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Apoptosis is induced by β-amyloid in cultured central nervous system neurons

(Alzheimer/programmed cell death/neurodegeneration)

DERYK T. LOO, AGATA COPANI, CHRISTIAN J. PIKE, EDWARD R. WHITTEMORE, ANDREA J. WALENCEWICZ, AND CARL W. COTMAN*

Irvine Research Unit in Brain Aging and Departments of Psychobiology and Neurology, University of California, Irvine, CA 92717

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ABSTRACT **The molecular mechanism responsible for the neurodegeneration in Alzheimer disease is not known; however, accumulating evidence suggests that β-amyloid peptide (AβP) contributes to this degeneration. We now report that synthetic AβPs trigger the degeneration of cultured neurons through activation of an apoptotic pathway. Neurons treated with AβPs exhibit morphological and biochemical characteristics of apoptosis, including membrane blebbing, compaction of nuclear chromatin, and internucleosomal DNA fragmentation. Aurintricarboxylic acid, an inhibitor of nucleases, prevents DNA fragmentation and delays cell death. Our *in vitro* results suggest that apoptosis may play a role in the neuronal loss associated with Alzheimer disease.**

The accumulation of β-amyloid peptide (AβP) in senile plaques is a principal event in the neuropathology of Alzheimer disease (AD). AβP deposits are associated with both the presence of dystrophic neurites (1, 2) and the neuronal loss found in severely affected brain regions (3). A causal role of AβP in AD neurodegeneration is supported by molecular studies that have revealed mutations in the AβP precursor protein (AβPP)—clustered around the AβP region—that are linked with some forms of familial AD (4–6). These mutations have been hypothesized to promote the production of AβP. This idea is consistent with the fact that cultured cells that express a familial AD-linked mutated form of AβPP produce severalfold more AβP than cells expressing the normal AβPP (7, 8).

In vitro and *in vivo* studies further support the hypothesis that AβP directly contributes to neurodegeneration in AD. In primary neuronal cultures, aggregates of AβP induce dystrophic neurite morphology (9) and neuronal loss (10–12). Further, *in vivo* studies have shown that intracerebral injection of β-amyloid plaque cores and AβP aggregates induce neuronal degeneration in adult rats and aged primates (13–17). Although experimental studies confirm the predicted neurotoxicity of AβP, the mechanism(s) and molecular events of AβP-induced neuronal cell death have not been identified.

Several lines of evidence suggest that cell death occurs by one of two general pathways, necrosis or apoptosis (18–20). Necrosis is associated with nonphysiological conditions that disrupt cellular homeostasis (e.g., hypoxia, ischemia, and excitotoxicity) (18–20). By contrast, apoptosis is a type of regulated cellular self-destruction that functions in the normal control of development and tissue homeostasis (18) and may be regulated by various factors throughout life (19). Cells undergoing apoptosis exhibit focal cell surface protrusions (or blebs), nuclear and cytoplasmic condensation, and dense aggregation of chromatin that abuts the nuclear membrane. A biochemical hallmark of cells undergoing apoptosis is internucleosomal cleavage of DNA into oligonucleosome-length fragments (21). Additionally, cells undergoing apoptosis preserve their membrane integrity until late in degeneration (22). While several events are known to initiate apoptosis in nonneural cells (19), only a few specific insults have been associated with apoptotic degeneration of cultured neurons (23–26).

We and others have hypothesized that apoptosis may play a role in neurodegenerative diseases (27). Apoptosis occurs asynchronously within a population of cells, affecting individual, scattered cells, which is consistent with the progression of neurodegenerative diseases. However, *in vivo* detection of apoptosis is difficult since nonfunctional cells are subsequently removed via phagocytosis. To explore the mechanism by which neurons degenerate following exposure to AβPs, we studied the biochemical and morphological characteristics of cultured cortical and hippocampal neurons treated with the 42-amino acid synthetic peptide (β1–42) and the shorter β25–35 fragment. We report that cultured neurons treated with AβPs exhibit classical morphological and biochemical characteristics of apoptosis. Our *in vitro* results suggest that apoptosis may play a role in the neuronal loss associated with AD.

MATERIALS AND METHODS

Cell Culture. Cortical cultures were derived from mouse embryos (embryonic day 14–15) and plated (2×10^5 cells per cm^2) in Eagle's minimal essential medium (EMEM; GIBCO) supplemented with 10% (vol/vol) horse serum and 10% (vol/vol) fetal bovine serum as described (28); 24 h prior to treatment, cultures were shifted into EMEM supplemented with a modification (10) of B18 components (29). Young cultures were used at 1–2 days *in vitro* (DIV); mature cultures were used at 11–14 DIV. Hippocampal cultures were prepared from rat embryos (embryonic day 18–19) and plated (1.25×10^5 cells per cm^2) in Dulbecco's modified Eagle's medium supplemented with N2 components (30) as described (12).

Analysis of Cell Viability. Cell viability was assessed by morphological criteria (10), trypan blue exclusion (12), lactate dehydrogenase (LDH) activity (31, 32), and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (33) as described. Each condition was represented in three or four wells per experiment and repeated 3 or 4 times in independent experiments. Raw data from each experiment was normalized, combined, and analyzed using either an analysis of variance with Fisher test or *t* test.

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Abbreviations: AD, Alzheimer disease; AβP, β-amyloid peptide; DIV, day(s) *in vitro*; LDH, lactate dehydrogenase; ATA, aurintricarboxylic acid; EAA, excitatory amino acid; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

*To whom reprint requests should be addressed at: Irvine Research Unit in Brain Aging, University of California, Irvine, CA 92717.

Electron Microscopy. Neurons grown on glass coverslips were examined by scanning electron microscopy (SEM) as described (9). For transmission electron microscopy (TEM), neurons were postfixed in 1% buffered OsO_4, dehydrated in graded alcohols, and embedded in Epon (PolyBed 812). Sections were stained with uranyl acetate and lead citrate and then examined with a JEOL 100 microscope.

Analysis of DNA Fragmentation. Total genomic DNA was isolated from cell pellets as described (34). DNA samples (18 μg per lane) were electrophoresed through a 1.2% agarose gel and visualized by ethidium bromide staining.

Peptide Synthesis. Peptides were synthesized by solid-phase fluoren-9-ylmethoxycarbonyl (Fmoc) amino acid substitution and purified by reverse-phase HPLC as described elsewhere (35). The peptides were solubilized in deionized water as described (36) and exhibited aggregation consistent with our previous reports (10, 12).

RESULTS

AβPs Induce Degeneration of Cultured Neurons. Cortical neurons at 1–2 DIV were exposed to AβPs at 25 μM, a concentration previously established to induce neuronal degeneration in our cultures (10). Viability was quantified by morphological criteria, release of LDH, and mitochondrial activity. Over a period of 24 h after AβP exposure, neurons degenerated asynchronously, exhibiting small, condensed and irregularly shaped cell bodies with dystrophic-like neurites (Fig. 1*B*). Although we observed some variability in the time course of morphological degeneration, it generally appeared within 4–6 h of AβP treatment and continued for 24 h. The number of degenerating cells did not increase by 48 h (Fig. 1*C*). Neuronal degeneration was not induced by the reverse sequence or a scrambled sequence (37) of the β25–35 peptide ($P = 0.46$).

Previous work demonstrated a strict correlation between LDH release and glutamate-mediated necrotic degeneration (31). Interestingly, in the presence of AβPs, the amount of LDH released into the culture media did not correlate with the extent of morphological degeneration observed (Fig. 1*C*). At 24 h the LDH release in β1–42-treated cultures was not significant ($P = 0.2402$), indicating that membrane integrity was still preserved. However, LDH release increased to 40% above control level by 48 h posttreatment ($P = 0.0142$), likely reflecting late lysis of the degenerating neurons. Similarly, by 24 h only a small increase in LDH release was observed in β25–35-treated cultures, whereas the amount of LDH release increased to $\approx$70% above control level by 48 h (Fig. 1*C*).

The mitochondrial activity in AβP-treated cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. After a 24-h exposure to β25–35, only a small decrease in mitochondrial activity (16.5% reduction relative to control; $P = 0.0009$) was observed compared to the extent of morphological degeneration. The observed pattern of AβP-induced degeneration is consistent with an apoptotic pathway, which is characterized by asynchronous somal shrinkage, followed by impairment of mitochondria and late membrane lysis. To explore this possibility further, SEM and TEM were carried out to determine if AβP-treated neurons also exhibit ultrastructural changes consistent with apoptosis.

AβPs Induce Ultrastructural Features of Apoptosis in Cultured Neurons. One characteristic of apoptotic degeneration is an extensive blebbing of the plasma membrane (18). Cell blebbing, an indicator of cytoskeletal network rearrangement, leads to the formation of spheroid, sealed membrane-bounded bodies, which may contain nuclear fragments and intact organelles in addition to cytoplasm (38). Neurons examined by SEM 24 h after exposure to AβPs exhibited

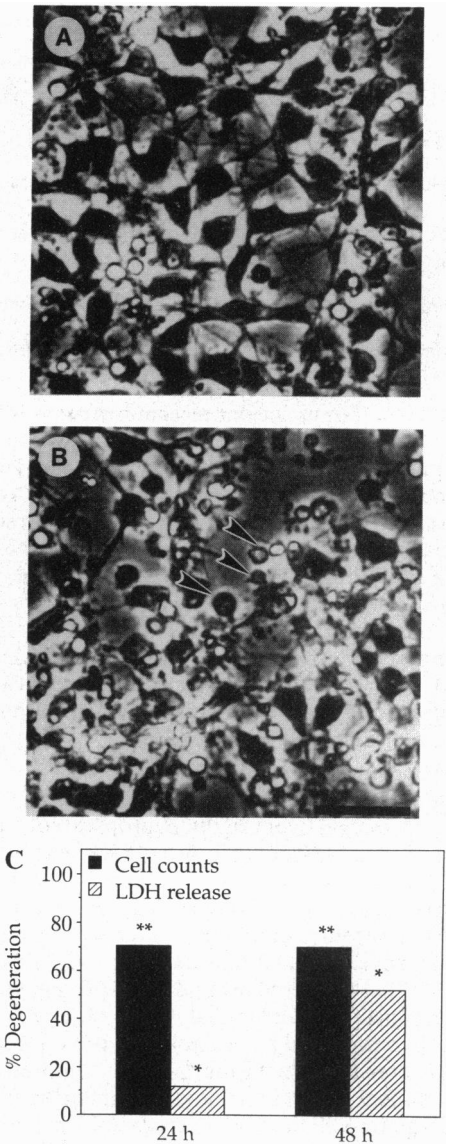

FIG. 1. (*A* and *B*) Representative phase-contrast micrographs of 2-DIV cortical neurons. (*A*) Control culture. (*B*) Culture exposed to 25 μM β25–35 for 24 h. Treated cultures undergo asynchronous cell degeneration; degenerating neurons show shrunken and irregularly shaped cell bodies (arrowheads). Similar results were obtained with the β1–42 peptide. (Bar = 25 μm.) (*C*) Neurodegeneration, measured by cell count and LDH release, at 24 h and 48 h after exposure to 25 μM β25–35. Bars represent the mean value expressed as a percentage above control. *, Different from control at $P < 0.05$; **, different from control at $P < 0.001$. Control groups at 24 h and 48 h were not significantly different. At 3 DIV, hippocampal and cortical populations were determined to be at least 90% neuronal by immunostaining (12).

severe surface blebbing (Fig. 2*B*). Shrunken neurons with few remaining attached blebs were also observed (Fig. 2*C*).

A primary ultrastructural feature of apoptosis is the compaction of nuclear chromatin in degenerating cells (18). TEM examination of β25–35-treated hippocampal neurons revealed compact patches of condensed nuclear chromatin with no apparent plasma membrane disruption or organelle breakdown (Fig. 3), suggesting that chromatin condensation may precede these degenerative events. Neurons in various stages of degeneration were observed, which is consistent with the asynchronism observed by phase-contrast microscopy. Membrane-bound pyknotic nuclei lacking cytoplasm were also present. Neurons in untreated cultures displayed normal

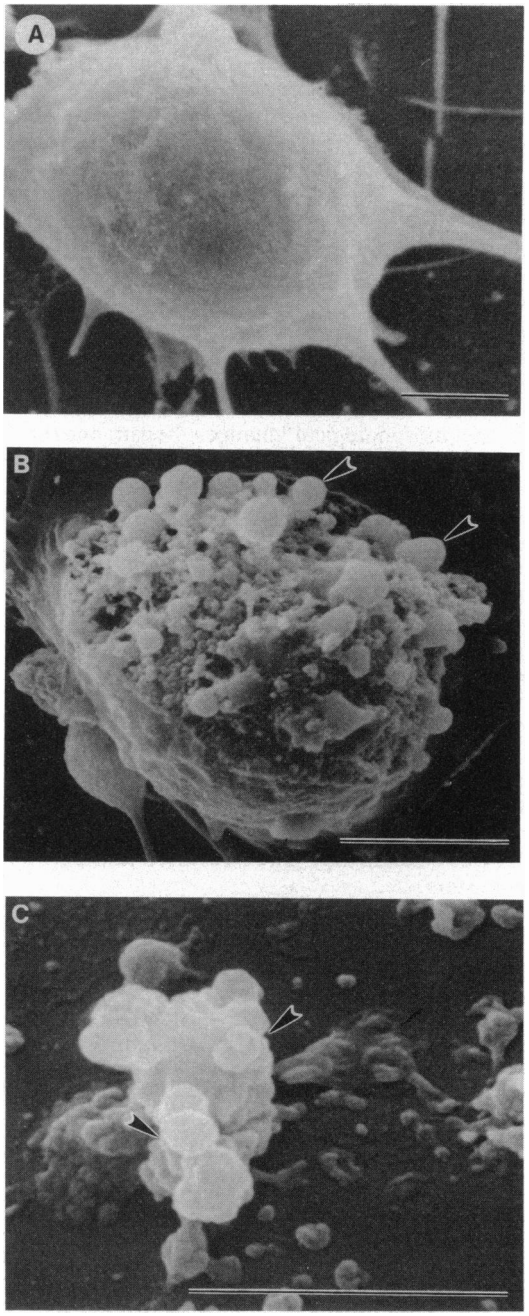

FIG. 2. SEM of 2-DIV hippocampal neurons. (*A*) Untreated neuron exhibiting a round, smooth cell body with neuritic processes. (*B*) Neuron from a 24-h β1–42-treated culture, showing shrinkage of the cell body, blebbing of the membrane surface (arrowheads), and loss of neuritic processes. (*C*) Shrunken apoptotic neuron, treated as in *B*, with few remaining attached blebs (arrowheads). (Bars = 2 μm.)

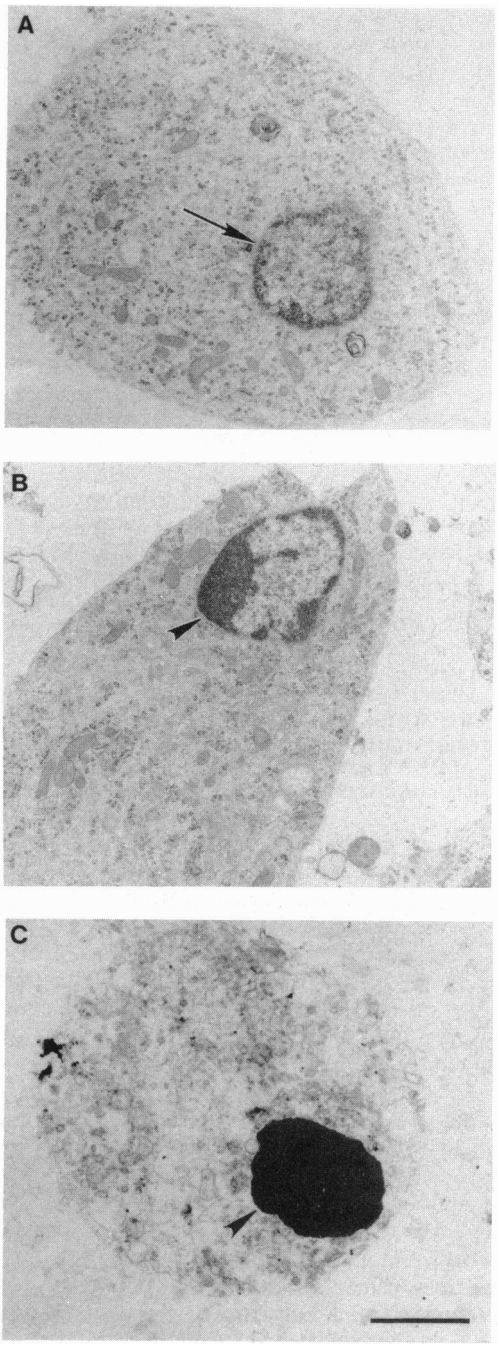

FIG. 3. TEM of 2-DIV hippocampal neurons. (*A*) Untreated neuron showing well-preserved organelles and abundant cytoplasm. The nucleus contains dispersed chromatin (arrow). (*B*) Neuron from 24-h β25–35-treated culture, exhibiting early nuclear changes. Patches of condensed chromatin lie against the nuclear membrane (arrowhead). Note preservation of cellular membranes and maintenance of organelle structure. (*C*) Neuron from 24-h β25–35-treated culture, showing a late stage of degeneration. The nucleus exhibits uniform, highly condensed chromatin and irregular membrane perimeter (arrowhead). The cytoplasm contains abundant vacuoles and dilated organelles. (Bar = 2 μm.)

ultrastructure. Thus, AβP induced ultrastructural changes in cultured neurons characteristic of apoptosis. We next sought biochemical evidence that AβP-treated neurons degenerate through an apoptotic pathway.

Cultured Neurons Undergo DNA Fragmentation After Exposure to AβPs. A definitive biochemical feature of many cell types undergoing apoptosis is fragmentation of DNA into oligonucleosome-length fragments (18). DNA was extracted from young cortical cultures treated with AβPs and analyzed by agarose gel electrophoresis. After a 24-h exposure to AβPs, a ladder of oligonucleosome-length fragments of DNA was observed (Fig. 4*A*). The DNA ladder was not appreciably detectable until 24 h after AβP treatment and increased in intensity by 48 h (data not shown). Similarly, mature cortical neurons exposed to β25–35 also underwent endonucleolytic DNA cleavage (Fig. 4*B*).

DNA fragmentation in other apoptosis paradigms has been correlated with endonuclease activation (18, 21). Aurintricarboxylic acid (ATA), an inhibitor of nucleases (39), suppressed AβP-induced DNA fragmentation when added to the

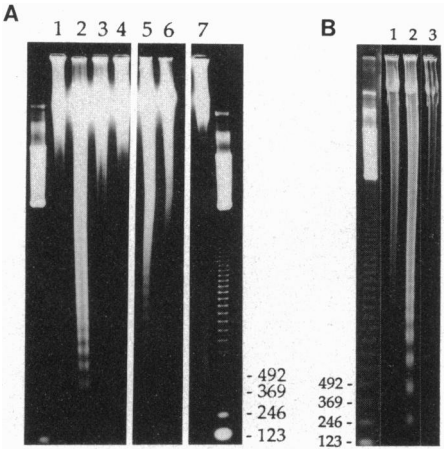

FIG. 4. (*A*) Agarose gel electrophoresis of DNA fragmentation in 2-DIV cortical neurons. Lane 1, control; lane 2, β25–35 for 24 h; lane 3, β25–35 + ATA for 24 h; lane 4, β25–35 for 24 h + ATA added 8 h after β25–35; lane 5, β1–42 for 24 h; lane 6, β1–42 + ATA for 24 h; lane 7, A23187 for 24 h. The culture displayed 63% degeneration after an 8-h exposure to β25–35 (P = 0.0004, 2-tail t-test). The β25–35 concentration was 25 μM, the ATA concentration was 100 μM, and the A23187 concentration was 1 μM. (*B*) Agarose gel electrophoresis of DNA fragmentation in 14-DIV cortical neurons. Lane 1, control; lane 2, β25–35 (25 μM) for 48 h; lane 3, β25–35 + ATA (100 μM) for 48 h. A 123-bp DNA ladder is shown for comparison. Untreated cultures occasionally displayed trace amounts of DNA fragmentation. The gels shown are representative of data obtained in three independent experiments.

cultures either simultaneously with AβPs or after the appearance of significant degenerative changes (Fig. 4). ATA also significantly delayed cell lysis, as measured by the exclusion of trypan blue dye at 48 h (63% increase in viability relative to β25–35 alone; P = 0.0001). The lysosomal inhibitor chloroquine (1 μM) did not prevent β25–35-induced DNA fragmentation, indicating that this process was not dependent on lysosomal nucleases (data not shown).

To exclude the possibility that DNA breakdown is a nonspecific consequence of neuronal injury, cortical cultures were exposed to the calcium ionophore A23187. Under these conditions, neuronal swelling occurred within 4 h, and almost complete cell lysis was observed 2 h later (+108.8% LDH release above controls; P = 0.0001). Analysis of DNA extracted from A23187-treated cultures showed a diffuse smear consistent with random DNA cleavage in cells undergoing necrosis rather than apoptosis (18) (Fig. 4*A*). Furthermore, neurodegeneration induced by excitatory amino acids (EAAs) does not appear to result in DNA fragmentation associated with apoptosis (40, 41).

DISCUSSION

The present study reports the finding that exposure of cultured neurons to AβPs induces degeneration and cell death that occurs via an apoptotic pathway. Neuronal cultures exposed to AβPs degenerated asynchronously, exhibited nuclear chromatin condensation and plasma membrane blebbing, and maintained cellular membrane integrity until late in degeneration. This pattern of AβP-induced degeneration is consistent with apoptosis observed in other culture systems (18). Peptide-induced degeneration was not observed with the reverse sequence or a scrambled sequence of the β25–35 peptide or an aggregated peptide corresponding to a region of the amyloidogenic islet amyloid protein (36, 12). This suggests that the induction of apoptosis by AβPs is sequence and conformation specific.

Most strikingly, neurons exposed to AβPs underwent internucleosomal DNA fragmentation, a hallmark of apoptosis. Comparable AβP-induced neuronal degeneration and DNA fragmentation was observed in both young, developing cultures and mature cultures. Thus, *in vitro* activation of the apoptotic program by AβP does not appear to be restricted by developmental stage.

ATA has been reported to suppress DNA fragmentation and block apoptosis in a variety of cell types (42, 43). ATA was effective in blocking AβP-induced DNA fragmentation and also delayed cell lysis when added simultaneously with AβPs. This suggests that DNA breakdown contributes to the process of neuronal degeneration. Furthermore, ATA blocked DNA fragmentation when added to cultures displaying significant AβP-induced morphological degeneration. Thus, while it is difficult to establish an absolute time at which the morphological changes began and/or became significant, it appears that they precede the onset of DNA breakdown. Additionally, our preliminary studies indicate that the protein synthesis inhibitor cycloheximide protects neurons from AβP-induced degeneration, suggesting that protein synthesis may play a role in AβP-induced neurodegeneration.

In both neuronal and nonneuronal cell types, alterations in the level of intracellular calcium may be involved in the initiation of apoptosis (44, 45). Accordingly, we used fura-2 imaging (46) to examine the possibility that AβP-induced apoptosis involves changes in intracellular free calcium. In agreement with Mattson *et al.* (37), we found that the resting level of intracellular free calcium in AβP-treated cultures did not differ significantly from those in control cultures over a 24-h exposure. This suggests that large alterations in intracellular free calcium may not be required to trigger apoptosis in this system.

In addition to the direct neurodegeneration induced by AβPs, recent evidence suggests that sublethal doses of AβPs compromise neuronal control of homeostasis, increasing the vulnerability of cells to subsequent challenges. In support of this view, AβPs potentiate the neurotoxicity of EAAs on cultured neurons (32, 37) and increase the susceptibility of cultured neurons to injury by glucose deprivation (28). The increased vulnerability of AβP-treated neurons to subsequent insults may result from destabilization of cellular calcium homeostasis (37). Taken together, these findings suggest a potential link between apoptotic cell death and susceptibility to other insults.

A speculative scheme of the role of AβP in AD neurodegeneration encompasses two mechanisms that contribute to neuronal loss. First, as our *in vitro* data suggest, the accumulation of lethal doses of AβP may drive select, uncompromised neuronal populations into apoptotic pathways. Second, sublethal insults of AβP increase the vulnerability of neuronal populations to subsequent insults. Challenges, such as glucose deprivation, ischemia, EAAs, and stress, which healthy neurons are able to overcome, may become lethal to neurons compromised by AβP. Our results and others suggest that the mechanism of cell death in instances where cells are put at risk by AβP may be related to the secondary challenge and, in the case of EAAs, may follow a necrotic pathway (32, 37).

Our results provide initial evidence that AβP-induced degeneration of cultured neurons proceeds through an apoptotic pathway and predict that activation of an apoptotic pathway may contribute to the neuronal loss associated with AD. The brain is a highly complex, integrated structure, the function of which is acutely dependent on precisely organized architecture of neuronal networks and support systems. Apoptosis may in fact serve a beneficial purpose in the mature brain by providing a controlled mechanism through which dysfunctional neuronal elements can be selectively

removed without evoking inflammatory responses or the release of harmful cellular products, which could endanger the surrounding network. Thus, apoptosis may contribute to the global maintenance, fine structuring, and plasticity of neuronal networks. AD neurodegeneration may result in part from the accumulation of AβP, which leads to the localized activation of a series of apoptotic events. A role for apoptosis in slow progressive neurodegenerative diseases is noteworthy, as it implies that interruption of the apoptotic pathway may be possible. Defining the precise steps in the apoptotic pathway and possible regulatory targets may allow for the development of strategies that will delay the progression of neurodegeneration in AD.

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