growth medium. The cells were triturated three times through a 23 gauge needle attached to a 10 cc syringe to provide a single cell suspension or, at most, small cell aggregates. Trypan blue (Polyscience Inc.) dye exclusion indicated over 99% viable cells.

PC12 cells for the toxicity assays were grown in the presence of 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Sigma), 5% heat-inactivated horse serum (HyClone), and 10% fetal bovine serum (HyClone) containing 50 ng/ml β-NGF (gift of Dr. William Mobley). Cells were seeded at 10^4 cells/well in polylysine-treated 24-well plates (Costar). The medium was replaced every 2 d. When the pH dependence of the toxicity was examined, a given pH was maintained by adding 25 mM HEPES to the medium and adjusting the medium to the desired pH at 36°C (the temperature at which the cells were maintained). The pH of the medium in the wells was rechecked during the course of the experiment. Four or more replicates of each experiment were plated; one field in each well containing 100–300 cells on day 0 was defined for cell counts. We returned to the same field for cell counts on subsequent days. Viability of the NGF-treated cells was determined on the basis of phase brightness of cell bodies, intactness of neurites (some cells became phase-dark due to spreading on the polylysine substrate but extended neurites in response to NGF; these cells were counted as viable as long as they had neurites), and adherence to substrate. All the
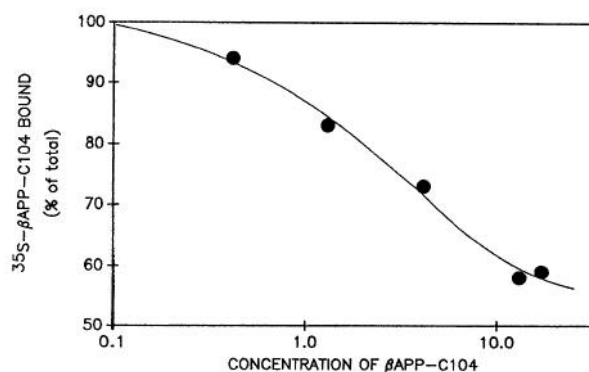

Figure 2. Representative curve showing the inhibition of ³⁵S-βAPP-C104 binding to NGF-treated (5 d) PC12 cells by βAPP-C104.

transfected cells were regularly examined for the presence of the transfected RNA (neo in the case of the DO transfectants; βAPP-C104-neo fusion in the case of the βAPP-C104 transfectants; see Yankner et al., 1989).

To assay the toxicity of conditioned media from NIH 3T3 transfectants, the growth medium of the transfectants (DMEM plus 5% bovine calf serum; HyClone) was replaced with the PC12 medium for 2 d when the 3T3 cell transfectants were at approximately 50% confluence. At the time that the conditioned media were harvested, RNA was isolated from the 3T3 cell transfectants and analyzed by Northern blots to confirm that all the transfectants were expressing equivalent amounts of mRNA from the appropriate transfected recombinant. PC12 cells were seeded at 10^4 cells/well in polylysine-coated 24-well plates (Costar) and the conditioned media (diluted 1:1 with PC12 medium) containing 50 ng/ml β-NGF were applied and replaced every other day. To test the ability of tachykinins to inhibit βAPP-C104 toxicity, each tachykinin analyzed was included in the conditioned medium at 20 μM, and replaced each time the conditioned medium was replaced.

Preparation and characterization of βAPP-C104 and ³⁵S-βAPP-C104. The BglII-SmaI fragment of the βAPP-695 cDNA (bp 1769–2959 according to the sequence of Kang et al., 1987), or of the appropriate mutant, was cloned into BamHI-SmaI–digested pGEM3 (Promega Biotec). The resulting recombinant was linearizied with ClaI (to make sense βAPP-C104 RNA using SP6 RNA polymerase) or SalI (to make antisense βAPP-C104 RNA using T7 RNA polymerase) and used as template to make 5'-capped RNA *in vitro* using Promega Biotec reagents and protocol. RNA (20 μg) was typically synthesized in a 50 μl reaction using 5 μg of template. Two micrograms of this RNA were used in a 50 μl wheat germ lysate *in vitro* translation reaction together with 3.8 μCi of ³⁵S-methionine (Amersham; 1200 Ci/mmol). The concentration of βAPP-C104 in the lysates was determined by SDS-polyacrylamide gel electrophoresis of an aliquot of the *in vitro* translation product, followed by excision of the radiolabeled band from the dried and autoradiographed gel, solubilization of the band in H_2O_2, and quantitation of the radioactive protein in the band. The concentration of βAPP-C104 in the band was extrapolated from these data, using the assumption that during the *in vitro* synthesis of ³⁵S-βAPP-C104, ³⁵S-methionine was incorporated at all of the five methionine residue positions (specific activity, 11,000 Ci/mmol) in the polypeptide. The ³⁵S-methionine was replaced with 80 μM methionine to make unlabeled βAPP-C104. A 1/10 vol of 100 mM *N*-acetyl-D-glucosamine (Sigma) was added to each completed *in vitro* translation reaction to block the binding activity of wheat germ agglutinin before the reaction was aliquotted and stored at −80°C. The *in vitro* translation products were thawed only once for use in the binding assays. Multiple freeze-thaw cycles, which resulted in aggregation of *in vitro* translated βAPP-C104 (S. A. Fidel and R. L. Neve, unpublished observations), also resulted in loss of binding activity of the ³⁵S-βAPP-C104. ³⁵S-βAPP-C104 was 90% bindable to NGF-treated PC12 cells in conditions of binding site excess.

Receptor binding assay. For βAPP-C104 binding experiments, PC12 cells, grown and prepared as described above, were counted, collected by low-speed centrifugation ($138 \times g$), and resuspended in PBS (pH 7.4) with 1% BSA and 1% glucose (PBG). In the experiments in which the pH dependence of the binding was tested, pH values for the PBS ranged from 7.4 to 8.0. A 0.08 ml volume of the cell suspension, con-
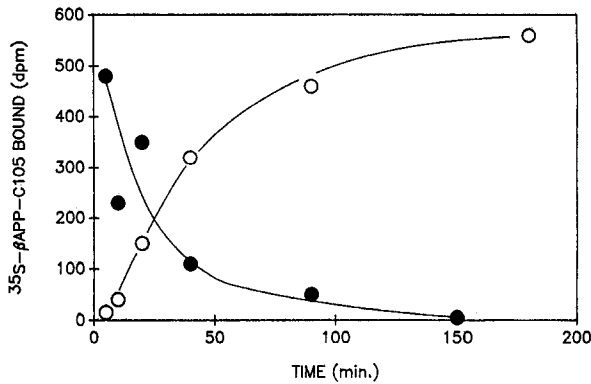
Figure 3. ^{35}S-βAPP-C104 association with (*open circles*) and dissociation from (*solid circles*) NGF-treated (5 d) PC12 cells. Data shown are from one of two experiments in which similar results were obtained. A K_D value was not determined with the kinetic data since a 0 time off rate could not be measured using the current binding protocol (see Materials and Methods).

Figure 4. Representative saturation isotherm (*top*) and Scatchard plot (*bottom*) of ^{35}S-βAPP-C104 binding to NGF-treated (6 d) PC12 cells. The *lines* were computer generated by the LUNDON 1 program (see Materials and Methods) and represent the best interpretation of the data.

taining 2 × 10^6 cells, was mixed with 0.01 ml of ^{35}S-βAPP-C104 and 0.01 ml of PBG, or PBG containing unlabeled βAPP-C104. For kinetic and inhibition experiments, the concentration of ^{35}S-βAPP-C104 was either 25 pM or 50 pM. For saturation experiments, the ligand concentration ranged from 15 pM to 6 nM. The mixture was incubated at 4°C for 3 hr, unless otherwise indicated. At the end of the incubation period, the cells were pelleted by centrifugation at 138 × g for 90 sec, the supernatant (containing the unbound ligand) was removed, and 0.2 ml of fresh, ice-cold PBG was added. The cells and fresh buffer were briefly agitated, and again centrifuged and suspended in fresh, ice-cold buffer as above. Finally, the cells were pelleted by centrifugation, filtered through BSA (1%)-treated filters using a Tomtec (Orange, CT) 96-well harvester, and washed with four 1 ml rinses of ice-cold PBS with 1% BSA. The duration of the period between resuspension of the cells in fresh, ice-cold buffer and filtration of the cells was less than 10 min. In the dissociation experiments and to collect a sample of bound ligand, the cells were incubated in the buffer added as the second wash for up to 3 hr to allow the bound ligand to dissociate. The cells were then pelleted by centrifugation and the supernatant (containing the ligand that had dissociated) was removed. The cells were then rewashed and harvested as described above. Radioactivity retained by the filters was measured by scintillation counting using a Betaplate counter (LKB). Nonspecific binding was taken as that occurring in the presence of excess (3.2–18 nM) βAPP-C104. Saturation data were analyzed using LUNDON 1 binding analysis software (Lundeen and Gordon, 1986). The binding model that best fitted the experimental data was determined by F test ($p < 0.05$).

In vitro mutagenesis of βAPP-C104. In order to make mutants of βAPP-C104, we used oligonucleotide-directed *in vitro* mutagenesis as described by Kunkel (1985). To change serine 603 to an alanine, a 20mer representing the reverse complement of bases 1795–1814 (according to the sequence of Kang et al., 1987) that directs the alteration of the initial T of the serine codon to a G was used. To change tyrosine 687 to a phenylalanine, we constructed a 19mer representing the reverse complement of bases 2053–2071 that directs the appropriate A to T alteration. The EcoRI-ClaI subfragment of βAPP-C104 was subcloned into the phagescript vector SK M13 (Stratagene) and introduced into the *Escherichia coli dut,ung* strain CJ236. The uracil-containing phage DNA produced in this strain was used as template in the *in vitro* mutagenesis reaction, which was transformed into *E. coli* MV1190 for selection against the parental strand. The mutated EcoRI-ClaI subfragment was used to replace the wild-type fragment in the DO recombinant, after which presence of the desired mutation was confirmed by sequence analysis.

Results

Synthesis and analysis of βAPP-C104

The portion of the βAPP cDNA encoding βAPP-C104 was cloned into the plasmid vector pGEM3, in which the cloning site is flanked by binding sites for SP6 and T7 RNA polymerases. The sense (or antisense control) transcript was synthesized and then
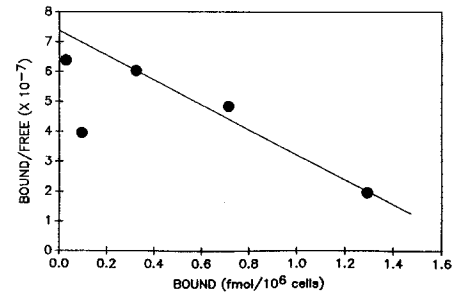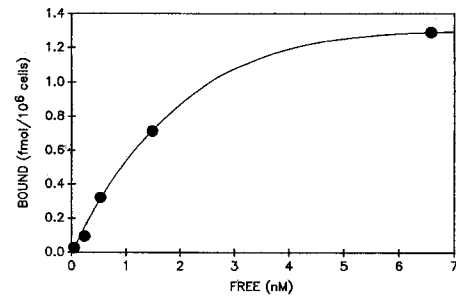
translated *in vitro* in a wheat germ lysate. SDS-PAGE (5–20% gradient) of ^{35}S-labeled βAPP-C104 made in this *in vitro* transcription–translation system revealed that the radiolabeled product migrated as an approximately 15 kDa band (Fig. 1A). To show that this band represented βAPP-C104, the *in vitro* translation extract was analyzed by immunoblot with an antibody (termed anti-BX6; Benowitz et al., 1989; Oltersdorf et al., 1990) directed against a bacterial fusion protein expressing βAPP-C104 (Fig. 1B). The anti-BX6 immunoreacted with an approximately 15 kDa band in an *in vitro* translation of the sense βAPP-C104 RNA (first lane); no reactivity was seen with secondary antibody alone (second lane).

Specific binding of βAPP-C104 to the surface of differentiated PC12 cells

Because βAPP-C104 is toxic to NGF-differentiated PC12 cells, we tested the binding of the *in vitro* synthesized βAPP-C104 fragment to the surface of PC12 cells that had been treated with NGF. ^{35}S-βAPP-C104 binding to the differentiated PC12 cells was inhibitable by unlabeled βAPP-C104 (Fig. 2). The inhibitable fraction of the binding accounted for 40–60% of the total binding. The IC_{50} value for the inhibitable binding was 1.7 ± 0.7 nM ($n = 5$). Inhibitable binding reached a maximum after 3 hr of incubation and was completely dissociable (Fig. 3). Saturation experiments indicated the presence of a single class of binding sites with a K_D value of 0.81 ± 0.37 nM, in approximate agreement with the IC_{50} value determined above, and a B_{max} value of 0.37 ± 0.05 fmol/10^6 cells ($n = 3$; Fig. 4).

Binding was not significantly inhibited by other peptides, including a number of tachykinins (Table 1). Non-sense ^{35}S-labeled material synthesized from antisense βAPP-C104 RNA did not bind to NGF-treated PC12 cells.

To assess the stability of ^{35}S-βAPP-C104 in the assay conditions, samples of both free ligand at the end of the incubation
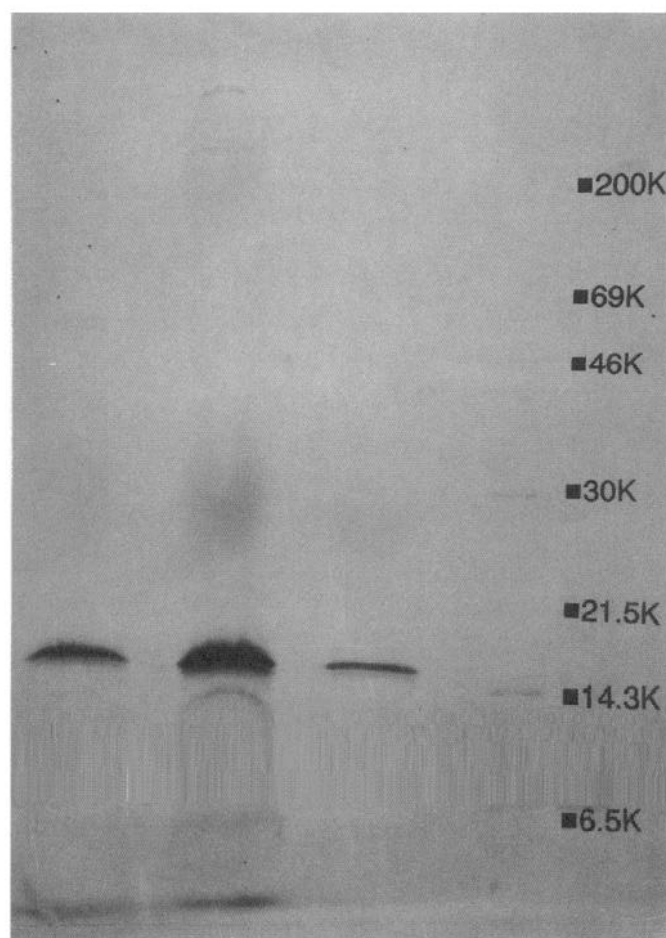
Figure 5. Characterization of ³⁵S-βAPP-C104 by SDS-PAGE analysis. The *left lane* shows the ligand at the end of the binding assay. The *middle lane* gives the initial profile of the ligand. The *right lane* shows the profile of the ligand that had dissociated from binding to the receptor (see Materials and Methods). The *last two lanes* contain ¹⁴C-labeled commercial molecular weight markers (Amersham). The molecular masses of the markers are also shown.

period (unbound) and ligand that had bound to the cells and been released (see Materials and Methods) were examined by SDS-PAGE. Before addition to the assay, most of the radioactivity was contained in a single band with an apparent mo-
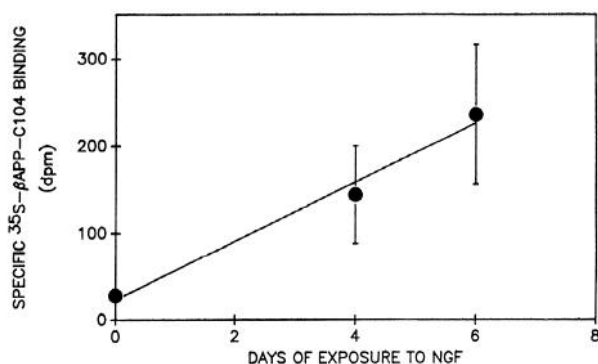

Figure 6. Increase in ³⁵S-βAPP-C104 binding to NGF-treated PC12 cells as a function of the duration of the exposure to NGF. Values are mean ± SEM of two to four experiments. Values from some experiments run at lower ligand concentrations were normalized to the standard concentration (100 pM). Time 0 represents cells that were not exposed to NGF.

**Table 1. Inhibition of ³⁵S-βAPP-C104 binding by various agents**

Inhibitor	IC$_{50}$
βAPP-C104 fragments	
βAPP-C104	1.7 nM
βAPP-C104 MutY	Inactive at 14 nM
Tachykinins	
Substance P	Inactive at 1 μM
Eledoisin	"
Physalaemin	"
Kassinin	"
Neurokinin A	"
Neurokinin B	"
Other peptides	
ACTH	Inactive at 10 μM
Bradykinin	"
Endothelin-1	"
Neurotensin	"
Oxytocin	"
Pentagastrin	"
Vasoactive intestinal peptide	"
Neuropeptide Y	"
Somatostatin	"
Glucagon	"
Fibroglast growth factor	"
Secretin	"
Leupeptin	"
Helodermin	"
Peptide YY	"

All substances were tested in triplicate.

lecular mass of 15 kDa (Fig. 5). This represents monomer βAPP-C104. A small amount of material was also present in two additional diffuse bands. One migrated at approximately twice the molecular weight of the major band and possibly represents a dimer of βAPP-C104. The other band had a much higher molecular weight and may be an aggregate. The gel profile of the unbound ligand at the end of the incubation period was identical to that of the ligand before incubation, indicating that the ligand had not been degraded. Similarly, the ligand released from binding had the same profile as the ligand originally added, with the possible exception of a slight reduction in the amount of high molecular weight material. These data show that the binding of βAPP-C104 ligand does not cause its modification.

Inhibition of binding of ³⁵S-βAPP-C104 to NGF-treated PC12 cells by conditioned medium from βAPP-C104–transfected PC12 cells

Having identified a binding site for ³⁵S-βAPP-C104 on the surface of NGF-treated PC12 cells, we then carried out a series of experiments to ascertain whether the binding of βAPP-C104 to these sites could account for its neurotoxicity. Because the degeneration caused by βAPP-C104 occurs gradually over a period of 11 d, it was not feasible to synthesize the amount of *in vitro*–translated βAPP-C104 necessary to maintain a reasonable concentration of the peptide in the cultures for the full course of each experiment. Hence, we carried out, in parallel with the binding studies, cell culture studies utilizing PC12 cells stably transfected with a retroviral recombinant expressing βAPP-C104
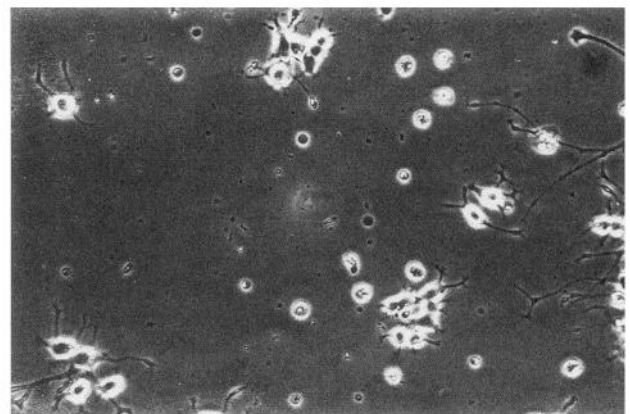
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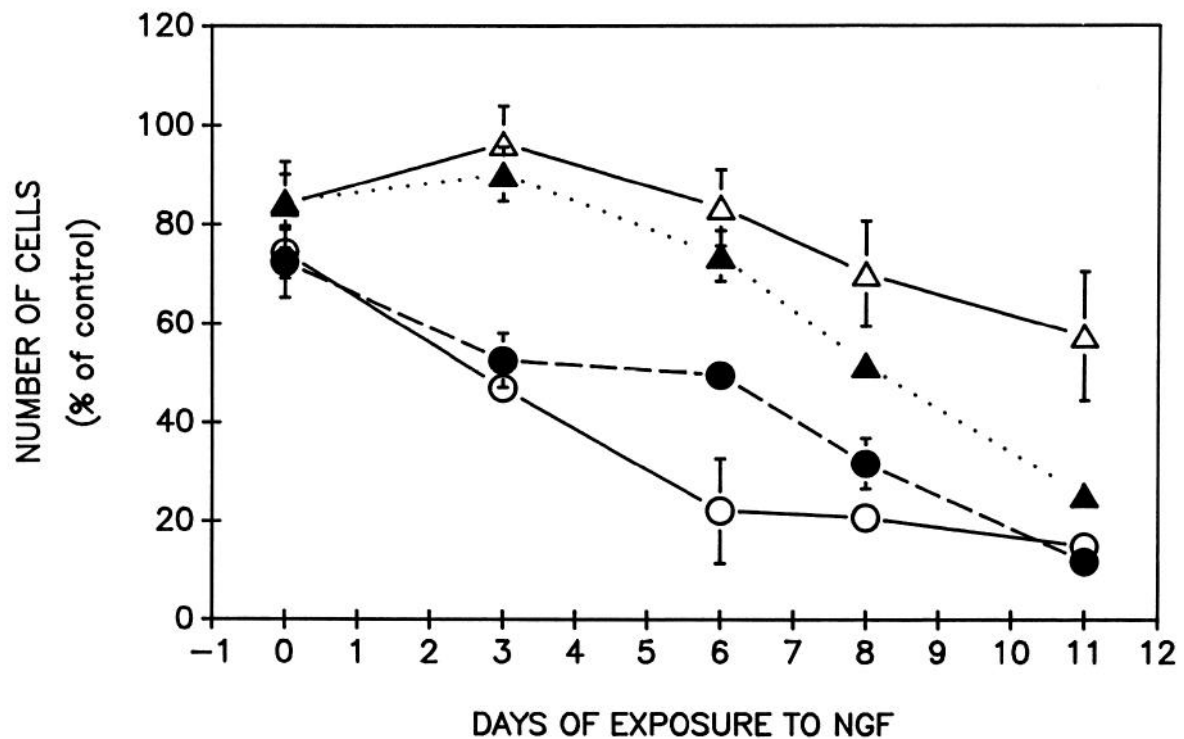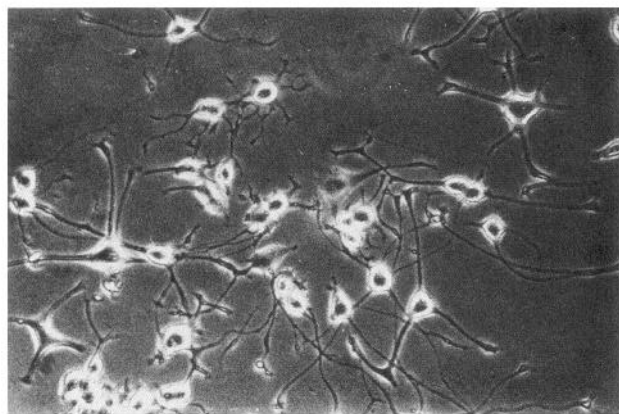
DO - PC12

β APP - C104 - PC12#1

B

Figure 7. *A,* NGF-induced cell loss in two independently isolated βAPP-C104 PC12 cells transfectants, #1 (*open symbols*) and #2 (*solid symbols*). The cells were cultured in serum-containing medium (*circles*) or serum-free medium (*triangles*). Values are mean ± SEM ($n = 4$) cell number as a percentage of control (DO-PC12 cells). Control cell number increased from 256 ± 7 on day 0 to either 681 ± 3 (serum-containing medium) or 429 ± 18 (serum-free medium) on day 11. In all cases, the decline in cell number in the βAPP-C104 transfected cell lines was statistically significant (*t* test, $p < 0.05$). *B,* Depiction of control (DO-PC12) and βAPP-C104 PC12 #1 cells after 6 d of treatment with NGF, showing cell loss and retraction of neurites in the βAPP-C104 transfected PC12 cells.

(Yankner et al., 1989) or using conditioned medium from cells expressing βAPP-C104 (see Materials and Methods).

We first showed that binding of ³⁵S-βAPP-C104 to NGF-treated PC12 cells was inhibited by conditioned medium from two different isolates of βAPP-C104–transfected PC12 cells, with half-maximal displacement of inhibitable binding occurring at dilutions of 1:10 to 1:32. Medium from cells transfected with the vector alone did not inhibit binding.

Dependence of both binding and neurotoxicity on exposure of cells to NGF

We had previously reported (Yankner et al., 1989) that PC12 cells stably transfected with a retroviral recombinant expressing βAPP-C104 degenerate when caused to differentiate into neuronal cells with NGF. Thus, the neurotoxicity appears only after treatment of the cells with NGF. We therefore tested the effect
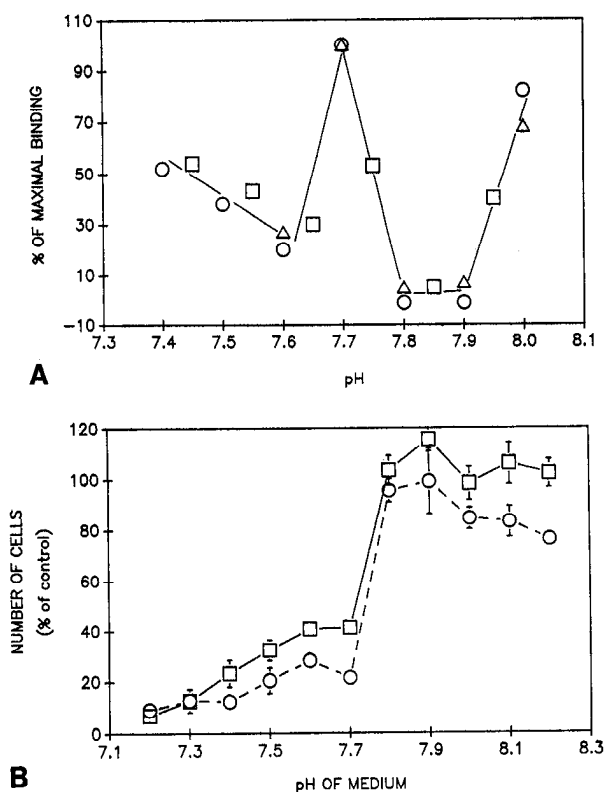
Figure 8. *A,* Dependence of ³⁵S-βAPP-C104 binding to NGF-treated (6 d) PC12 cells on pH. Results from three experiments are shown separately (*different symbols*). pH values were adjusted by 0.1 pH unit or less to bring the curves into register. Maximal binding was 498 dpm in two experiments and 110 dpm in the third (*triangles*). *B,* Effect of pH on NGF-induced cytotoxicity in βAPP-C104 PC12 #1 cells (*circles*) and βAPP-C104 PC12 #2 cells (*squares*) cells. Values are as in Figure 7. Cell number is expressed as a percentage of control (DO-PC12) cell number after 11 d culturing in the presence of NGF. Significant toxicity was found at pH values of 7.7 or less (isotonic regression, $p < 0.05$), but not at pH values of 7.8 or greater. Control cell number on day 11 (603 ± 14) did not vary with pH.

of NGF on βAPP-C104 binding to PC12 cells, and found that the amount of inhibitable ³⁵S-βAPP-C104 binding was dependent upon the duration of exposure of the PC12 cells to NGF. PC12 cells that were not exposed to NGF showed little inhibitable binding, whereas those cultured in the presence of NGF for 4 or 6 d showed progressively greater amounts of binding (Fig. 6).

To confirm and extend our earlier results (Yankner et al., 1989) demonstrating the neurotoxicity of βAPP-C104 in culture, we evaluated the effects of NGF on βAPP-C104–transfected cells in serum-containing and serum-free medium. Two independently isolated βAPP-C104 PC12 cell transfectants (termed AB1-PC12 #1 and #2 in Yankner et al., 1989) and a control vector-transfected PC12 cell line (termed DO-PC12 and described in Yankner et al., 1989) were treated with NGF in serum-containing medium (see Materials and Methods) or in serum-free medium (Ex-Cell 301, JRH Biosciences), both at pH 7.3. The results, shown in Figure 7, show that βAPP-C104–transfected PC12 cells, but not DO-transfected PC12 cells, gradually degenerate in the presence of NGF. The degeneration of the βAPP-C104–transfected PC12 cells was characterized by the

appearance of vacuolar inclusions in the cell body, retraction of neurites, and loss of attachment of the cells to the substrate. In many cases (see Fig. 9B), degenerating cells clumped together before floating into the medium; extensive fasciculation of processes was also evident among the remaining population of cells that did not show retraction of neurites.

Dependence of both binding and neurotoxicity on pH

A second variable affecting binding was pH. A marked effect of pH on binding was observed between pH 7.7 and 7.9 (Fig. 8A). Between pH 7.4 and 7.6, binding was relatively constant, or slightly declining. At approximately pH 7.7, there was a sharp increase in binding followed by a total loss at pH 7.8 to 7.9. Inhibitable binding was again seen at pH 8.0.

Because the ³⁵S-βAPP-C104 binding to NGF-treated PC12 cells was pH dependent, we examined the pH dependence of NGF-induced degeneration of βAPP-C104–transfected cells. Our initial studies of neurotoxicity in these cells had been carried with the medium at pH 7.3 (see above). We then compared the βAPP-C104 neurotoxicity in medium at pH 7.2 with that in medium at pH 8.2 (Fig. 9). Because we had noted that cell loss was more rapid in serum-containing than in serum-free medium, we chose to carry out these and subsequent experiments in the presence of serum. βAPP-C104 transfectants displayed no detectable degeneration at pH 8.2 even after 9 d of treatment with NGF, whereas neurodegeneration of the transfectants was apparent by day 6 of NGF treatment at pH 7.2. Because HEPES was used to maintain the 8.2 pH, we included a HEPES control at pH 7.2 to show that HEPES alone is not sufficient to inhibit βAPP-C104 neurotoxicity.

To examine in greater detail the pH dependence of βAPP-C104 toxicity, we exposed control and experimental transfected PC12 cell lines to NGF in media at pH ranging from pH 7.2 (medium at pH lower than 7.2 was slightly toxic to control cells) to pH 8.2 in 0.1 pH unit increments (Fig. 8B). It is clear that the neurotoxicity of βAPP-C104 is dependent upon pH and is almost completely inhibited at pH 7.8 or above. Notably, binding of βAPP-C104 to NGF-treated PC12 cells is virtually lost at pH 7.8 and 7.9 (Fig. 8A).

Lack of inhibition of βAPP-C104 binding and neurotoxicity by tachykinins

As stated above, binding of βAPP-C104 to NGF-treated PC12 cells was not significantly inhibited by a number of tachykinins (Table 1). We tested the ability of the tachykinins listed in Table 1 (with the exception of neurokinin A) to inhibit βAPP-C104 neurotoxicity. We had previously transfected NIH 3T3 cells with the same βAPP-C104 retroviral recombinants and control vector that we used to transfect the PC12 cells (Yankner et al., 1989). We had shown that conditioned medium from the βAPP-C104 transfected 3T3 cells, but not from the vector-transfected 3T3 cells, was toxic to NGF-differentiated PC12 cells. To examine the effect on βAPP-C104 neurotoxicity of the tachykinins, we added βAPP-C104 NIH 3T3–conditioned medium containing NGF, or control conditioned medium containing NGF together with 20 μM of each tachykinin (as described in Yankner et al., 1990), to PC12 cells. The cells exposed to βAPP-C104–conditioned medium, but not the cells exposed to control conditioned medium, degenerated over a period of 8 d; the degeneration was not inhibited by the presence of any of the tachykinins tested ($n = 12$ for each condition; data not shown).

A
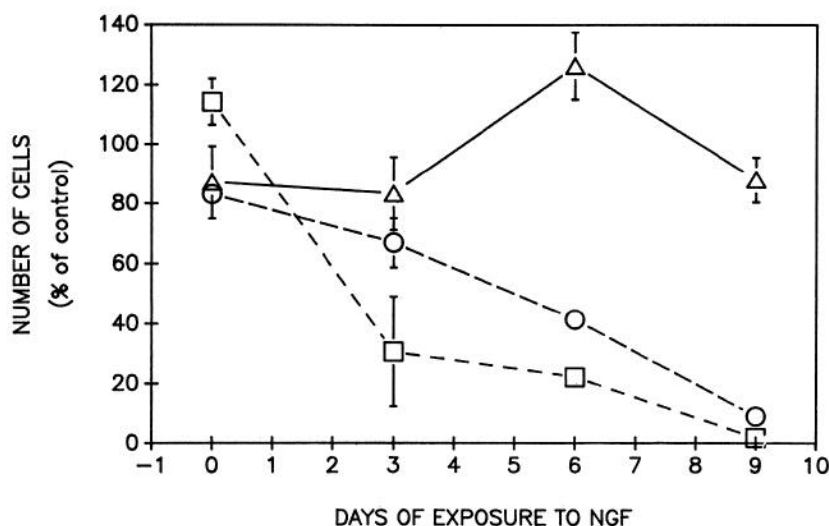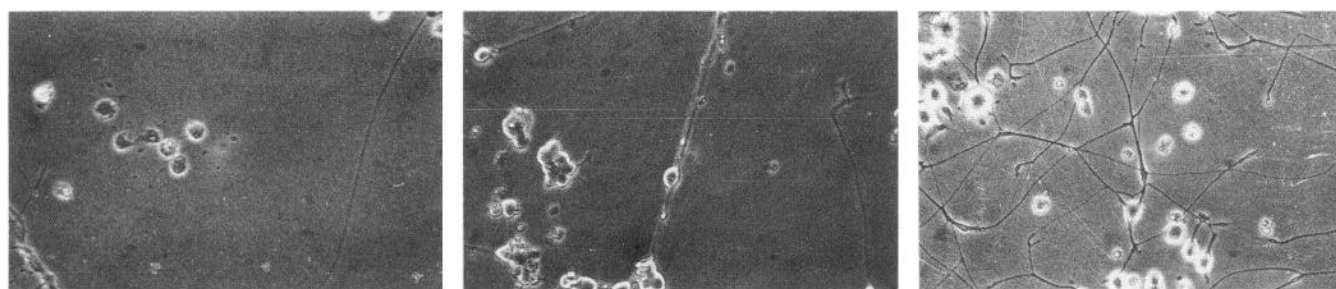
β APP - C104 - PC12 #1 pH 7.3   β APP - C104 - PC12 #2 pH 7.3   β APP - C104 - PC12 #1 pH 8.2

B

Figure 9. *A,* Comparison of NGF-induced toxicity in βAPP-C104 transfected cells grown at pH 7.2 versus pH 8.2. βAPP-C104 PC12 #1 cells were cultured in the presence of NGF at either pH 7.2 in phosphate-buffered medium (*circles*) or HEPES-buffered medium (*squares*), or at pH 8.2 in HEPES-buffered medium (*triangles*). Values are as in Figure 8. There was a significant decline in cell number with time in both buffers at pH 7.2 (t test, $p < 0.05$), but no change at pH 8.2. Similar results were obtained with βAPP-C104 PC12 #2 cells. Control cell (DO-PC12) numbers increased from 263 ± 22 (phosphate-buffered medium), 156 ± 20 (HEPES, pH 7.2), and 269 ± 12 (HEPES, pH 8.2) on day 0 to $353 + 8$, 221 ± 55, and 821 ± 115, respectively, on day 9. *B,* Depiction of βAPP-C104 PC12 #1 and #2 at pH 7.3 (HEPES), and of βAPP-C104 PC12 #1 at pH 8.2 (HEPES) after 9 d of exposure to NGF.

Mutational analysis of βAPP-C104 neurotoxicity and binding

By comparing the sequence of βAPP-C104 with that of other molecules with known functions, we identified at least two sites within βAPP-C104 that may play a role in its binding and neurotoxicity. The amino terminus of βAPP-C104 contains the tripeptide aspartic acid–serine–glycine (residues 603–605 of βAPP-695), which is conserved in the active site of serine proteases (Hartley, 1970). Roberts (1986) has suggested that β/A4 or a similar βAPP fragment may have serine protease activity and cause neurodegeneration by virtue of such activity. If βAPP-C104 has serine protease activity, and if this activity is implicated in its neurotoxicity, it should be possible to neutralize its neurotoxicity by removing the serine residue in the putative serine protease active site. We used oligonucleotide-directed *in vitro* mutagenesis to change the serine at residue 604 (using the numbering system of Kang et al., 1987, for βAPP-695) to an alanine. The mutant was termed βAPP-C104 MutS. Two independent stable NIH 3T3 cell transfectants expressing this mutant mRNA at levels equivalent to or exceeding the βAPP-C104

mRNA levels in nonmutated transfectants were isolated and their conditioned media assayed for neurotoxicity by adding the media to NGF-treated PC12 cells (see Materials and Methods). The conditioned media from the βAPP-C104 MutS 3T3 cells was as toxic to NGF-treated PC12 cells as was the βAPP-C104–conditioned medium ($n = 8$ for each condition; data not shown). Thus, the replacement of serine 604 with an alanine in βAPP-C104 has no effect on its toxicity.

The carboxy terminus of βAPP-C104 contains a tyrosine (687 in βAPP-695) that lies within a sequence homologous to that surrounding a phosphorylated tyrosine in integrins and some plasma membrane receptors (reviewed by Tamkun et al., 1986). This homology suggests that tyrosine 687 in βAPP-C104 might be important to its function. We used site-directed mutagenesis to replace the tyrosine with a phenylalanine, and selected two stable NIH 3T3 cell transfectants (termed βAPP-C104 MutY) expressing the mutant mRNA at appropiate levels. The conditioned medium of these transfectants was not toxic to NGF-treated cells, although conditioned medium of a nonmutated βAPP-C104 3T3 cell transfectant tested at the same time was
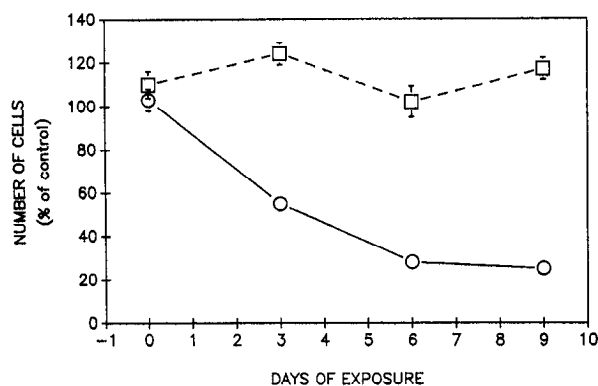
Figure 10. Comparison of the cytotoxicity to NGF-treated PC12 cells of conditioned medium from NIH 3T3 cells transfected with either βAPP-C104 (*circles*) or βAPP-C104 MutY (*squares*). Values are as in Figure 8. Control (DO-PC12) cell number increased from 158 ± 14 on day 0 to 236 ± 16 on day 9. Conditioned medium from a second βAPP-C104 MutY NIH 3T3 transfectant gave similar results (data not shown).

toxic to the NGF-treated cells (Fig. 10). In accordance with these data, βAPP-C104 MutY made in an *in vitro* transcription–translation system did not significantly inhibit ³⁵S-βAPP-C104 binding to NGF-treated PC12 cells at concentrations up to 14 nM ($n = 3$; Table 1), indicating that this mutant is at least 2.5 log units less potent in its binding affinity than is βAPP-C104.

Discussion

The present results show that the ³⁵S-βAPP-C104 binding site expressed by NGF-treated PC12 cells has several receptorlike properties. First, it binds the ligand reversibly, as shown in the dissociation experiments. Second, it specifically recognizes the ligand in that binding is inhibited by unlabeled βAPP-C104 but not inhibited by other peptides, including tachykinins. Furthermore, a small modification of βAPP-C104 (the mutation of tyrosine 687) abolishes binding activity. Third, βAPP-C104 is not altered after binding as shown by SDS-PAGE analysis of the ligand, suggesting that the binding site is not an enzyme or, at least, not an enzyme that metabolizes βAPP-C104 under these assay conditions. Finally, it has high (nanomolar) affinity and low capacity, as would be expected for a receptor.

Three of the present results provide circumstantial evidence that this binding site mediates the toxic effects of βAPP-C104 on differentiated PC12 cells. First, the binding site is much more prevalent on PC12 cells rendered susceptible to the toxic effects of βAPP-C104 by treatment with NGF than on nontreated cells, to which the peptide is not toxic (Yankner et al., 1989). The increase in βAPP-C104 binding following NGF treatment is not due to the increased cell surface area that accompanies differentiation since, in the present study, the cells are not attached and therefore do not extend neurites. Second, a loss of both binding and toxicity occurs near pH 7.8. Third, a mutation that eliminates the neurotoxic efficacy of βAPP-C104 (tyrosine 687) also abolishes its ability to bind to the site identified in this study.

The relationship between binding and pH may be due to a pH-related aggregation of the ligand. The increase in βAPP-C104 binding to NGF-treated PC12 cells at pH 7.7 could result from low-order aggregation of the ligand, which would have little effect on receptor occupancy but would result in the binding

of small aggregates of ligand at each site. If the extent of aggregation increases with increased pH, the binding domains of the ligand may become inaccessible, resulting in decreased receptor occupancy. Finally, when the aggregates become large enough, the effect of low receptor occupancy would be offset by the binding of large aggregates of the ligand at each site, resulting in the return of measurable amounts of binding.

It is surprising that the binding site for βAPP-C104 does not recognize tachykinins. β/A4 (the amyloid encoding sequence of βAPP) has also been reported to be neurotoxic and to have its toxicity reversed by various tachykinin peptides (Yankner et al., 1990). Because β/A4 is contained within the βAPP-C104 domain, it seems reasonable to expect that both peptides would have a similar site of action and therefore would be inhibited by similar agents. The present data do not support this idea. Tachykinins inhibit neither βAPP-C104 binding nor its neurotoxicity to NGF-treated PC12 cells.

Mutational analysis of βAPP-C104 neurotoxicity and binding indicates that alteration of a serine in a putative serine protease active site within the β/A4 coding sequence, at the amino terminus of βAPP-C104, does not affect its neurotoxicity. However, alteration of a tyrosine located nine residues from the carboxy terminus of βAPP-C104 (tyrosine 687) abolishes both the neurotoxicity of the molecule and also its ability to inhibit binding of ³⁵S-βAPP-C104 to NGF-treated PC12 cells. This tyrosine is contained within a sequence of amino acids homologous to those surrounding phosphotyrosines in integrins and some growth factor receptors, suggesting that phosphorylation of tyrosine 687 may be a requirement for the binding and neurotoxic activities of βAPP-C104.

Degeneration of PC12 cells exposed to βAPP-C104 is characterized by the appearance of vacuolar inclusions in the cell body, accompanied by retraction of neurites and loss of adherence of cells to the substrate. The cells that remain tend to clump together, and to display fasciculation of processes. Primary rat cortical or hippocampal neurons treated with βAPP-C104–conditioned medium exhibit the same pattern of degeneration (A. Spanoyannis and R. L. Neve, unpublished observations). These characteristics suggest that βAPP-C104 may cause cell loss by virtue of its interference with cell-substrate interactions during process outgrowth. Isolation and identification of the cell surface receptor to which βAPP-C104 binds will aid us in defining the mechanism by which this molecule causes neurodegeneration.

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