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Amidation of β-Amyloid Peptide Strongly Reduced the Amyloidogenic Activity Without Alteration of the Neurotoxicity

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Abstract: β-Amyloid accumulates in cerebral deposits in Alzheimer's disease, so to test the correlation between the neurotoxic and fibrillogenic capacity of β-amyloid, we synthesized a peptide homologous to fragment 25–35 of β-amyloid (β25–35) and amidated at the C-terminus (β25-35-NH_2). As the amidation strongly reduced the amyloidogenic capacity of β25–35, we compared its neurotoxic activity in the amidated (β25–35-NH_2) and nonamidated forms. The viability of primary cultures from fetal rat hippocampus was reduced in a dose-related manner (10–100 μ*M*) similarly by β25–35 and β25–35-NH_2, whereas a scrambled peptide, amidated or nonamidated, did not alter the neuronal viability. The neurotoxic activity of β25–35-NH_2 is mediated by apoptosis as demonstrated by morphological and biochemical investigations. Electron microscopy examination of culture media with β25–35 or β25–35-NH_2 incubated with neuronal cells for 7 days confirmed the high level of fibrillogenic activity of β25–35 and the almost total absence of fibrils in the solution with β25–35-NH_2. Furthermore, staining with thioflavine S was used to identify amyloid fibrils, and only the cultures exposed to β25–35 exhibited intense staining associated with neuronal membranes. These data indicate that the neurotoxic activity of the β-amyloid fragment is independent of the aggregated state of the peptide. **Key Words:** β-Amyloid peptide—Amidation—Alzheimer's disease—Thioflavine S—Apoptosis—Hippocampal cultures.
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Alzheimer's disease (AD) is characterized neuropathologically by senile plaques (SP), tangles, and cortical atrophy. β-Amyloid (βA) is the major component of SP and is deposited in cortical and meningeal blood vessels of the AD brain. βA derives from a larger transmembrane glycoprotein precursor [amyloid precursor protein (APP)] with multiple isoforms generated by differential splicing of a gene mapped on the human chromosome 21 and highly conserved across the species (Selkoe, 1991; Forloni et al., 1996). Numerous studies have drawn attention to βA and its essential role in the development of AD: Linkage studies have shown an association between APP gene mutations and some cases of familial early-onset AD (Goate et al., 1991); a neurotoxic effect of synthetic peptides homologous to βA and its fragments has been described in vitro; and mutations of the APP gene have been found in early-onset AD families and neurodegenerative transgenic mice carrying mutated human APP (Games et al., 1995) or βA (LaFerla et al., 1995) have been bred.

In vitro studies using synthetic peptides homologous to βA and its fragments showed that their neurotoxicity was associated with the self-aggregation capacity of peptides and that the βA fragment 25–35 (β25–35) retained the biological activity of whole βA (Yankner et al., 1990; Abe and Kimura, 1996; Yan et al., 1996). We and others have demonstrated that the neuronal cell death induced by βA is associated with the stereotyped events characteristic of apoptosis (Forloni et al., 1993*b*; Loo et al., 1993), and electron microscopy examination of negatively stained preparations shows that peptide homologues to β25–35 assembled into long unbranched fibrils (Forloni et al., 1993*b*).

These findings were in agreement with numerous studies supporting the concept that amyloid aggregate formation is vital in the development of neuropathological signs of AD. Morphological examination supported the association between amyloid fibrils and neurodegeneration. Neuronal death was found in combination with Congo red-stained deposits of βA around the cells (Busciglio et al., 1992). Roher et al. (1991)

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Abbreviations used: ACT, α1-antichymotrypsin; AD, Alzheimer's disease; APP, amyloid precursor protein; βA, β-amyloid; β25–35, βA fragment 25–35; β25–35-NH_2, amidated β25–35; β25–35sc, scrambled β25–35; SP, senile plaques.

reported the presence of βA around the neuronal soma and collected along the pathological neuritic arbor in sympathetic neurons exposed for 2–4 days to amyloid plaque core proteins purified from AD brain. Pike et al. (1992) observed that cultured hippocampal neurons exposed for 2 days to aggregates of synthetic βA acquired a dystrophic appearance. The degenerative changes, including beading, fragmentation, and terminal swelling, are similar to those observed in neurites associated with SP. However, recent studies have cast doubt on the correlation between neurotoxicity and the aggregate state. Heparin and chondroitin sulfate glycosaminoglycan, two proteoglycans localized in SP and believed to promote βA aggregation, attenuated the toxicity of β25–35 (Woods et al., 1995). Preaggregation of β1–42 abolishes the peptide's ability to induce degeneration of human cerebrovascular smooth muscle cells (David-Salinas and Van Nostrand, 1995; David-Salinas et al., 1995).

To test directly the effect of the conformational state of βA peptide in neurotoxicity, we synthesized β25–35 peptide amidated at the C-terminus (β25–35-NH_2); according to Terzi et al. (1994*a,b*), the amidation strongly reduces the peptide's fibrillogenic activity. We verified the low level of self-aggregation activity in a solution of this peptide compared with that of β25–35 by electron microscopy after 7 days of incubation. The presence of fibrillar material associated with neuronal cells exposed to β25–35-NH_2 or β25–35 peptide was investigated by thioflavine S staining. The neurotoxicities of β25–35-NH_2 and the normal peptide were compared by exposing rat hippocampal cells to the peptides for 5–7 days.

MATERIALS AND METHODS

Peptide synthesis

β25–35 (GSNKGAIIGLM) and scrambled peptide (INMAGGKSLGI) were synthesized using solid-phase chemistry with a 430A instrument (Applied Biosystems). FMOC (9-fluorenylmethoxycarbonyl) was used as the protective group for amine residues, and 1-hydroxybenzotriazole and *N,N*-dicyclohexylcarbodiimide as activators of carboxylic residues. Peptides were purified by crystallization and analyzed by HPLC. Their composition was determined by amino acid analysis using a Beckman system 6300 amino acid analyzer and amino acid sequencing in a 477A microsequencer (Applied Biosystems). The amidated form of β25–35 and scrambled β25–35 (β25–35sc; in which the carboxylic residue was blocked with a nonionizable amide group) were synthesized starting with a 4-methylbenzhydrylamine resin as an anchor for the carboxylic group. The solutions of peptides were dissolved in sterile water at the concentration of 2 m*M* immediately before the application to culture preparation.

Tissue culture

Brains were removed from fetal rats on embryonic day 17. Hippocampal cells were dissociated in serum-free medium containing 0.1% trypsin (Difco) and 25 μg/ml deoxyribonuclease for 5 min at room temperature and plated (5×10^5 cells/ml) in Primaria (Falcon) 15-mm dishes or 96-well dishes, precoated with poly-D-lysine (50 μg/ml; Sigma). The cells were cultured in basal medium Eagle (BME-Hanks' salt; GIBCO) supplemented with 10% fetal calf serum (GIBCO) and glutamine (2 m*M*). Cultures were kept at 37°C in a humidified CO_2 atmosphere. After 5 and 7 days in vitro, nonneuronal cell division was halted by exposure to 10^{-5} *M* cytosine arabinoside, an inhibitor of mitosis, to prevent overgrowth of glial cells. The neuronal cell cultures are characterized elsewhere (Forloni et al., 1993*b*).

For a single exposure to β25–35, a sterilized solution of the peptide was added to the medium after 10 days of culture and the effect was observed for the next 4 days. Chronic treatment with β25–35 or unrelated peptides started on the first plating day and was repeated every 2 days. Neuronal cell death was assessed quantitatively using crystal violet (0.5% in water/methanol, 4:1). After washing, the cells were dried and dissolved in sodium citrate/ethanol (1:1) and analyzed spectrophotometrically at 540 nm with an automated micro plate reader (Perkin–Elmer λ reader). The absorbance of the solution is proportional to the number of cells as shown by Andreoni et al. (1997). Apoptotic nuclei were visualized using Hoechst 33258 (Calbiochem). The cells, grown on a coverslip precoated with poly-D-lysine, were fixed in methanol/acetic acid (3:1), washed in phosphate-buffered saline, incubated at 37°C for 10 min with Hoechst 33258 (0.4 μg/ml), and washed in distilled water (Forloni et al., 1993*a*).

To evaluate the DNA fragmentation, neuronal cells from 15-mm wells were washed with phosphate-buffered saline, pH 7.4, lysed in ice-cold lysis buffer (10 m*M* Tris-HCl, pH 8, 20 m*M* EDTA, 0.5% Triton X-100), and kept on ice. After 15 min, samples were centrifuged (13,000 *g*, 20 min). The supernatant was transferred to other tubes and incubated with DNase-free RNase A (100 μg/ml); the pellets were incubated with proteinase K (75 μg/ml) in 50 m*M* Tris-HCl, pH 8, 10 m*M* EDTA, and 0.5% *N*-lauroylsarcosine overnight at 48°C. Both supernatants and pellets were then extracted with phenol/chloroform solutions, and the aqueous phase was precipitated in a solution of 0.5 *M* NaCl/isopropanol (1:1, vol/vol) overnight at −70°C. DNA was resuspended in 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA. DNA samples were electrophoresed through 1.2% TAE-agarose gel, visualized by staining with ethidium bromide (0.5 μg/ml in distilled water), and photographed with Polaroid 667 film.

Electron microscopy

Aggregation properties of the peptides were investigated directly in 500 μl of medium exposed to neuronal cells for 24 h or 7 days containing β25–35 or β25–35-NH_2 at 50 μ*M*. The solutions were centrifuged [8,000 rpm (4,600 *g*) × 10 min], and the pellets were resuspended in 70 μl of distilled water. Ten microliters of the suspension was applied to carbon-stabilized Formvar-coated copper grids; after removal of the sample, the grids were negatively stained with 5% aqueous uranyl acetate. Grids were examined under a transmission electron microscope (CM10 Philips) operating at 80 kV.

Thioflavine S staining

To identify fibrillar material associated with cell membranes, neurons cultured in chamber slides (Nunc, Germany)

and exposed to the peptides for 7 days were fixed with alcohol (100%) and stained with thioflavine S (1% in aqueous solution) (Moechars et al., 1996). After prolonged washing with water, the cultures were mounted under a coverslip with glycerine/water (3:1) and examined with a fluorescent microscope (Axiophot, Zeiss, Germany).

RESULTS

In our cell culture conditions, β25–35 induced neuronal death only after chronic exposure (5–7 days) to micromolar concentrations (Forloni et al., 1993*b*). We compared the neurotoxic activity of the amidated form of β25–35 with that of β25–35. In preliminary experiments (data not shown), we tested the effect of β25–35-NH_2 at different exposure times, and the results were similar to those with β25–35 (Cafè et al., 1996). There was no real reduction in cell viability after 24 h or 3 days of exposure, but a dramatic dose-related effect was observed after 7 days. (Fig. 1): β25–35 significantly reduced neuronal survival starting from 25 μ*M* (40%), and the maximal effect was evident at 100 μ*M* with a reduction of ~70%. Similar neurotoxic activity was seen with β25–35-NH_2, and the effect of the amidated peptide was indistinguishable from that of the normal peptide, although the reduction of cell viability was significant starting from 10 μ*M*.

To test the effect of amidation per se on neuronal survival, we synthesized a β25–35 scrambled and amidated on the C-terminus. As shown in Fig. 2, after 7 days of exposure, β25–35 and β25–35-NH_2 (50 μ*M*) had similar effects on cell death, whereas β25–35sc or β25–35sc-NH_2 did not alter cell viability.

We investigated whether the neuronal death induced by β25–35-NH_2 was mediated by an apoptotic mechanism, as shown for β25–35 (Forloni et al., 1993*b*). Hippocampal cells were exposed to β25–35 or β25–35-NH_2, and apoptosis was studied with morphological

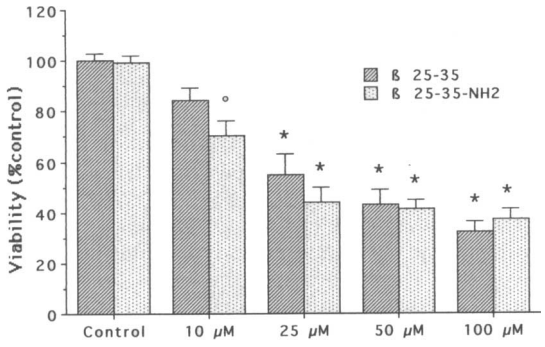

FIG. 1. Dose–response relationship of the neurotoxic effect of β25–35 or β25–35-NH_2. Primary rat hippocampal cells were exposed to the peptides for 7 days starting from the first day of culture. Cell viability was determined by densitometric analysis of surviving cells stained with crystal violet. The data are the means ± SE of six to eight determinations. °$p < 0.05$, *$p < 0.01$, versus respective control (Dunnett's test).

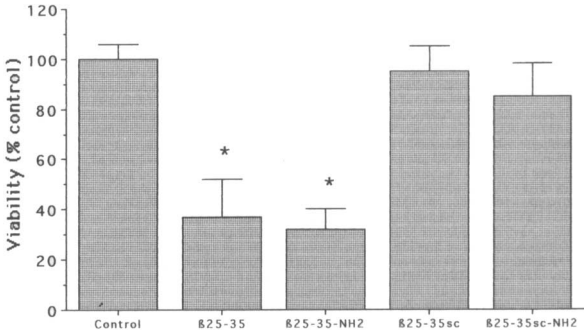

FIG. 2. Similar neurotoxic effects of β25–35 and β25–35-NH_2 and the scrambled peptides. Primary rat hippocampal cells were exposed to the peptides for 7 days starting from the first day of culture. Cell viability was determined by densitometric analysis of surviving cells stained with crystal violet. The data are the means ± SE of six to eight determinations. *$p < 0.01$, versus respective control (Dunnett's test).

and biochemical methods. Agarose gel electrophoretic analysis of DNA extracted from cultured cells after 7 days of treatment with β25–35 or β25–35-NH_2 showed a characteristic pattern of fragmentation in both conditions, visualized by ethidium bromide, resulting from cleavage of nuclear DNA in cells of internucleosomal regions (Fig. 3D). Morphological examination of cell nuclei stained with DNA-binding fluorochrome showed that several hippocampal neurons chronically exposed to β25–35 or β25–35-NH_2 presented similar apoptotic morphology with condensation of chromatin and fragmentation of the nucleus, whereas in the control condition this phenomenon was absent (Fig. 3A–C). β1–40 also induced cell death mediated by apoptosis (Loo et al., 1993) consistently with the activation of cytoplasmic Ca^{2+} and oxidative stress induced by βA peptides (Mattson and Rydel, 1992; Cafè et al., 1996). In fact, the increase of intracellular calcium has been interpreted as a primary signal in several models of apoptosis (McConkey et al., 1989), and the excess of reactive oxygen species has often been associated with programmed cell death (Buttke and Sandstrom, 1994). The similar capacity to activate apoptosis for amyloidogenic and nonamyloidogenic β25–35 peptide indicates a common mechanism of neurotoxicity independent of the conformational state of peptide.

Although previous physicochemical data showed the low level of fibrillogenic activity of β25–35-NH_2 (Terzi et al., 1994*a*,*b*), we directly investigated the presence of peptide fibrils in our cell culture condition. Media containing β25–35 or β25–35-NH_2 at 50 μ*M* were examined by electron microscopy after 7 days of exposure to hippocampal cells. The electron micrographs in Fig. 4 show the abundance of fibrils in the medium with β25–35, whereas in the solution with β25–35-NH_2 only very rare fibrils were seen. These results confirm the low level of amyloidogenic activity

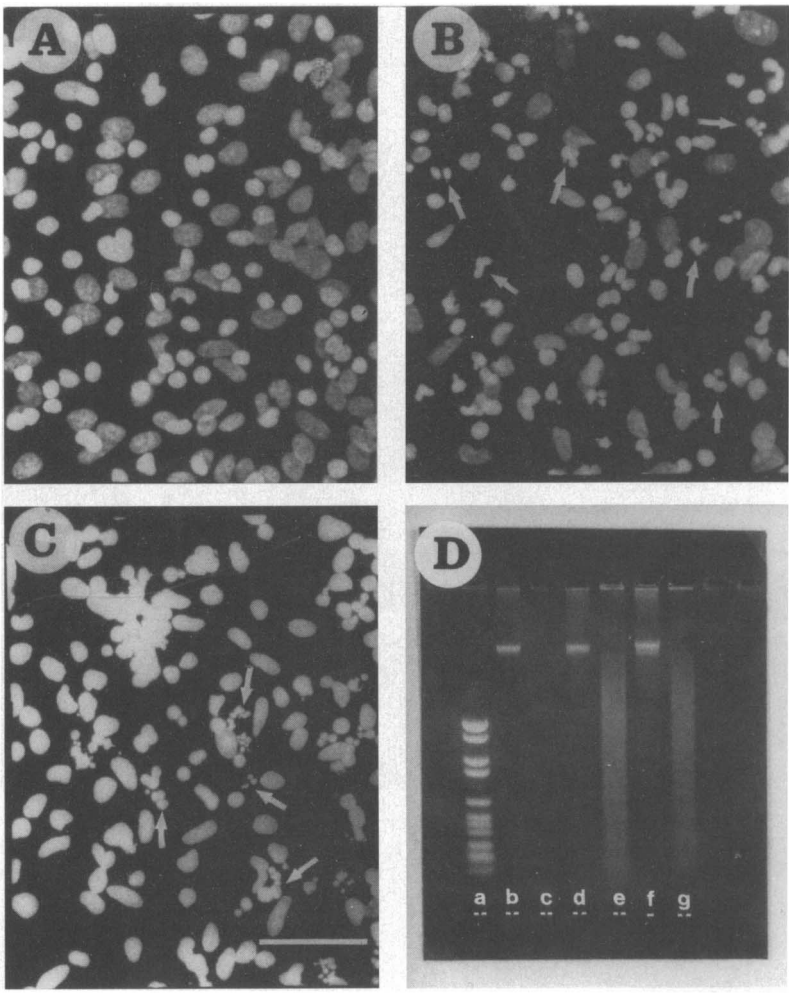

FIG. 3. Rat hippocampal nuclei visualized using Hoechst 33258. Cells were treated chronically (5 days) with saline (**A**) or with β25–35 (**B**) or β25–35-NH_2 (**C**) at 50 μ*M*. Several fragmented nuclei with apoptotic features (arrows) are evident in B and C, but absent in the control condition (A). **D:** The laddering of DNA is shown. Lane a, DNA standards; DNA from neurons treated with vehicle (lanes b and c), β25–35 at 50 μ*M* (lanes d and e), or β25–35-NH_2 at 50 μ*M* (lanes f and g). The DNA fragmentation observed in lanes e and g is comparable. Bar = 50 μm

of β25–35-NH_2 compared with β25–35. The formation of amyloid fibrils in cultures was examined by staining with thioflavine S (Mazur-Kolecka et al., 1995) in neuronal cells exposed to β25–35 or β25–35-NH_2. Positive thioflavine S staining surrounding cell bodies was evident in neuronal cells exposed to β25–35, whereas in the cells treated with β25–35-NH_2, the staining was indistinguishable from the control condition (Fig. 5). As batch-to-batch differences in fibrillogenic and neurotoxic activity of βA peptides have been shown (Busciglio et al., 1992; May et al., 1992; Howlett et al., 1995), three batches of both preparations, β25–35 and β25–35-NH_2, were tested and the results were similar. The neurotoxic activity was comparable for β25–35 and β25–35-NH_2, whereas β25–35-NH_2 exhibited a lower level of amyloidogenic activity.

DISCUSSION

The data indicate that the fibrillogenic capacity of β25–35 does not influence its neurotoxic activity. This finding is at variance with the ample data accumulated in the last 5 years that showed that the self-aggregation activity of βA fragments is correlated with their neurotoxic activity. This correlation was suggested originally by Pike et al. (1991), who showed that hippocampal cells were insensitive to freshly prepared β1–42, whereas their viability decreased when they were exposed to β1–42 previously stored for 2–4 days at 37°C. Later, the close relationship between the neurotoxic and fibrillogenic activity of βA fragments was supported by a structure–activity study showing that alteration of β33–35 residues disrupts the β-sheet structure of β1–42 and, consequently, its aggregation capacity and neurotoxicity, whereas other substitutions or deletions of β1–42 and β25–35 residues never dissociated the neurotoxicity and self-aggregation activity (Pike et al., 1995). These results are supported by neuropathological observations, neuronal alterations being evident in SP with amyloid deposits, whereas the "preamyloid" noncongophilic formations containing soluble βA were not associated with dystrophic neurites (Tagliavini et al., 1988). However, some data

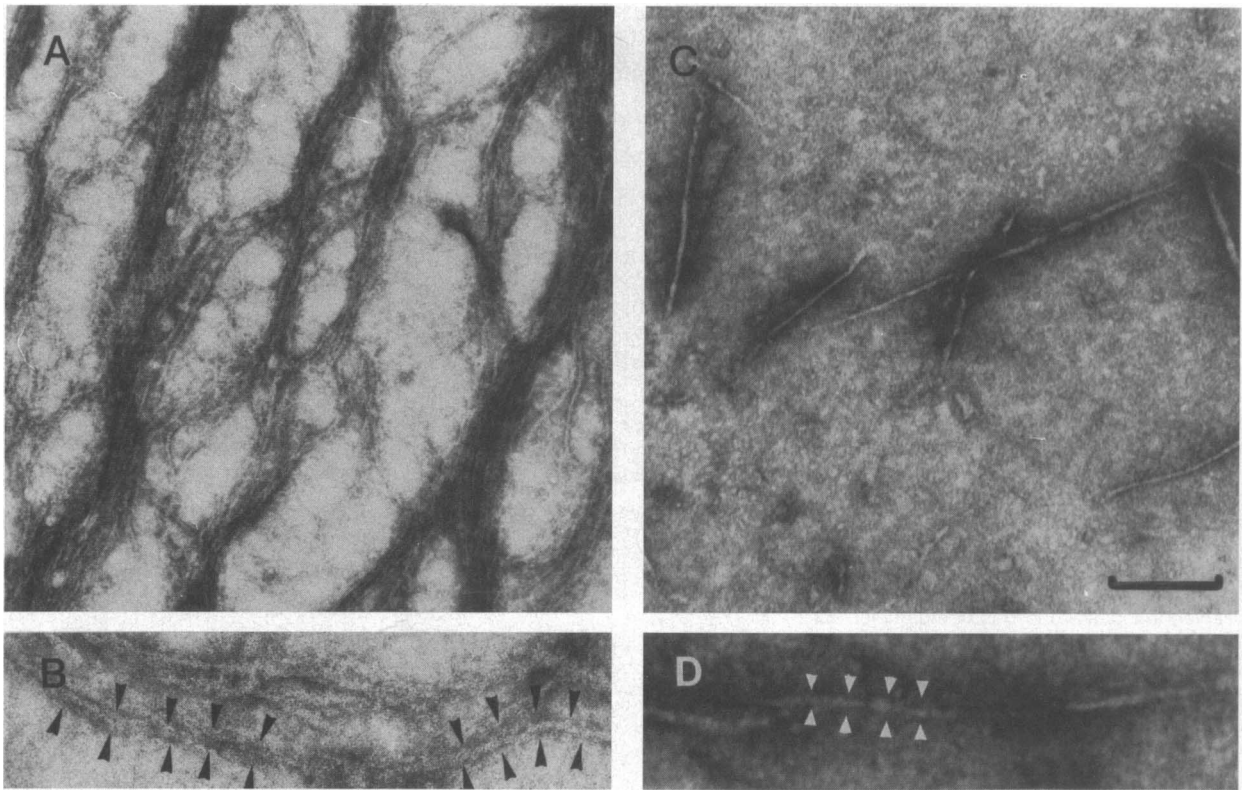

FIG. 4. Electron micrographs of negatively stained fibrils of β25–35 (**A** and **B**) and β25–35-NH_2 (**C** and **D**). Culture media containing β25–35 or β25–35-NH_2 at 50 μ*M* were incubated with rat hippocampal cells for 7 days and then examined (see Materials and Methods). β25–35 assembled in a dense network (A) of filaments, shown at higher magnification in B; filaments formed by β25–35-NH_2 were isolated and rarely present in the preparation (C), with ultrastructural differences (D) from the β25–35 filaments. Bar = 50 nm in A and C and 20 nm in B and D.

have emerged against the association of fibrillogenic activity and neurotoxicity: β25–35 toxicity was attenuated by heparin and chondroitin sulfate glycosaminoglycan, two proteoglycans localized in SP believed to promote βA aggregation (Woods et al., 1995); preaggregation of β1–42 abolishes the peptide's ability to induce the degeneration of human cerebrovascular smooth muscle cells (David-Salinas and Van Nostrand, 1995; David-Salinas et al., 1995). Aksenov et al. (1996*a*) reported that glutamine synthetase enhanced βA neurotoxicity, while preventing fibril formation. Several treatments have been proposed to reduce βA toxicity by an antiamyloidogenic mechanism that prevents the self-aggregation of βA (Camilleri et al., 1994; Tomiyama et al., 1994; Pollack et al., 1996). However, the neuroprotective activity of two of these, rifampicin and sulfated compounds, was independent of their antiamyloidogenic activity. The neuroprotective activity of rifampicin may involve its scavenging ability on hydroxyl free radicals generated by βA peptides (Tomiyama et al., 1996), whereas the sulfated compounds, including Congo red, reduced βA toxicity by inhibition of the association of βA with cells, independently of the inhibition of βA aggregation (Sadler et al., 1996). The serine protease inhibitor α1-antichymotrypsin (ACT), which colocalizes with amyloid deposits in AD and aged human brain (Abraham et al., 1988), interacts with β1–40 and β1–42 apparently with opposite effects on the fibrillar capacity of the peptides. Studies by Abraham et al. (1988) have demonstrated that ACT promotes the β1–42 aggregation, whereas an inhibition of β1–40 fibrillization by ACT has been shown by Ma et al. (1994). However, in contrast with a direct relationship between neurotoxicity and fibril formation, ACT reduces the neurotoxicity induced by β1–42 (Aksenov et al., 1996*b*) and does not affect the β1–40 toxicity (Aksenova et al., 1996).

Thus, although the aggregation of βA peptides is crucial in the development of neuropathological events in AD, these data indicate that the aggregated state of βA peptides is not essential for their neurotoxic activity. The assembled peptide appears to interact better with the lipid membrane than the monomeric form (Good and Murphy, 1995). However the monomeric form may pass through the membrane so the state of aggregation only has a positive influence on this passage, rather than the entire βA fibril interacting with the membrane and triggering the biological response.

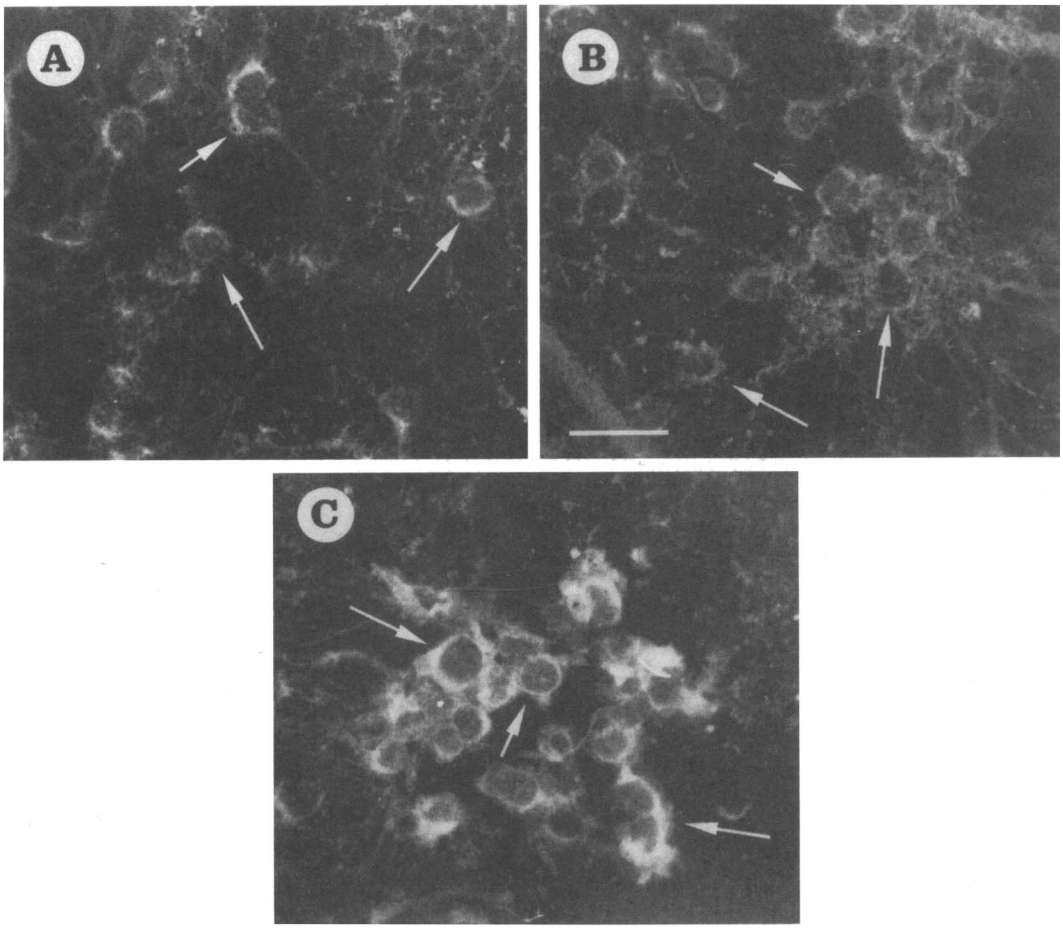

FIG. 5. Photomicrographs of cortical neurons chronically exposed to vehicle (**A**), β25–35-NH_2 (**B**), and β25–35 (**C**) and stained with thioflavine S. The arrows indicate some cell bodies nonspecifically labeled in A and B and positively stained in C. The presence of fibrillar material in C appears associated with external cellular surfaces. Bar = 100 μm.

According to this hypothesis, x-ray diffraction analysis indicates that β25–35 in monomeric form has a strong hydrophobic interaction with the membrane hydrocarbon core (Mason et al., 1996). Furthermore, the recent identification of RAGE ("receptors for advanced glycation end products") as a receptor that partially mediates the effects of βA peptides (β1–42 and β25–35) on neurons and microglia did not clarify whether neurotoxicity is activated by the monomer or aggregated form (Yan et al., 1996).

Although it is necessary to test the neurotoxicity of the nonamyloidogenic forms β1–40 and β1–42 before drawing any final conclusion on the whole βA, our findings dissociate the neurotoxic activity from its amyloidogenic capacity. This must be considered in studies of AD pathogenesis and in the creation of experimental models to investigate neuroprotective drugs.

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