

# Involvement of Endoplasmic Reticulum $\text{Ca}^{2+}$ Release Through Ryanodine and Inositol 1,4,5-Triphosphate Receptors in the Neurotoxic Effects Induced by the Amyloid- $\beta$ Peptide

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Studies with in-vitro-cultured neurons treated with amyloid- $\beta$  (A $\beta$ ) peptides demonstrated neuronal loss by apoptosis that is due, at least in part, to the perturbation of intracellular  $\text{Ca}^{2+}$  homeostasis. In addition, it was shown that an endoplasmic reticulum (ER)-specific apoptotic pathway mediated by caspase-12, which is activated upon the perturbation of ER  $\text{Ca}^{2+}$  homeostasis, may contribute to A $\beta$  toxicity. To elucidate the involvement of deregulation of ER  $\text{Ca}^{2+}$  homeostasis in neuronal death induced by A $\beta$  peptides, we have performed a comparative study using the synthetic peptides A $\beta_{25-35}$  or A $\beta_{1-40}$  and thapsigargin, a selective inhibitor of  $\text{Ca}^{2+}$  uptake into the ER. Incubation of cortical neurons with thapsigargin (2.5  $\mu\text{M}$ ) increased the intracellular  $\text{Ca}^{2+}$  levels and activated caspase-3, leading to a significant increase in the number of apoptotic cells. Similarly, upon incubation of cortical cultures with the A $\beta$  peptides (A $\beta_{25-35}$ , 25  $\mu\text{M}$ ; A $\beta_{1-40}$ , 0.5  $\mu\text{M}$ ), we observed a significant increase in  $[\text{Ca}^{2+}]_i$ , in caspase-3-like activity, and in number of neurons exhibiting apoptotic morphology. The role of ER  $\text{Ca}^{2+}$  release through ryanodine receptors (RyR) or inositol 1,4,5-trisphosphate receptors (IP $_3$ R) in A $\beta$  neurotoxicity has been also investigated. Dantrolene and xestospongine C, inhibitors of ER  $\text{Ca}^{2+}$  release through RyR or IP $_3$ R, were able to prevent the increase in  $[\text{Ca}^{2+}]_i$  and the activation of caspase-3 and to protect partially against apoptosis induced by treatment with A $\beta_{25-35}$  or A $\beta_{1-40}$ . In conclusion, our results demonstrate that the release of  $\text{Ca}^{2+}$  from the ER, mediated by both RyR and IP $_3$ R, is involved in A $\beta$  toxicity and can contribute, together with the activation of other intracellular neurotoxic mechanisms, to A $\beta$ -induced neuronal death. This study suggests that A $\beta$  accumulation may have a key role in the pathogenesis of AD as a result of deregulation of ER  $\text{Ca}^{2+}$  homeostasis. © 2004 Wiley-Liss, Inc.

**Key words:** Alzheimer's disease; amyloid- $\beta$  peptide; apoptosis; endoplasmic reticulum;  $\text{Ca}^{2+}$  homeostasis; thapsigargin

A defining feature of Alzheimer's disease (AD), the most common form of senile dementia, is the accumulation of amyloid- $\beta$  (A $\beta$ ; Mattson, 1997), a 39–43-amino-acid peptide derived from the amyloid precursor protein (APP; Wisniewski et al., 1997). The importance of A $\beta$  in the pathogenesis of AD is suggested by several findings. Notably, mutations in APP or presenilin, two proteins that are implicated in familial forms of AD, lead to an increase in the amyloidogenic forms of A $\beta$  (Selkoe, 1999). In addition, A $\beta_{1-40}$  and A $\beta_{1-42}$  (the principal products resulting from the sequential proteolytic processing of APP by  $\beta$ - and  $\gamma$ -secretases) and the toxic fragment A $\beta_{25-35}$  have been shown to be toxic to a wide variety of cells, including neurons (Yankner, 1996). The mechanism(s) by which these peptides lead to neuronal loss is not well understood, so greater understanding is required and is necessary to future therapeutic intervention. Studies with in-vitro-cultured neurons treated with amyloidogenic A $\beta$  peptides demonstrated neuronal loss by an apoptotic pathway (Loo et al., 1993; Estus et al., 1997). Furthermore, there is good agreement that A $\beta$ -induced apoptosis is due, at least in part, to the perturbation of intracellular  $\text{Ca}^{2+}$  homeostasis (Scorziello et al., 1996; Huang et al., 2000).

Several studies have suggested that changes in intracellular  $\text{Ca}^{2+}$  homeostasis play an important role in the modulation of apoptosis. Many cell death stimuli are known to alter the concentration of  $\text{Ca}^{2+}$  in the cytosol and the storage of  $\text{Ca}^{2+}$  in the intracellular organelles (Baffy et al., 1993; Bian et al., 1997). In addition, com-

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pounds that directly affect the intracellular  $\text{Ca}^{2+}$  homeostasis have been shown to induce apoptosis (Jiang et al., 1994; Reynolds et al., 1996; Wei et al., 1998).  $\text{Ca}^{2+}$  release channels in the endoplasmic reticulum (ER), i.e., ryanodine receptors (RyR) or inositol 1,4,5-trisphosphate receptors ( $\text{IP}_3\text{R}$ ), seem to participate in the signal transduction pathway of apoptosis (Guo et al., 1997; Jayaraman and Marks, 1997; Pan et al., 2000).

It was recently demonstrated a novel ER-specific apoptosis pathway in which caspase-12 acts as the initiator caspase in response to a toxic insult to the ER (Nakagawa et al., 2000). Caspase-12 is specifically involved in apoptosis that results from stress in the ER and seems to be activated upon perturbation of ER  $\text{Ca}^{2+}$  homeostasis (Nakagawa et al., 2000; Nakagawa and Yuan, 2000). ER stress has received growing attention because it is considered a cause of pathologically relevant apoptosis, and it is implicated particularly in neurodegenerative disorders (Paschen and Frandsen, 2001). Recent evidence points to the involvement of the ER in AD pathogenesis (Ferri and Kroemer, 2000; Mattson and Chan, 2001; Mattson et al., 2001) owing to the fact that it is an important site for generating  $\text{A}\beta_{1-40}$  and  $\text{A}\beta_{1-42}$  in neurons (Hartmann et al., 1998) and that both presenilin proteins are localized predominantly in this cellular compartment (Walter et al., 1996).

The goal of the present study was to determine the role of ER  $\text{Ca}^{2+}$  release in the neurotoxic effects induced by the AD-associated  $\text{A}\beta$  peptides. For that purpose, primary cultures of rat brain cortical neurons were exposed to the synthetic peptides  $\text{A}\beta_{25-35}$  or  $\text{A}\beta_{1-40}$ , which have been shown to be toxic to a wide variety of cells, including neurons (Mattson, 1997; Pereira et al., 1999; Cardoso et al., 2002; Casley et al., 2002). In addition, cortical neurons were treated with thapsigargin, a selective ER  $\text{Ca}^{2+}$ -ATPase inhibitor that leads to the loss of  $\text{Ca}^{2+}$  homeostasis and to apoptotic cell death (Jiang et al., 1994). Agents that suppress ER  $\text{Ca}^{2+}$  release through the RyR or the  $\text{IP}_3\text{R}$  were used to investigate the involvement of ER  $\text{Ca}^{2+}$  release channels in the deregulation of  $\text{Ca}^{2+}$  homeostasis and in the apoptotic death process that occurs upon  $\text{A}\beta$  exposure. The results support a mechanistic link among  $\text{A}\beta$  accumulation, ER dysfunction, and apoptotic neuronal death in AD.

## MATERIALS AND METHODS

### Materials

Neurobasal medium and B27 supplement were purchased from Gibco BRL, Life Technologies (Paisley, United Kingdom). Trypsin, DNase I, trypsin inhibitor type II-S-soybean, dantrolene, and thapsigargin were obtained from Sigma Chemical Co. (St. Louis, MO). SYTO-13, propidium iodide (PI), and Indo-1 acetoxymethyl ester (Indo-1/AM) were purchased from Molecular Probes (Leiden, The Netherlands). The synthetic  $\text{A}\beta_{25-35}$  and  $\text{A}\beta_{1-40}$  peptides and the respective reverse sequences  $\text{A}\beta_{35-25}$  and  $\text{A}\beta_{40-1}$  were from Bachem (Bubendorf, Switzerland). Ionomycin and xestospongine C were purchased from Calbiochem (Darmstadt, Germany). The colorimetric sub-

strate for caspase-3 [N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA)] was also from Calbiochem. All the other chemicals were obtained from Sigma or from Merck kgaA (Darmstadt, Germany).

### Primary Cortical Neuronal Cultures and Experimental Treatments

Primary cultures of cortical cells were prepared from 15–16-day embryos of Wistar rats according to the method described by Hertz et al. (1989), with some modifications (Agostinho and Oliveira, 2003). Briefly, the neocortices of embryos were dissected and placed in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free Krebs buffer (in mM): NaCl 120, KCl 4.8,  $\text{KH}_2\text{PO}_4$  1.2, glucose 13, Hepes 10 (pH 7.4), supplemented with 0.3% (wt/vol) bovine serum albumin (BSA). Minced cortical tissues were washed and incubated in Krebs buffer containing 0.05% (wt/vol) trypsin and 0.004% (wt/vol) DNase I, for 10 min at 37°C. The digestion was stopped with Krebs buffer containing 0.05% (wt/vol) trypsin inhibitor (type II-S) and 0.004% (wt/vol) DNase I, and the tissue was centrifuged at 140g for 5 min. After the pellet was washed once with Krebs buffer, the cells were dissolved in fresh Neurobasal medium supplemented with 2 mM L-glutamine, 2% (vol/vol) B27 supplement, penicillin (100 U/ml), and streptomycin (100  $\mu\text{g}/\text{ml}$ ) and were dissociated mechanically. Under these culture conditions, 90% of the cells are neurons as evaluated using microtubule-associated protein-2 (MAP-2) and glial fibrillary acidic protein (GFAP) immunoreactivity (data not shown). Cortical cells were plated on poly-L-lysine (0.1 mg/ml)-coated plates or coverslips at a density of  $0.10 \times 10^6$  cells/ $\text{cm}^2$  for fluorescence microscopy experiments or at  $0.50 \times 10^6$  cells/ $\text{cm}^2$  for measurement of  $[\text{Ca}^{2+}]_i$  and caspase-3-like activity. The cultures were maintained at 37°C in a humidified atmosphere of 5%  $\text{CO}_2/95\%$  air and were used for experiments after 5–7 days in vitro. Cortical neurons were treated with  $\text{A}\beta_{25-35}$  or  $\text{A}\beta_{35-25}$  (25  $\mu\text{M}$ ),  $\text{A}\beta_{1-40}$  or  $\text{A}\beta_{40-1}$  (0.5  $\mu\text{M}$ ), or thapsigargin (2.5  $\mu\text{M}$ , a concentration that induces 50% decrease in cell viability at 24 hr) in serum-free Neurobasal medium supplemented with B27. The concentrations of  $\text{A}\beta$  peptides used were chosen based on previous results demonstrating that 25  $\mu\text{M}$   $\text{A}\beta_{25-35}$  and 0.5  $\mu\text{M}$   $\text{A}\beta_{1-40}$  induced a maximal toxic effect (50% decrease in cell viability) on cortical neurons. Before the addition of  $\text{A}\beta$  peptides, cells were preincubated for 1 hr with dantrolene (10  $\mu\text{M}$ ) or xestospongine C (1  $\mu\text{M}$ ), concentrations that were shown to exert maximal protective effects, without being toxic by themselves (data not shown).

### Assessment of Neuronal Injury

After treatment of cortical cells with thapsigargin or with  $\text{A}\beta$  peptides, neuronal injury was assessed by analysis of the nuclear morphology by fluorescence microscopy of live cells using the stains PI, which fluoresces red and enters the cells only when membrane integrity is lost, and SYTO-13, which fluoresces green and enters the cells with intact cytoplasmic membrane (Rego et al., 2001). Cortical cells cultured on glass coverslips were treated with thapsigargin or with  $\text{A}\beta$  peptides in the absence or in the presence of dantrolene or xestospongine C. After washing, cells were incubated in sodium medium (in

mM): NaCl 132, KCl 4,  $\text{NaH}_2\text{PO}_4$  1.2,  $\text{MgCl}_2$  1.4, glucose 6, HEPES-Na 10, and  $\text{CaCl}_2$  1, pH 7.4, containing 3.8  $\mu\text{M}$  SYTO-13 and 2.5  $\mu\text{g}/\text{ml}$  of PI for 3 min at 37°C. Viable, necrotic, or apoptotic neurons were visualized by nuclear morphology criteria. Viable neurons display a normal nuclear size and green fluorescence. Necrotic neurons manifest red fluorescence without chromatin condensation. Scored apoptotic neurons include neurons that display pyknotic nuclei with condensed or fragmented chromatin and fluoresce green and secondary necrotic neurons that display condensed or fragmented chromatin and fluoresce red. Cells were examined and scored with a Nikon Diaphot TMP microscope. All experiments were performed in duplicate, and at minimum 300 cells were scored for each coverslip. The number of viable, necrotic, and apoptotic cells was expressed as the percentage of the total number of cells in the microscope field.

### Measurement of Intracellular $\text{Ca}^{2+}$ Concentration

Control or treated cortical cells cultured on glass coverslips were incubated with 3  $\mu\text{M}$  Indo-1/AM in a sodium medium containing (in mM): NaCl 132, KCl 4,  $\text{CaCl}_2$  1,  $\text{MgCl}_2$  1.4, glucose 6, HEPES-Na 10, pH 7.4, for 45 min at 37°C. The cells were further incubated in sodium medium for 15 min at 37°C to ensure a complete hydrolysis of the acetoxymethyl ester of Indo-1. After being washed, cells were mounted in a special holder, and the Indo-1 fluorescence was measured with excitation of 335 nm and 410 nm emission. The free intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) was calculated as previously described (Bandeira-Duarte et al., 1990).

### Caspase-3-Like Activity Assay

Untreated or treated cortical cells were lysed with a buffer containing (in mM): HEPES-Na 25,  $\text{MgCl}_2$  2, EDTA 1, EGTA 1, supplemented with 100  $\mu\text{M}$  phenylmethylsulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), and a protease inhibitor cocktail (containing 1  $\mu\text{g}/\text{ml}$  leupeptin, pepstatin A, chymostatin, and aprotinin). The cellular suspension was rapidly frozen/thawed three times and then centrifuged for 10 min at 20,200g. The supernatant was collected and assayed for protein content by using the Bio-Rad protein dye assay reagent. To measure caspase-3-like activity, aliquots of cell extracts containing 25  $\mu\text{g}$  of protein were incubated for 2 hr at 37°C, in a reaction buffer containing 25 mM HEPES-Na, 10 mM DTT, 10% (wt/vol) sucrose, and 0.1% (wt/vol) CHAPS (pH 7.4), with 100  $\mu\text{M}$  Ac-DEVD-pNA, a chromogenic substrate for caspase-3 (Cregan et al., 1999). Caspase-3-like activity was determined by measuring substrate cleavage at 405 nm with a microplate reader. Results were expressed as the increase above control absorbance at 405 nm.

### Statistical Analysis

Results are expressed as means  $\pm$  SEM of the number of experiments indicated in the figure legends. Statistical significance was analyzed by analysis of variance (ANOVA), followed by Dunnett's post hoc tests for multiple comparisons or by the unpaired two-tailed Student's *t*-test.  $P < .05$  was considered statistically significant.

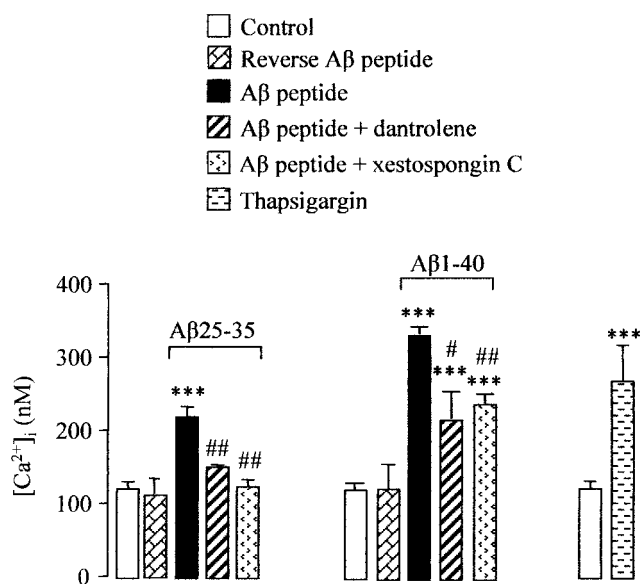


Fig. 1. Effect of  $\text{A}\beta_{25-35}$ ,  $\text{A}\beta_{1-40}$ , and thapsigargin on basal intracellular  $\text{Ca}^{2+}$  levels in cortical neurons. Cells cultured on coverslips were treated, for 24 hr, with  $\text{A}\beta_{25-35}$  (25  $\mu\text{M}$ ),  $\text{A}\beta_{1-40}$  (0.5  $\mu\text{M}$ ), in the absence or in the presence of dantrolene or xestospongine C, or with thapsigargin (2.5  $\mu\text{M}$ ). The fluorescence of Indo-1-loaded cells was measured, and the  $[\text{Ca}^{2+}]_i$  was calculated as described in Materials and Methods. The results are the means  $\pm$  SEM of values corresponding to at least three experiments, each value being the mean of duplicate assays. \*\*\* $P < .001$  with respect to control values. # $P < .05$ , ## $P < .01$  with respect to  $\text{A}\beta$  addition.

## RESULTS

### Intracellular Calcium Levels Are Increased in Cortical Neurons Treated With $\text{A}\beta_{25-35}$ , $\text{A}\beta_{1-40}$ , and Thapsigargin; Dantrolene and Xestospongine C Prevent $\text{A}\beta$ -Induced $[\text{Ca}^{2+}]_i$ Rise

To analyze the involvement of ER  $\text{Ca}^{2+}$  release in the toxic effects of  $\text{A}\beta_{25-35}$  or  $\text{A}\beta_{1-40}$  on cortical neuronal cultures, the intracellular  $\text{Ca}^{2+}$  levels were determined in the absence or in the presence of dantrolene or xestospongine C, which inhibit the release of  $\text{Ca}^{2+}$  through the ER RyR or IP<sub>3</sub>R, respectively. In addition, cytosolic  $\text{Ca}^{2+}$  concentration was determined in neurons exposed to thapsigargin, a known inhibitor of the ER  $\text{Ca}^{2+}$ -ATPase that deregulates ER  $\text{Ca}^{2+}$  homeostasis.

Treatment of cells with 25  $\mu\text{M}$   $\text{A}\beta_{25-35}$  increased the intracellular  $\text{Ca}^{2+}$  levels, as shown in Figure 1. After 24 hr, the  $[\text{Ca}^{2+}]_i$  increased from  $122.60 \pm 9.78$  nM in control to  $201.90 \pm 20.65$  nM in  $\text{A}\beta_{25-35}$ -treated cells. When cells were preincubated for 1 hr with dantrolene (10  $\mu\text{M}$ ) or xestospongine C (1  $\mu\text{M}$ ) before  $\text{A}\beta_{25-35}$  addition, the intracellular  $\text{Ca}^{2+}$  levels decreased significantly and were similar to those determined for controls (Fig. 1). The  $[\text{Ca}^{2+}]_i$  measured after incubation with  $\text{A}\beta_{25-35}$  during 24 hr, in the presence of dantrolene, was  $152.30 \pm 3.11$  nM and the  $[\text{Ca}^{2+}]_i$  measured after  $\text{A}\beta_{25-35}$  exposure during 24 hr, in the presence of xestospongine C, was

125.60  $\pm$  9.64 nM. Cells treated with A $\beta_{1-40}$  (0.5  $\mu$ M) also showed an increment in intracellular Ca<sup>2+</sup> levels in comparison with controls (Fig. 1). After treatment for 24 hr with A $\beta_{1-40}$ , [Ca<sup>2+</sup>]<sub>i</sub> increased 2.7-fold above control values (Ca<sup>2+</sup> levels increased from 122.60  $\pm$  9.78 nM in controls to 336.10  $\pm$  9.37 nM in cells treated with A $\beta_{1-40}$ ). When cells were pretreated with dantrolene (10  $\mu$ M) or xestospongion C (1  $\mu$ M) before A $\beta_{1-40}$  addition, intracellular Ca<sup>2+</sup> levels decreased significantly (Fig. 1). Intracellular Ca<sup>2+</sup> levels decreased from 336.10  $\pm$  9.37 nM to 219.50  $\pm$  36.90 nM vs. 240.80  $\pm$  12.60 nM in cells treated with A $\beta_{1-40}$  in the absence or in the presence of dantrolene vs. xestospongion C, respectively. Incubation of cortical neurons with dantrolene or xestospongion C alone during 24 hr did not affect the [Ca<sup>2+</sup>]<sub>i</sub> compared with control. Unlike A $\beta_{25-35}$  and A $\beta_{1-40}$ , the reverse peptides A $\beta_{35-25}$  (25  $\mu$ M) and A $\beta_{40-1}$  (0.5  $\mu$ M) were not able to increase intracellular free Ca<sup>2+</sup> levels.

When cells were treated with 2.5  $\mu$ M thapsigargin, an increase in intracellular Ca<sup>2+</sup> levels was also observed (Fig. 1). After incubation of cortical neurons with thapsigargin during 24 hr, the [Ca<sup>2+</sup>]<sub>i</sub> increased 2.2-fold above control values (Ca<sup>2+</sup> levels increased from 122.60  $\pm$  9.78 nM in controls to 269.30  $\pm$  8.69 nM in thapsigargin-treated cells at 24 hr).

### Caspase-3 Activation Occurs After A $\beta_{25-35}$ or A $\beta_{1-40}$ and Thapsigargin Treatment; Dantrolene and Xestospongion C Protect From A $\beta$ -Induced Caspase-3 Activation

Cells treated with A $\beta_{25-35}$  (25  $\mu$ M) for 24 or 48 hr showed a significant increase in caspase-3-like activity (Fig. 2), this increase being higher after 24 hr than after 48 hr of treatment. A 3.58-fold increase above control in the activity of caspase-3 was determined in cells exposed to A $\beta_{25-35}$  during 24 hr, whereas an increment of 2.23-fold was measured in cells treated with this peptide during 48 hr. When cells were treated with A $\beta_{1-40}$  (0.5  $\mu$ M), a significant increase in caspase-3 activation was observed at 48 hr of treatment, but no caspase-3 activation was demonstrated after 24 hr of incubation (Fig. 2). A $\beta_{1-40}$  (0.5  $\mu$ M) induced a 1.67-fold increase above control values. Caspase-3-like activity was not significantly affected by treatment with the reverse peptides (A $\beta_{35-25}$ , 25  $\mu$ M, and A $\beta_{40-1}$ , 0.5  $\mu$ M).

Cells treated with thapsigargin also demonstrated caspase-3 activation (Fig. 2). An activation of 7.20-fold was determined for caspase-3 upon 24 hr incubation with 2.5  $\mu$ M thapsigargin. No significant differences were detected in caspase-3 activity between 24 and 48 hr incubation in the presence of thapsigargin.

Because caspase-3 activation is higher after incubation for 24 hr than for 48 hr in the presence of A $\beta_{25-35}$  (25  $\mu$ M) and because caspase-3 is only activated upon exposure of A $\beta_{1-40}$  (0.5  $\mu$ M) for 48 hr, the protective effect of dantrolene and xestospongion C against A $\beta$ -induced caspase-3 activation was analyzed under these experimental conditions. As shown in Figure 3A, preincubation of cortical cells with dantrolene or xestospongion

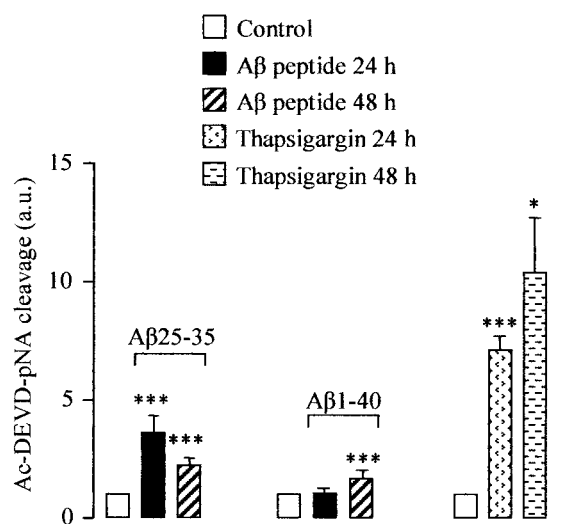


Fig. 2. Caspase-3 activation in cortical neurons induced by A $\beta_{25-35}$ , A $\beta_{1-40}$ , or thapsigargin. After incubation for 24 or 48 hr with 25  $\mu$ M A $\beta_{25-35}$ , 0.5  $\mu$ M A $\beta_{1-40}$ , or 2.5  $\mu$ M thapsigargin, the cultured cortical cells were lysed, and the protein was extracted. Caspase-3-like activity was determined by measuring the cleavage of the chromogenic substrate Ac-DEVD-pNA at 405 nm, as described in Materials and Methods. The results, expressed as the increase above control values, are the means  $\pm$  SEM of values corresponding to at least to three experiments, each value being the mean of duplicate assays. \* $P$  < .05, \*\*\* $P$  < .001 with respect to control values.

C before A $\beta_{25-35}$  (25  $\mu$ M) addition significantly reduced caspase-3 activation induced by this peptide (Ac-DEVD-pNA cleavage decreased from 3.58  $\pm$  0.72 to 1.53  $\pm$  0.20 vs. 1.48  $\pm$  0.04 in cells treated with A $\beta_{25-35}$  in the absence or in the presence of dantrolene vs. xestospongion C, respectively). Cells pretreated with dantrolene or xestospongion C before A $\beta_{1-40}$  addition showed a significant decrease in caspase-3 like activity (Ac-DEVD-pNA cleavage decreased from 1.67  $\pm$  0.33 to 1.01  $\pm$  0.05 vs. 0.86  $\pm$  0.14 in cells treated with A $\beta_{1-40}$  in the absence or in the presence of dantrolene vs. xestospongion C, respectively; Fig. 3B).

### A $\beta_{25-35}$ , A $\beta_{1-40}$ , and Thapsigargin Reduce Cell Viability and Induce Apoptosis; Dantrolene and Xestospongion C Protect From A $\beta$ Toxicity

In cortical neurons, the number of viable, necrotic, and apoptotic cells upon exposure to A $\beta$  peptides or thapsigargin was analyzed by fluorescence microscopy after SYTO-13/PI labeling. Exposure of cortical neurons to A $\beta_{25-35}$  (25  $\mu$ M) revealed a reduction in the number of viable cells after 48 hr of incubation (Fig. 4A). Dantrolene (10  $\mu$ M) and xestospongion C (1  $\mu$ M) protected cortical neurons against A $\beta_{25-35}$ -induced toxicity (the number of viable cells was 46.89%  $\pm$  1.94%, 71.3%  $\pm$  2.22%, and 81.78%  $\pm$  3.01% in cells treated with A $\beta_{25-35}$  alone, with A $\beta_{25-35}$  plus dantrolene, or with A $\beta_{25-35}$  plus xestospongion C, respectively). Incubation of cortical neurons with A $\beta_{25-35}$  peptide during 48 hr increased significantly the

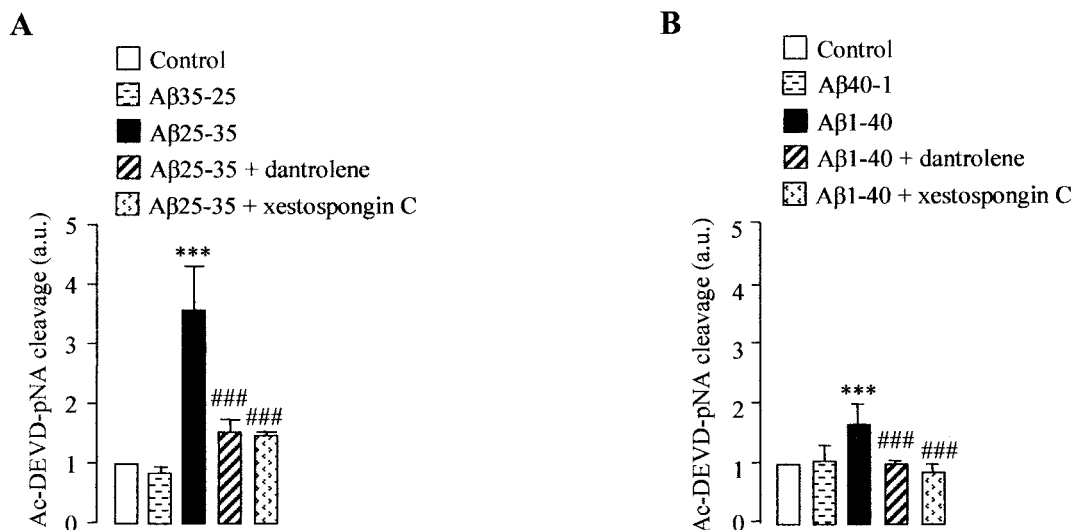


Fig. 3. Effect of dantrolene and xestospongine C on the increase in caspase-3-like activity induced by  $A\beta_{25-35}$  (A) or  $A\beta_{1-40}$  (B). After incubation for 24 hr with the peptide  $A\beta_{25-35}$  (25  $\mu$ M) and incubation for 48 hr with the peptide  $A\beta_{1-40}$  (0.5  $\mu$ M), the cultured cortical cells were lysed, and the protein was extracted. Caspase-3-like activity was determined by measur-

ing Ac-DEVD-pNA cleavage at 405 nm, as described in Materials and Methods. The results, expressed as the increase above control values, are the means  $\pm$  SEM of values corresponding to at least to three experiments, each value being the mean of duplicate assays. \*\*\* $P$  < .001 with respect to control values. ### $P$  < .001 with respect to  $A\beta$  addition.

number of necrotic cells, the increase being prevented by dantrolene or xestospongine C ( $35.24\% \pm 2.39\%$ ,  $15.68\% \pm 3.01\%$ , and  $9.26\% \pm 2.76\%$  in cells treated with  $A\beta_{25-35}$  alone, with  $A\beta_{25-35}$  plus dantrolene, or with  $A\beta_{25-35}$  plus xestospongine C, respectively). Furthermore, in the presence of  $A\beta_{25-35}$  a higher number of neurons exhibiting apoptotic morphology was observed (from  $2.76\% \pm 0.47\%$  in controls to  $25.68\% \pm 1.48\%$  in  $A\beta_{25-35}$ -treated cells; Fig. 4A). Dantrolene was shown to prevent significantly, although partially, the  $A\beta_{25-35}$ -induced increase in the number of apoptotic cells ( $8.21\% \pm 1.15\%$  or  $25.68\% \pm 1.48\%$  apoptotic cells after treatment with  $A\beta_{25-35}$  in the presence or in the absence of dantrolene). Similarly, xestospongine C prevented the increase in the number of apoptotic cells induced by  $A\beta_{25-35}$  ( $8.51\% \pm 0.91\%$  or  $25.68\% \pm 1.48\%$  apoptotic cells after treatment with  $A\beta_{25-35}$  in the presence or in the absence of xestospongine C). Treatment of cortical cells with  $A\beta_{35-25}$  (25  $\mu$ M) for 48 hr did not affect the number of viable, necrotic, or apoptotic cells in comparison with the control situation (Fig. 4A).

Similar analysis was performed for the peptide  $A\beta_{1-40}$  (0.5  $\mu$ M). As observed for the  $A\beta_{25-35}$  peptide, the SYTO-13/PI labeling also revealed a reduction in cell viability in cortical neurons treated with  $A\beta_{1-40}$  (0.5  $\mu$ M) for 48 hr and the protective effect of dantrolene and xestospongine C (the number of viable cells was  $50.95\% \pm 2.43\%$ ,  $77.74\% \pm 1.98\%$ , or  $73.33\% \pm 3.00\%$  in cells treated with  $A\beta_{1-40}$  alone, with  $A\beta_{1-40}$  plus dantrolene, or with  $A\beta_{1-40}$  plus xestospongine C, respectively; Fig. 4B). Furthermore, the number of necrotic cells after  $A\beta_{1-40}$  exposure ( $28.09\% \pm 1.46\%$ ) is higher compared with control ( $13.95\% \pm 1.24\%$ ). The incubation with

dantrolene and  $A\beta_{1-40}$  or with xestospongine C and  $A\beta_{1-40}$  reduced the number of necrotic cells, exerting its protective effect over cortical neurons ( $10.05\% \pm 1.45\%$  or  $11.25\% \pm 0.65\%$ , respectively). Likewise, upon treatment of cortical neurons with  $A\beta_{1-40}$ , the number of apoptotic cells increased above control ( $2.76\% \pm 0.48\%$  and  $20.96\% \pm 1.80\%$  in control cells and  $A\beta_{1-40}$ -treated cells, respectively). Once again, both dantrolene and xestospongine C show a protective effect evident in the reduced number of apoptotic cells ( $5.68\% \pm 0.76\%$  or  $5.71\% \pm 0.95\%$  with  $A\beta_{1-40}$  in the presence of dantrolene or xestospongine C, respectively). Treatment of cortical cells with the reverse peptide,  $A\beta_{40-1}$  (0.5  $\mu$ M), for 48 hr did not affect the number of viable, necrotic, or apoptotic cells (Fig. 4B). Incubation of cortical neurons with dantrolene or xestospongine C alone did not affect the SYTO-13/PI fluorescence in comparison with controls.

Finally, data obtained also demonstrated a decrease in the number of viable cells and an increase in the number of apoptotic cells after incubation with thapsigargin (Fig. 4C). The number of viable cells decreased from  $79.36\% \pm 2.26\%$  in controls to  $55.32\% \pm 4.47\%$  or  $35.05\% \pm 2.54\%$  in thapsigargin-treated cells and the number of apoptotic cells increased from  $2.76\% \pm 0.47\%$  in controls to  $24.25\% \pm 2.67\%$  or  $54.40\% \pm 2.54\%$  upon treatment with thapsigargin during 24 or 48 hr, respectively. The number of necrotic cells was not significantly affected upon thapsigargin treatment.

## DISCUSSION

Our results with primary cultures of rat brain cortical neurons showed that incubation with the  $A\beta_{25-35}$  or  $A\beta_{1-40}$  peptides significantly increases the  $[Ca^{2+}]_i$ , activates caspase-3, and leads to apoptotic cell death. On the

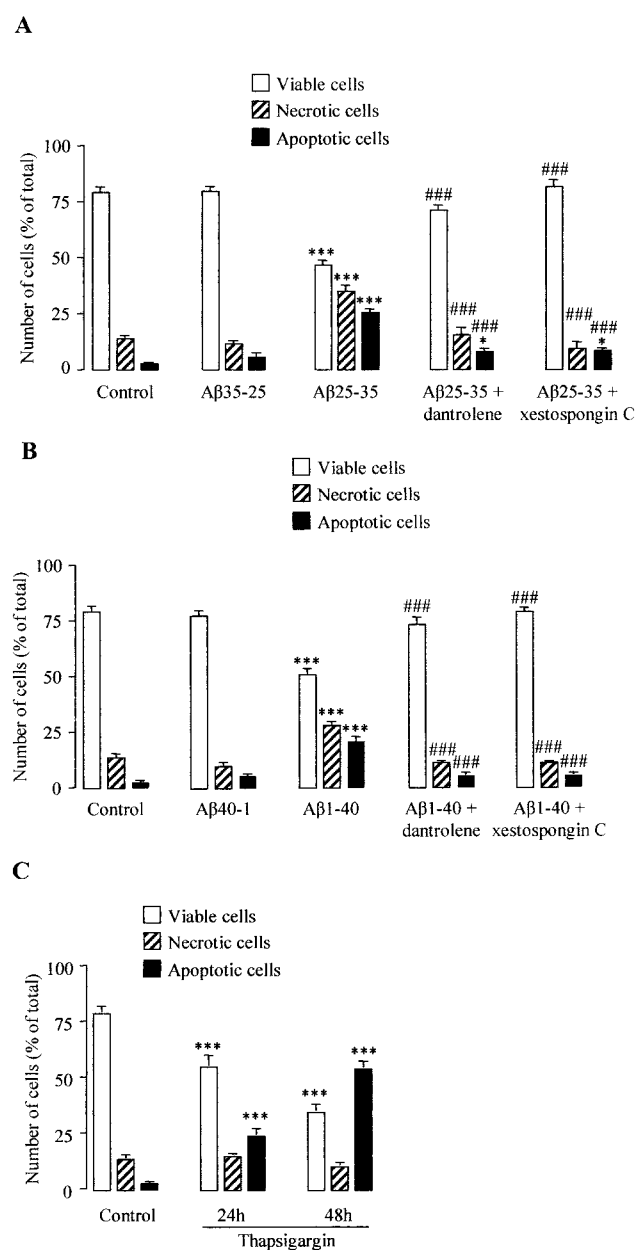


Fig. 4. DNA fragmentation induced by A $\beta_{25-35}$ , A $\beta_{1-40}$ , or thapsigargin. Cultured cortical neurons were treated, for 48 hr, with A $\beta_{25-35}$  (25  $\mu$ M; **A**) or A $\beta_{1-40}$  (0.5  $\mu$ M; **B**), in the absence or in the presence of dantrolene or xestospongin C, or with thapsigargin (2.5  $\mu$ M) for 24 and 48 hr (**C**). The cells were then incubated with SYTO-13 and PI to visualize nuclei morphology and were scored with a fluorescence microscope. The results, expressed as the percentage of the total number of cells, are the means  $\pm$  SEM of values corresponding to at least to three experiments, each value being the mean of duplicate assays. \* $P$  < .05, \*\*\* $P$  < .001 with respect to control values. #### $P$  < .001 with respect to A $\beta$  addition.

other hand, the reversed peptides, A $\beta_{35-25}$  or A $\beta_{40-1}$ , were not able to induce neuronal injury. The inhibitors of the ER Ca<sup>2+</sup> release through the RyR and IP<sub>3</sub>R, dantrolene and xestospongin C, respectively, prevented the

[Ca<sup>2+</sup>]<sub>i</sub> rise, the activation of caspase-3, or the increase in number of cells exhibiting apoptotic morphology triggered by the A $\beta$  peptides. Therefore, the present study demonstrates that Ca<sup>2+</sup> release through the ER RyR or IP<sub>3</sub>R is induced by the A $\beta$  peptides and is involved in A $\beta$ -induced apoptotic death. The comparative study performed with thapsigargin, a selective ER Ca<sup>2+</sup>-ATPase inhibitor, which was shown to lead to the loss of Ca<sup>2+</sup> homeostasis, to caspase-3 activation, and to apoptotic cell death, further supports this hypothesis.

Several studies have suggested that changes in intracellular Ca<sup>2+</sup> homeostasis play an important role in the modulation of apoptosis. Compounds that directly affect the intracellular Ca<sup>2+</sup> homeostasis have been shown to induce apoptosis (Jiang et al., 1994; Reynolds et al., 1996). The antiapoptotic protein Bcl-2 protects against thapsigargin-induced increases of Ca<sup>2+</sup> and apoptosis and was shown to suppress Ca<sup>2+</sup> release from ER (Guo et al., 1997; Wei et al., 1998). Recently, degradation of caspase-3, cleavage of poly(ADP-ribose) polymerase, DNA fragmentation, and cell death were observed in human neuroblastoma SH-SY5Y cells treated with thapsigargin (Kitamura et al., 2003). Previous studies with in-vitro-cultured neurons treated with A $\beta$  peptides demonstrated neuronal loss by apoptosis (Loo et al., 1993; Estus et al., 1997) that was due at least in part to the perturbation of intracellular Ca<sup>2+</sup> homeostasis (Scorziello et al., 1996; Huang et al., 2000). However, the routes of Ca<sup>2+</sup> homeostasis deregulation in A $\beta$ -treated cells remain controversial. Ca<sup>2+</sup> influx through voltage-sensitive Ca<sup>2+</sup> channels has been reported to be involved in the neurotoxic effects of A $\beta_{25-35}$  (Guo et al., 1996; Ekinici et al., 2000). Others have hypothesized that the ability of A $\beta$  peptides to form amyloid channels, and the subsequent disruption of Ca<sup>2+</sup> homeostasis, could be the molecular basis of the neurotoxicity of these peptides (Kawahara and Kuroda, 2000). Guo et al. (1996, 1997) have demonstrated that Ca<sup>2+</sup> release from RyR is involved in A $\beta_{25-35}$  toxicity in PC12 cells. In the present study, with cortical neurons in culture, we have shown that, in addition to Ca<sup>2+</sup> release through RyR, IP<sub>3</sub>R also contributes to A $\beta_{25-35}$ - and also A $\beta_{1-40}$ -induced cell death, insofar as the increase in [Ca<sup>2+</sup>]<sub>i</sub> and the induction of apoptotic cell death were significantly prevented by dantrolene and also by xestospongin C, a specific inhibitor of IP<sub>3</sub>R-mediated Ca<sup>2+</sup> release. IP<sub>3</sub>-mediated Ca<sup>2+</sup> release from the ER was previously demonstrated to trigger apoptosis in response to diverse signals (Distelhorst and Roderick, 2003). Many cell death stimuli are known to alter the concentration of Ca<sup>2+</sup> in the cytosol and the storage of Ca<sup>2+</sup> in the intracellular organelles (Baffy et al., 1993; Bian et al., 1997). Lam et al. (1993) showed that glucocorticoids induce release of calcium from ER, which is correlated with subsequent DNA fragmentation and apoptosis. Moreover, Ca<sup>2+</sup> release channels in the ER, i.e., IP<sub>3</sub>R and RyR, seem to participate in the signal transduction pathway of apoptosis (Guo et al., 1997; Jayaraman and Marks, 1997; Pan et al., 2000). Dubrez et al. (2001) have

shown that caffeine, a well-known inducer of  $\text{Ca}^{2+}$  release through ryanodine-sensitive  $\text{Ca}^{2+}$  channels expressed in ER, induces translocation of the proapoptotic protein Bax to the mitochondria, leading to caspase-3 activation and apoptotic cell death. Recent studies indicate that  $\text{IP}_3\text{R}$ -mediated  $\text{Ca}^{2+}$  spikes trigger opening of the mitochondrial permeability transition pore and, in turn, the release of cytochrome c (Szalai et al., 1999; Brustovetsky et al., 2002). The effect of added extracellular A $\beta$  peptides on intracellular  $\text{Ca}^{2+}$  stores located at the ER could involve a direct action of A $\beta$  on surface membrane constituents, which could modulate the activity of intracellular constituents. It was previously demonstrated that A $\beta$  toxicity involves the early induction of membrane lipid peroxidation, which liberates 4-hydroxynonenal that binds to ion channels at the ER and thereby alters their activities, leading to the perturbation of ER  $\text{Ca}^{2+}$  homeostasis (Mattson, 1998). Furthermore, A $\beta$  peptides could be internalized and act directly on the ER  $\text{Ca}^{2+}$  channel, increasing its  $\text{Ca}^{2+}$  sensitivity, as occurs with caffeine (Zhao et al., 2001), or could form a pore permeable to  $\text{Ca}^{2+}$  in the ER membrane.

ER  $\text{Ca}^{2+}$  release has been shown to have immediate effects on mitochondrial function, leading to rapid  $\text{Ca}^{2+}$  accumulation in mitochondria, which promotes cytochrome c release and activation of downstream caspase pathways in cells exposed to proapoptotic agents (Nutt et al., 2002). The A $\beta_{25-35}$ - or A $\beta_{1-40}$ -induced release of  $\text{Ca}^{2+}$  from ER might stimulate mitochondrial  $\text{Ca}^{2+}$  uptake, leading to cytochrome c release and activation of a caspase-dependent apoptotic cascade. In our laboratory, it was recently demonstrated that incubation of cortical neurons with A $\beta_{25-35}$  (25  $\mu\text{M}$ ) increases mitochondrial cytochrome c release and activates caspase-3 (Agostinho and Oliveira, 2003). Moreover, mitochondrial dysfunction was previously demonstrated upon A $\beta_{25-35}$  or A $\beta_{1-40}$  treatment (Pereira et al., 1998). Taken together, these observations suggest that ER stress induced by the disruption of  $\text{Ca}^{2+}$  homeostasis in neuronal cells exposed to A $\beta$  peptides may be associated with mitochondrial dysfunction. The disruption of  $\text{Ca}^{2+}$  homeostasis mediated through  $\text{Ca}^{2+}$  release by ER  $\text{IP}_3\text{R}$  and RyR may be intermediate between ER stress and mitochondrial dysfunction. In human neuroblastoma cells, both ER- and mitochondrial-dependent pathways were shown to be involved in thapsigargin-induced apoptosis (Kitamura et al., 2003).

One of the mechanisms involved in the activation of apoptotic cell death upon A $\beta_{25-35}$ - and A $\beta_{1-40}$ -induced  $\text{Ca}^{2+}$  release from ER could be related to calcineurin activation. It was recently demonstrated in our laboratory that this  $\text{Ca}^{2+}$ /calmodulin-dependent phosphatase is involved in A $\beta_{25-35}$  toxicity in cortical neurons (Agostinho and Oliveira, 2003). Calcineurin has previously been proposed as a link between  $\text{Ca}^{2+}$  deregulation and neuronal cell death (Wang et al., 1999; Foster et al., 2001). Jayaraman and Marks (2000) have shown that calcineurin is downstream of the  $\text{IP}_3\text{R}$  in the apoptotic pathway, sup-

porting the involvement of ER  $\text{Ca}^{2+}$  homeostasis deregulation in calcineurin-mediated apoptosis. When activated, calcineurin dephosphorylates the proapoptotic BAD protein, allowing its translocation to the mitochondria, where it binds to antiapoptotic Bcl-2 family members and induces cytochrome c release (Springer et al., 2000). In the cytosol, cytochrome c binds Apaf-1 and dATP to form a complex that activates caspase-9, which can activate the executioner caspase-3 (Desagher and Martinou, 2000).

Several studies have shown that caspase-3 activation is an initial event in neurodegeneration induced by A $\beta_{25-35}$  (Mattson et al., 1998; Harada and Sugimoto, 1999; Xiao et al., 2002). Under our experimental conditions, caspase-3 activity increased upon treatment with A $\beta_{25-35}$  or A $\beta_{1-40}$ , this increase being prevented by the ER  $\text{Ca}^{2+}$  release inhibitors dantrolene and xestospongine C, which suggests that perturbation of ER  $\text{Ca}^{2+}$  homeostasis mediated by  $\text{Ca}^{2+}$  release from RyR and  $\text{IP}_3\text{R}$  is involved in the activation of caspase-3. Furthermore, thapsigargin significantly increased caspase-3 activity, supporting the activation of this caspase executioner of apoptosis under conditions of perturbed ER  $\text{Ca}^{2+}$  homeostasis. Increased  $\text{Ca}^{2+}$  release from ER has been previously associated with caspase-3 activation (Kim et al., 2002). In addition, activation of caspase-3 has been shown to be completely suppressed in cells overexpressing Bcl-2 targeted specifically to the ER (Häcki et al., 2000).

In conclusion, our results show that A $\beta_{25-35}$  and A $\beta_{1-40}$  induce  $\text{Ca}^{2+}$  homeostasis deregulation, caspases activation, and apoptotic neuronal death. Dantrolene and xestospongine C, inhibitors of the ER  $\text{Ca}^{2+}$ -release channels RyR and  $\text{IP}_3\text{R}$ , respectively, prevented the A $\beta_{25-35}$ - or A $\beta_{1-40}$ -induced rise in  $[\text{Ca}^{2+}]_i$ , caspases activation, and apoptotic cell death, indicating that  $\text{Ca}^{2+}$  release through the ER RyR and  $\text{IP}_3\text{R}$  is involved in the neurotoxicity induced by these peptides. This hypothesis is further supported by the data obtained with thapsigargin, an inhibitor of the ER  $\text{Ca}^{2+}$ -ATPase. Therefore, ER stress resulting from deregulation of ER  $\text{Ca}^{2+}$  homeostasis seems to play a crucial role in neurodegeneration triggered by the AD-associated A $\beta$  peptide. However, because of the partial protective effect of dantrolene and xestospongine C against A $\beta$ -induced neuronal death, it cannot be ruled out the hypothesis that other intracellular neurotoxic mechanisms could be triggered by the A $\beta$  peptides and contribute to apoptotic death, in a manner independent of or synergistic with  $\text{Ca}^{2+}$ -sensitive ER stress. Given the role of perturbed ER function in AD (Mattson and Chan, 2001; Mattson et al., 2001), the regulation of ER  $\text{Ca}^{2+}$  homeostasis can be a target for the treatment of this neurodegenerative disorder.

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