Available online at www.sciencedirect.com

ScienceDirect

Biochimica et Biophysica Acta 1773 (2007) 157 – 165

Biochimica et Biophysica Acta BBA

www.elsevier.com/locate/bbamcr

The amyloid precursor protein potentiates CHOP induction and cell death in response to ER Ca^{2+} depletion

Ekaterini Copanaki [a], Tina Schürmann [b], Anne Eckert [c], Kristina Leuner [c], Walter E. Müller [c], Jochen H.M. Prehn [a,d,1], Donat Kögel [a,*,1]

[a] *Department of Neurosurgery, Johann Wolfgang Goethe University Clinics, D-60590 Frankfurt, Germany*
[b] *Interdisciplinary Center for Clinical Research, University Münster Clinics, D-48149 Münster, Germany*
[c] *Department of Pharmacology and Toxicology, Biocenter, Johann Wolfgang Goethe University, D-60439 Frankfurt, Germany*
[d] *Department of Physiology and RCSI Neuroscience Research Centre, Royal College of Surgeons in Ireland, 123, St. Stephen's Green, Dublin 2, Ireland*

Received 22 March 2006; received in revised form 20 September 2006; accepted 10 October 2006
Available online 19 October 2006

Abstract

Here we investigated the role of the amyloid precursor protein (APP) in regulation of Ca^{2+} store depletion-induced neural cell death. Ca^{2+} store depletion from the endoplasmic reticulum (ER) was induced by the SERCA (Sarco/Endoplasmic Reticulum Calcium ATPase) inhibitor thapsigargin which led to a rapid induction of the unfolded protein response (UPR) and a delayed activation of executioner caspases in the cultures. Overexpression of APP potently enhanced cytosolic Ca^{2+} levels and cell death after ER Ca^{2+} store depletion in comparison to vector-transfected controls. GeneChip^{R} and RT-PCR analysis revealed that the expression of classical UPR chaperone genes was not altered by overexpression of APP. Interestingly, the induction of the ER stress-responsive pro-apoptotic transcription factor CHOP was significantly upregulated in APP-overexpressing cells in comparison to vector-transfected controls. Chelation of intracellular Ca^{2+} with BAPTA-AM revealed that enhanced CHOP expression after store depletion occurred in a Ca^{2+}-dependent manner in APP-overexpressing cells. Prevention of CHOP induction by BAPTA-AM and by RNA interference was also able to abrogate the potentiating effect of APP on thapsigargin-induced apoptosis. Application of the store-operated channel (SOC)-inhibitors SK & F96365 and 2-APB downmodulated APP-triggered potentiation of cytosolic Ca^{2+} levels and apoptosis after treatment with thapsigargin. Our data demonstrate that APP significantly modulates Ca^{2+} store depletion-induced cell death in a SOC- and CHOP-dependent manner, but independent of the UPR.

Keywords: Alzheimer's disease; Apoptosis; Calcium store depletion; RNA interference; Microarray

1. Introduction

The β-Amyloid Precursor Protein (APP) and its metabolism play fundamental roles in the pathophysiology of familial and sporadic Alzheimer's disease (AD) [1–3]. Aβ, the major constituent of amyloid plaques in the brain of AD patients, is generated by sequential β- and γ-secretase cleavage of APP in the amyloidogenic pathway [3–5]. This pathway is promoted by mutations in APP, presenilin 1 and presenilin 2, and mutations in these three genes associated with familial forms of AD (FAD) [3,4,6]. In contrast, cleavage of APP within the Aβ domain precludes formation of Aβ peptides in the anti-amyloidogenic pathway. Although altered APP processing and aberrant expression of APP are known to play a crucial roles in the pathophysiology of AD and Down syndrome [4,7–11], much less is known about the non-pathological, physiological function of APP and its cleavage products [12]. However, accumulating evidence suggests that APP is implicated in regulation of gene expression [13–16].

Ca^{2+} is involved in a multitude of physiological functions in neurons, such as neurotransmitter release and neuronal excitability. On the other hand, intracellular Ca^{2+} overloading has

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; CHOP, C/EBP homologous protein-10; ER, endoplasmic reticulum; SERCA, sarcoplasmic ER Ca^{2+}-ATPase; UPR, unfolded protein response

* Corresponding author. Experimental Neurosurgery, Johann Wolfgang Goethe University Clinics, Theodor-Stern-Kai 7, HS 25 B, D-60590 Frankfurt am Main, Germany. Tel.: +49 69 6301 6940; fax: +49 69 6301 5575.
E-mail address: koegel@em.uni-frankfurt.de (D. Kögel).

[1] The authors share senior authorship.

0167-4889/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2006.10.002

been associated with cell injury and death. There is abundant evidence that dysregulation of Ca^{2+} homeostasis is critically involved in brain aging [17] and the pathophysiology of AD [18,19], and a contributory role of mutated presenilins and Aβ for the disruption of Ca^{2+} homeostasis observed in AD has been clearly established [2,18–20]. In addition to Aβ, further APP-derived proteolytic cleavage products have been implicated in disturbance of cellular Ca^{2+} homeostasis, including β-CTF (C99), and the APP-intracellular domain (AICD), which represent the C-terminal fragments of APP generated by β-secretase and γ-secretase activities, respectively [18,21,22]. There is compelling evidence for an intimate association between increased intracellular Ca^{2+} levels and the pathophysiological hallmarks of AD, i.e. a further accumulation of Aβ peptides, hyperphosphorylation of Tau, and neuronal apoptosis (reviewed in [22]).

The endoplasmic reticulum (ER) is considered to be the major intracellular store of Ca^{2+} in neurons and non-neuronal cells. The concentration gradients of Ca^{2+} in resting neurons between the extracellular space (~1–2 μM) and the cytoplasm (50–300 nM), as well as between the cytoplasm and the ER (100–500 μM) are steadily maintained by energy-dependent Ca^{2+} transport systems: the plasma membrane Ca^{2+} ATPases (PMCAs), and the sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCAs) [18,23]. Release of Ca^{2+} from the lumen of the ER into the cytosol requires two types of ligand-gated ion channels: the Inositol 1,4,5-trisphosphate (InsP3) receptors (InsP3Rs) and the ryanodine receptors (RyRs) [20]. Another major pool of Ca^{2+} resides in the extracellular space. Tight regulation of the influx of Ca^{2+} from the extracellular space plays a critical role in maintaining neuronal Ca^{2+} homeostasis, both in resting neurons and after Ca^{2+} store depletion. Acquisition of extracellular Ca^{2+} is achieved through the activity of ligand-dependent Ca^{2+} channels, voltage-dependent Ca^{2+} channels, and store-operated channels (SOCs) [20,24].

A number of previous studies have suggested that disturbances in Ca^{2+} homeostasis might play a crucial role in sensitizing neurons to degeneration and cell death in AD (reviewed in [2,18,20]). Since APP and its metabolism have been implied in regulation of Ca^{2+} homeostasis, we investigated the effect of overexpressed APP on the transcriptional stress response and cell death after ER Ca^{2+} store depletion. Our data reveal that overexpression of APP potentiates cytosolic Ca^{2+} levels after depletion of the ER stores by activating SOC-dependent entry of Ca^{2+} from the extracellular space. Furthermore, we show that APP-dependent elevation of cytosolic Ca^{2+} levels is associated with enhanced expression levels of the pro-apoptotic stress response gene *CHOP*, as well as CHOP-dependent cell death after Ca^{2+} store depletion.

2. Materials and methods

2.1. Materials

Thapsigargin was purchased from Alexis (Grünberg, Germany). Acetyl-DEVD-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was from Bachem (Heidelberg, Germany). All other biochemicals and chemicals came in analytical grade purity from Roche Diagnostics (Mannheim, Germany) or Roth (Karlsruhe, Germany). 5,5′-dimethyl BAPTA-AM (BAPTA-AM) was from Molecular Probes (Invitrogen, Karlsruhe, Germany). The SOC inhibitors 2-APB and SK & F96365 were from Sigma (Deisenhofen, Germany).

2.2. Cell culture

PC12 cell lines expressing equal amounts of human wild type APP (PC12 APPwt, clone M5), as well as vector-transfected control cells (PC12 vector, clone O1) have been described previously [25]. Cell lines were cultivated in DMEM supplemented with 10% heat-inactivated fetal calf serum, 5% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All experiments were performed in NGF-untreated cells.

2.3. Calcium imaging

The cells were plated on poly-L-lysine-coated cover slips. Intracellular free Ca^{2+} levels were quantified by fluorescence imaging of the calcium indicator dye fura-2 as described previously [26]. Briefly, cells were incubated for 30 min in the presence of 5 μM acetoxymethylester form of fura-2 (Molecular Probes, Amsterdam, The Netherlands), then washed twice with HBSS buffer and incubated for 30 min prior to imaging. Cells were imaged on a Zeiss Axiovert microscope (40× oil immersion objective) coupled to an Attofluor imaging system. The average Ca^{2+} concentration in 12–20 cell bodies per microscope field was quantified in 8–12 independent experiments (from at least four separate cultures) per treatment condition. Fluorescence was monitored prior to (baseline calcium) and after exposure of cells to vehicle or thapsigargin (1 μM; peak calcium) which were added to the bathing medium by dilution from a 2× stock. DMSO vehicle treatment did not alter basal fluorescence of fura-2. Fluorescence was quantified using the fluorescence ratio (R) of the emission (510 nm) measured following excitation at 340 nm divided by the emission following excitation at 380 nm.

2.4. Quantification of apoptosis

Measurement of caspase 3-like protease activity and analysis of nuclear apoptosis was performed as described previously [14].

2.5. Western blotting

Western blotting was essentially performed as described previously [14]. The blots were incubated with a mouse monoclonal anti-CHOP antibody (Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:1.000, a mouse monoclonal anti-GAPDH antibody (Acris, Hiddenhausen, Germany) diluted 1:10.000, a rabbit polyclonal anti-active p20 caspase 7 antiserum (New England Biolabs, Frankfurt, Germany) diluted 1:1000 or a rabbit polyclonal anti-active p20 caspase 3 antiserum (Merck-Frosst, Kirkland, Canada) diluted 1:1.000. To ensure equal loading of the samples, membranes were incubated with a mouse monoclonal anti-α-Tubulin antibody (clone DM 1 A; Sigma, Deisenhofen, Germany) diluted 1:10.000.

2.6. Semiquantitative RT-PCR

Semiquantitative RT-PCR and quantification of CHOP, BiP and GAPDH expression was performed as described previously [25].

2.7. Microarray analysis

Generation of cRNA probes and hybridization to RG-U34A arrays (Affymetrix) was performed as described elsewhere [25]. The Affymetrix Microarray Suite 5.0 was used to scan and analyze the relative abundance of each gene from the average difference of intensities. For each individual gene, the signal ratio between perfect match and mismatch probe cells was used to determine its "Absolute Call", indicating whether the corresponding transcript was present (P), absent (A), or marginal (M). For analysis of

differential target gene expression, the "Difference Call Decision Matrix" was employed. Individual transcript levels were ranked as either increased (I), marginally increased (MI), decreased (D), marginally decreased (MD) or not changed (NC). To be considered as differentially upregulated, the Absolute Call of individual transcripts had to be present, the Difference Call increased, and the fold induction had to be at least 3.0 fold in PC12 vector control cells.

2.8. Knockdown of CHOP

Annealed double-stranded siRNAs were obtained from Ambion (Huntingdon, United Kingdom). The sequence of the CHOP siRNA was follows: 5′-GGUCCUGUCCUCAGAUGAAdTdT-3′ (sense) and 5′-UUCAUCUGAGGACAGGACCdTdG-3′. Blast searches confirmed that these sequences were not homologous to any genes other than CHOP in rats. Cells were transfected with 30 nM siRNAs using siMPORTER™ (Biomol, Hamburg, Germany) or mock-transfected as described by the manufacturer. After incubation of the transfected cells for 20 h, they were treated with thapsigargin or vehicle (DMSO).

2.9. Statistics

Data are given as means±SEM. For statistical comparison, *t*-test or one-way ANOVA followed by Tukey test were employed. *P* values smaller than 0.05 were considered statistically significant.

3. Results

3.1. ER store depletion by thapsigargin leads to enhanced cytoplasmic Ca^{2+} levels and apoptosis in APP overexpressing cells

To analyze the role of APP as a modulator of Ca^{2+} homeostasis and Ca^{2+} store depletion-induced cell death, we employed rat PC12 cell lines overexpressing the wild type human APP695 isoform (PC12 APP) in comparison with the empty vector-transfected control cell line PC12 vector (PC12 vector)[25]. Of note, we have previously shown that overexpression of membrane proteins transported via the ER/Golgi secretory pathway per se does not affect the sensitivity of PC12 cells to apoptosis [25]. In initial experiments, we established the optimal working concentrations and incubation periods for activation of effector caspases after Ca^{2+} store depletion induced by thapsigargin in parental PC12 cells (data not shown). Overexpression of APP was associated with significantly enhanced effector caspase activity 20 h after treatment with 0.3 μM and 1 μM thapsigargin (Fig. 1A).

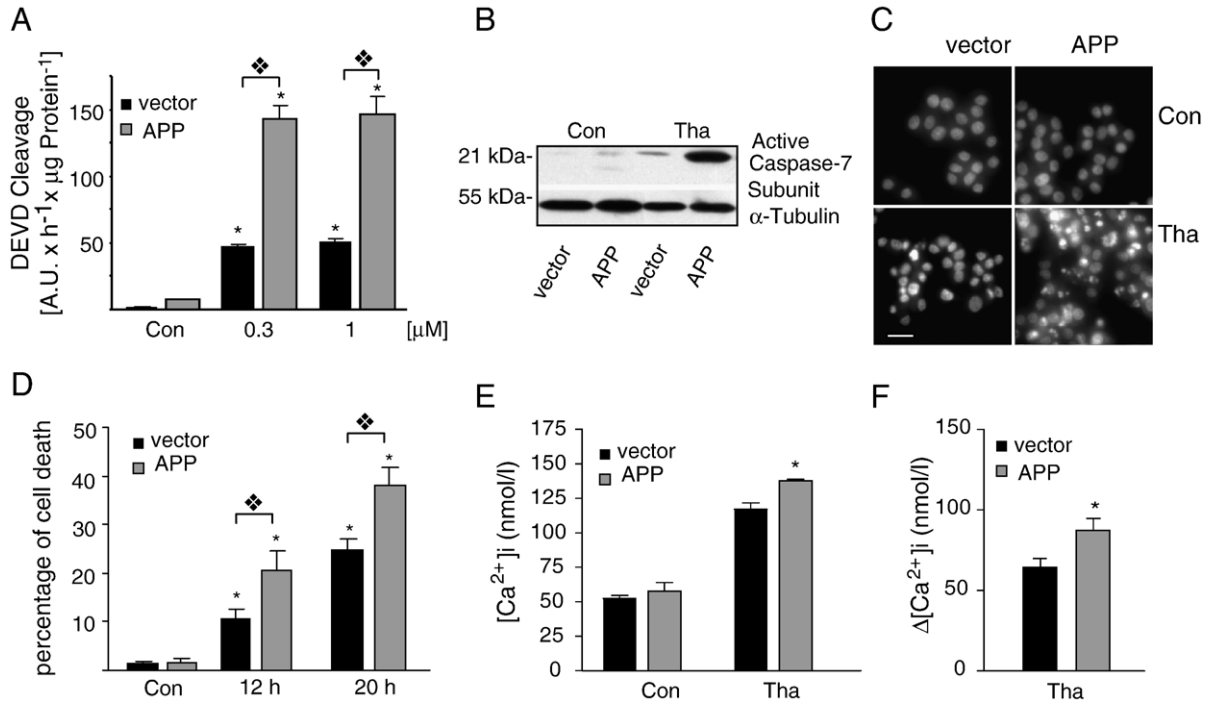


Fig. 1. Overexpression of wild type APP enhances thapsigargin-induced cytoplasmic Ca^{2+} levels and apoptosis in PC12 cells. (A) Analysis of effector caspase activation. Cultures of PC12 neo O1 (vector) and PC12 APPwt M5 (APP) cells were treated with the indicated concentrations of thapsigargin or vehicle (Con) for 20 h. Caspase 3-like activity was monitored over 1 h. Data are means±SEM from $n=4$ to 6 cultures per treatment. The experiment was repeated two times with similar results. A.U.=arbitrary fluorescence units. *$p<0.05$: Difference from vehicle-treated controls. ❖$p<0.05$: Difference from respective control cell line PC12 vector. (B) Activation of caspase 7 during thapsigargin-induced apoptosis. PC12 neo O1 (vector) and PC12 APPwt M5 (APP) cells were treated with 0.3 μM thapsigargin (Tha) or vehicle (Con) for 16 h. Active caspase 7 p20 subunits were detected by Western Blotting with α-tubulin serving as a loading control. Experiments were repeated two times with similar results. (C) Nuclear apoptosis visualized by Hoechst staining. Cultures of PC12 neo O1 (vector) and PC12 APPwt M5 (APP) cells were treated with 1 μM thapsigargin (Tha) or vehicle (Con) for 20 h. Scale bar: 25 μm. (D) Quantitative analysis of nuclear apoptosis visualized by Hoechst staining. Experiments were repeated two times with similar results. *$p<0.05$: Difference from vehicle-treated controls. ❖$p<0.05$: Difference from respective control cell line PC12 vector. (E and F) Analysis of cytoplasmic Ca^{2+} levels after store depletion with thapsigargin by fura-2 Calcium Imaging. PC12 neo O1 (vector) and PC12 APPwt M5 (APP) cells were treated with 1 μM thapsigargin or vehicle (DMSO). Absolute cytoplasmic Ca^{2+} levels (nmol/l) are shown in panel E. Data represent peaks of Ca^{2+} levels after stimulation with thapsigargin; *$p<0.05$ vs. untreated control (PC12 vector). The difference of cytoplasmic Ca^{2+} levels in comparison to the DMSO control in the respective cell lines after store depletion (nmol/l) obtained from the experiment shown in panel E are shown in panel F. *$p<0.05$ vs. control (PC12 vector).

Western blotting analysis furthermore revealed that thapsigargin-induced activation of effector caspase 7 was more prominent in the APP-overexpressing cell line in comparison to the PC12 vector control (Fig. 1B). Next, we visualized nuclear apoptosis in PC12 vector and PC12 APP cells by Hoechst staining on the single-cell level. Cultures of APP-overexpressing cells revealed an increased incidence of cells displaying an apoptotic nuclear morphology after treatment with 0.3 μM thapsigargin (Fig. 1C). Subsequent quantitative analysis by counting the number of apoptotic nuclei in the cultures revealed that the percentage of apoptotic cells in the thapsigargin-treated PC12 APP cultures was markedly higher than PC12 vector control cells (Fig. 1D). Analysis of APP-mediated effects on cytoplasmic [$Ca^{(2+)}$] after depletion of the ER Ca^{2+} stores with thapsigargin was performed by Fura2-Imaging (Fig. 1E and F). While there were no significant changes in the baseline values of cytoplasmic [$Ca^{(2+)}$] in vehicle-treated PC12 APP cells (Fig. 1E), overexpression of APP significantly enhanced cytoplasmic [$Ca^{(2+)}$] transients after treatment with thapsigargin (Fig. 1E and F).

3.2. APP modulates the transcriptional stress response to thapsigargin

Many ER resident chaperones require high ER Ca^{2+} concentrations for their activity. Thus, ER Ca^{2+} store depletion leads to accumulation of misfolded proteins in the ER, a condition known as ER stress. ER stress activates a conserved cellular stress response, the so called unfolded protein response (UPR). Thapsigargin has been shown to activate the unfolded protein response (UPR) [27] and our data clearly demonstrated that APP significantly enhances cell death induced by ER Ca^{2+} store depletion. To investigate the underlying transcriptional mechanisms of APP-mediated cell death potentiation, we performed gene expression microarray analysis of APP-mediated transcriptional changes in APP-overexpressing cells.

A

Gene identity	accession	vector	APP
sgk-1	**L01624**	**13.9**	**27.9**
NIPK	**H31287**	**9.9**	**13.0**
CHOP	**U30186**	**8.6**	**12.1**
BiP	S63521	9.9	8.0
NGFI-B	U17254	4.9	6.1
Erp70	M86870	5.3	4.6
grp94	S69316	4.3	4.0
Dnajb9	AI011998	3.7	3.7
grp58	AA892637	4.0	3.7
Nupr1	AF006617	3.3	3.7
Ifrd1	AI014163	3.7	3.5
ORP150	AI009098	3.0	3.5
Txndc7	AA892532	3.3	3.5
calnexin	AI010725	3.3	3.3
calreticulin	X53363	4.0	3.0
Sel1L	AA859897	4.0	2.8
PHAS-I	U05014	3.5	2.5
P4HB	M21476	3.0	1.9

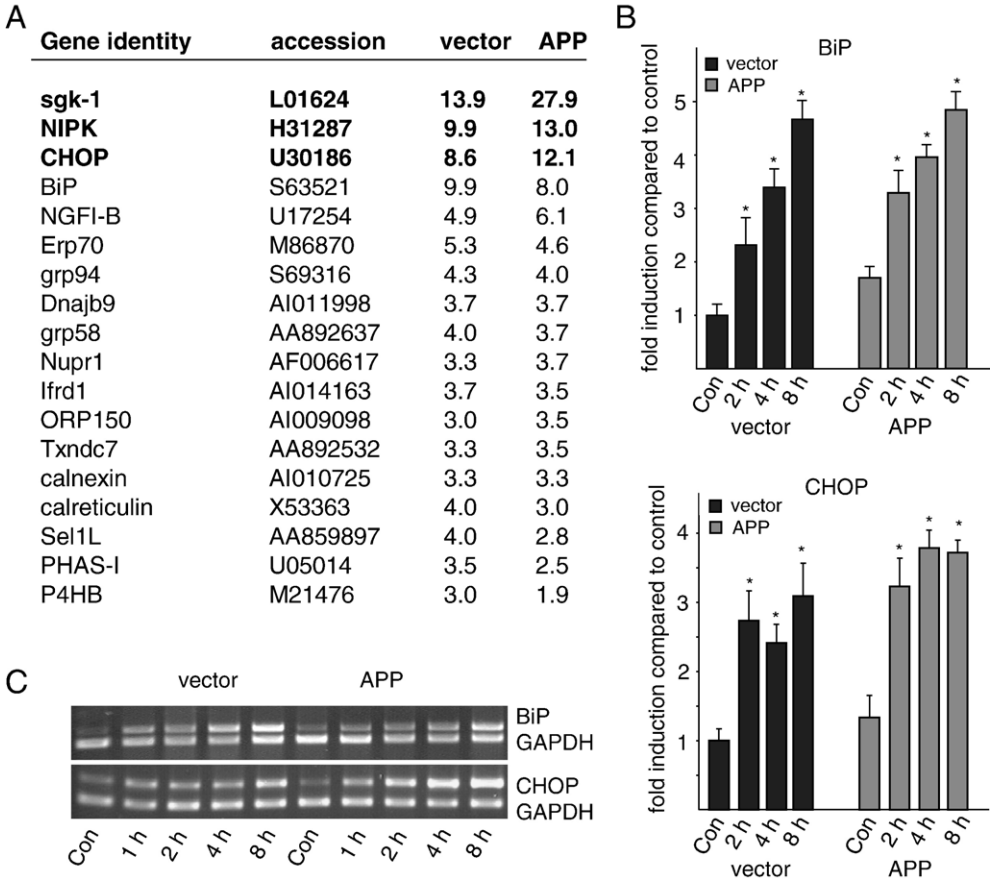


Fig. 2. APP regulates transcriptional events in response to ER Ca^{2+} store depletion. (A) Expression profiling by microarray analysis after induction of ER stress with thapsigargin. PC12 neo O1 (vector) and PC12 APPwt M5 (APP) cells were treated with 0.3 μM thapsigargin for 8 h. Controls were treated with vehicle (DMSO) for 8 h. cRNA was prepared and hybridized to Rat-U34A microarrays (Affymetrix) after which relative mRNA levels were quantified with the Affymetrix Microarray Suite 5.0 software (MAS 5.0) as described in Materials and methods. Genes found to be differentially expressed in both cell lines after thapsigargin-treatment are shown with fold-induction values (as determined with MAS 5.0) in comparison to the control (PC12 vector DMSO). (B and C) Thapsigargin-induced upregulation of CHOP expression is enhanced by APP. For analysis of CHOP and BiP expression, cDNA from PC12 neo O1 (vector) and PC12 APPwt M5 (APP) cells treated with vehicle (con) or 0.3 μM thapsigargin for 1 h, 2 h and 4 h and 8 h was amplified by 25 cycles of PCR with GAPDH serving as internal control. (C) PCR amplification products were separated by agarose gel electrophoresis and visualized with 0.1% ethidium bromide. (B) Band intensities relative to the GAPDH control bands were analyzed with the OneDScan software, and are shown as fold induction value relative to the control (PC12 vector treated with vehicle). Data are means ± SEM from $n=4$ cultures per treatment. *$p<0.05$ vs. control (PC12 vector DMSO).

GeneChip analysis of transcriptional changes after induction of ER stress with thapsigargin revealed that several UPR target genes were significantly upregulated in both PC12 vector and PC12 APP cells. In comparison to the PC12 vector control, most UPR target genes implicated in alleviation of ER stress (molecular chaperones such as BiP, grp94, Erp70 and calnexin) were induced to a comparable extent in PC12 APP cells (Fig. 2A). However, expression of serum/glucocorticoid-regulated kinase 1 (sgk1), the stress-responsive pro-apoptotic kinase NIPK and the pro-apoptotic transcription factor CHOP, was notably enhanced by overexpression of APP. Transcriptional induction of BiP and CHOP was also analyzed by semiquantitative RT-PCR after treatment with 0.3 μM thapsigargin for 1, 2 and 4 h (Fig. 2B and C). The kinetics and magnitude of *BiP* induction were very similar for both cell lines (PC12 vector O1 and PC12 APPwt M5). As predicted from the microarray data, RT-PCR analysis of the kinetics of *CHOP* induction revealed that thapsigargin elicited an increased expression of the *CHOP* gene in PC12 APP cells (Fig. 2B and C).

3.3. Chelation of intracellular Ca^{2+} with BAPTA-AM downmodulates CHOP induction and Ca^{2+} store depletion-induced cell death

In light of the facts that expression of CHOP has been suggested to be induced by activation of the UPR [28], but also by enhanced cytosolic Ca^{2+} levels [29], we next analyzed the effect of the Ca^{2+} chelator BAPTA-AM on the observed potentiation of thapsigargin-triggered CHOP induction in PC12 APP cells (Fig. 3A). Indeed, treatment with BAPTA-AM lead to a prominent downmodulation of CHOP induction in PC12 APP cells, whereas the effect of BAPTA-AM on CHOP expression in PC12 vector cells was less pronounced (Fig. 3A). To correlate these observations with APP-dependent modulation of apoptosis, we again performed DEVDase assays (Fig. 3B). Interestingly, chelation of intracellular Ca^{2+} with BAPTA-AM was able to downmodulate apoptosis in PC12 APP cells to a level comparable to that observed in PC12 vector cells (Fig. 3B).

3.4. RNA interference against CHOP abrogates the potentiating effect of APP on Ca^{2+} store depletion-induced cell death

Our data obtained so far suggested that APP significantly modulated CHOP expression and cell death after Ca^{2+} store depletion. To investigate the functional relevance of CHOP in the potentiating effect of APP on cell death triggered by Ca^{2+} store depletion, we knocked down expression of CHOP by siRNA. To monitor the silencing of CHOP we performed Western blot analysis after treatment with 1 μM Thapsigargin (Fig. 4A). In comparison to mock-transfected cultures, the thapsigargin-induced CHOP protein levels were significantly lowered by application of CHOP siRNA in both PC12 vector and PC12 APP cultures (Fig. 4A). Subsequent DEVDase assays revealed that silencing of CHOP markedly reduced thapsigargin-triggered apoptosis in PC12 APP to a level comparable to that observed in PC12 vector cells after Ca^{2+} store depletion (Fig. 4B).

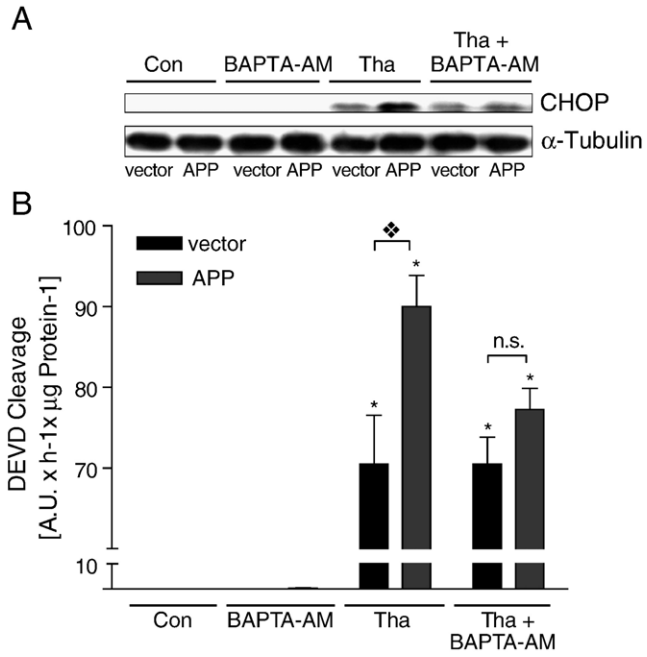


Fig. 3. Buffering of cytosolic Ca^{2+} with BAPTA-AM antagonizes the potentiating effect of APP on CHOP expression and cell death. PC12 vector (vector) and PC12 APP (APP) cells were treated with 1 μM thapsigargin in the presence or absence of 1 μM BAPTA-AM for 12 h. Controls were treated with vehicle (DMSO) in the presence or absence of BAPTA-AM (1 μM) for 12 h. (A) Protein expression levels of CHOP after treatment with 1 μM BAPTA-AM and treatment with 1 μM thapsigargin was analyzed by Western blotting with alpha-Tubulin expression levels serving as a loading control. Experiments were performed three times with similar results. (B) Analysis of effector caspase activation after thapsigargin treatment. Caspase 3-like activity was monitored over 1 h. Data are means±SEM from $n=4$ cultures per treatment. A.U.= arbitrary fluorescence units. Experiments were performed three times with similar results. *$p<0.05$ vs. untreated control (PC12 vector); ❖$p<0.05$: difference from respective control cell line PC12 vector. n.s.: not significant.

3.5. Inhibition of store-operated channels (SOCs) downmodulates APP-dependent potentiation of cytoplasmic Ca^{2+} levels and apoptosis after ER store depletion

Our initial Ca^{2+} imaging experiments in PC12 APP cells clearly demonstrated abnormally high intracellular Ca^{2+} levels after store depletion (Fig. 1E and F). On the one hand, these data might reflect elevated ER Ca^{2+} levels in untreated PC12 APP cells in comparison to PC12 vector control cells, leading to an increased release of ER Ca^{2+} into the cytosol after store depletion with thapsigargin. On the other hand, store depletion is also known to activate the influx of extracellular Ca^{2+} through SOCs, and this influx might be affected by overexpressed APP. To discriminate between these two possibilities, we next investigated a possible amplifying effect of APP on SOC entry of Ca^{2+}. To this end, we applied two different SOC-inhibitors, 1-{beta-[3-(4-methoxyphenyl)propoxyl]-4-methoxyphenethyl}-1*H*-imidazole (SK & F96365) and 2-Aminoethoxydiphenyl borate (2-APB). To prevent potential non-selective effects of these inhibitors on the Ca^{2+} release from the ER [30,31], we used both inhibitors at a final concentration of 10 μM. Fura2-Imaging again revealed potently enhanced cytoplasmic [$Ca^{(2+)}$] transients after treatment with thapsigargin in PC12 APP cells (Fig. 5A–C).

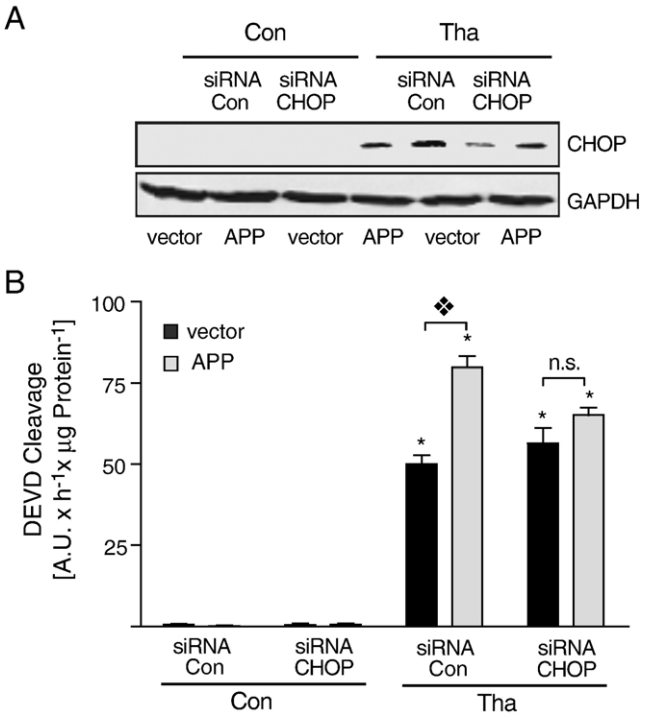


Fig. 4. Knockdown of CHOP with siRNA abrogates potentiation of Ca^{2+} store depletion-induced cell death in PC12 APP cells. Cultures of PC12 neo O1 (vector) and PC12 APPwt M5 (APP) cells were mock-transfected or transfected with 30 nM of CHOP siRNA, incubated for 16 h, and then treated with 1 μM thapsigargin (Tha) or vehicle (Con) for 12 h. (A) Protein expression levels of CHOP after treatment with CHOP siRNA and subsequent treatment with 1 μM thapsigargin was analyzed by Western blotting. GAPDH expression levels served as a loading control. Experiments were performed three times with similar results. (B) Analysis of effector caspase activation after thapsigargin treatment. Caspase 3-like activity was monitored over 1 h. Data are means± SEM from $n=4$ cultures per treatment. A.U.=arbitrary fluorescence units. Experiments were performed three times with similar results. *$p<0.05$ vs. untreated control (PC12 vector); ❖$p<0.05$: difference from respective control cell line PC12 vector. n.s.: not significant.

There was a pronounced downmodulating effect of SK & F96365 (Fig. 5A and B), as well as 2-APB (Fig. 5A and C) on cytoplasmic Ca^{2+} transients after store depletion in both cell lines, which however was much more potent in PC12 APP cells compared to PC12 vector cells (Fig. 5A–C). Indeed, the cytoplasmic [Ca($^{2+}$)] transients in the presence of both SOC-inhibitors (reflecting the ER Ca^{2+} release) reached very similar levels in PC12 APP and PC12 neo cells, indicating that APP-dependent Ca^{2+} overloading after store depletion occurred via amplified SOC entry of Ca^{2+}. We also investigated the effect of SOC-inhibition on thapsigargin-triggered apoptosis (Fig. 5D). Apoptosis was most potently inhibited after co-application of SK & F96365 and 2-APB with BAPTA-AM (Fig. 5D), indicating that Ca^{2+} release from the ER and SOC entry of Ca^{2+} act in a synergistic manner to promote apoptosis after treatment with thapsigargin. SK & F96365 and 2-APB alone were also able to significantly decrease the potentiating effect of APP on thapsigargin-triggered apoptosis (Fig. 5D), whereas the inhibitory effect on apoptosis in PC12 vector control cells was less pronounced (Fig. 5B).

4. Discussion

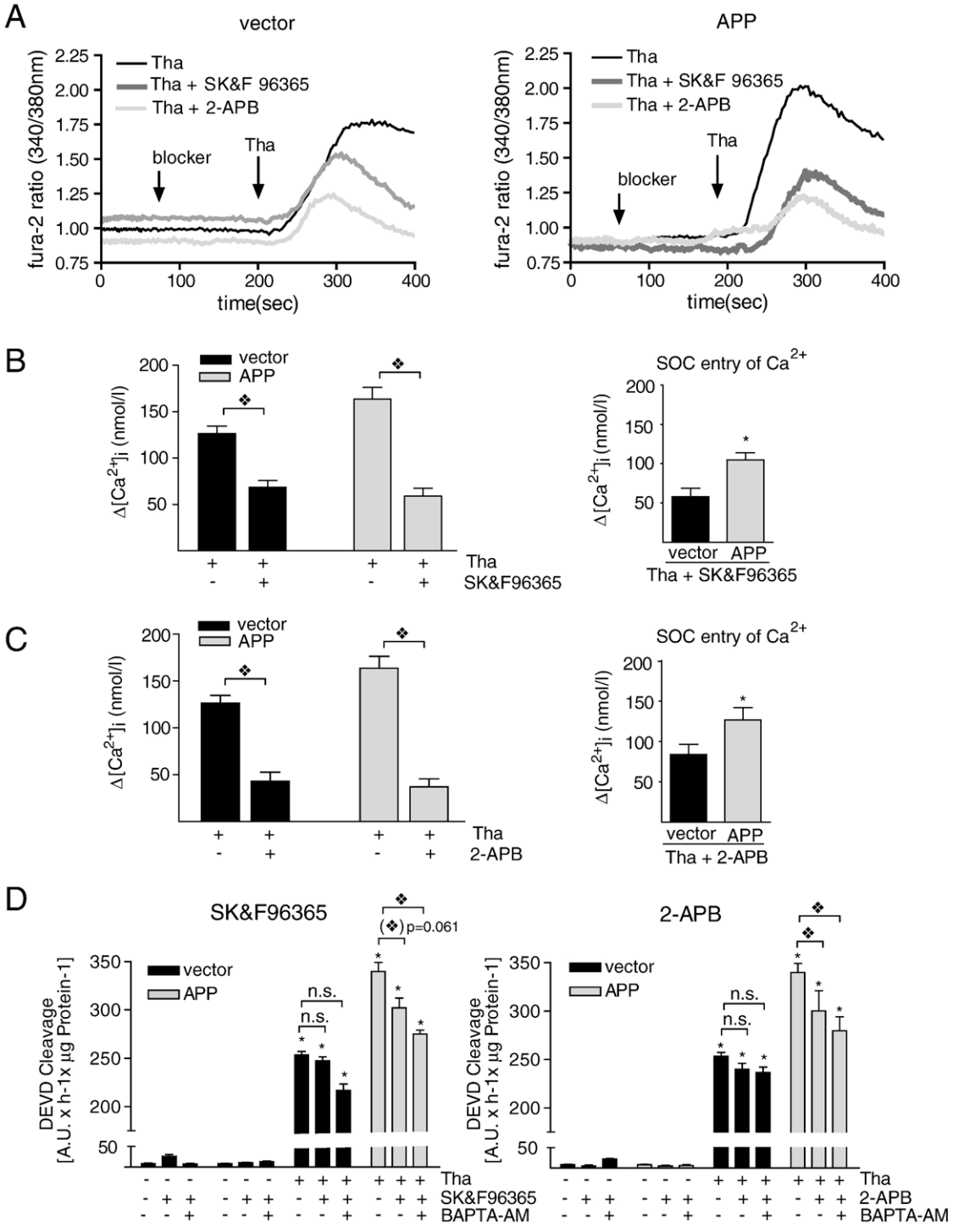
In this study, we investigated the effect of overexpressed APP on ER Ca^{2+} store depletion-induced apoptosis in rat PC12 cells. Interestingly, our data show that APP potentiates both cytoplasmic Ca^{2+} levels and subsequent apoptosis after store depletion. Since this potentiation could be completely abolished by the application of the two SOC inhibitors SK & F96365 and 2-APB, our data suggest that enhanced cytoplasmic Ca^{2+} transients and amplification of store depletion-triggered cell death by APP are probably not caused by ER Ca^{2+} overloading, but rather by an APP-dependent amplification of SOC entry of Ca^{2+} after store depletion. Interestingly, it has been proposed that full length APP might function as a cell surface receptor [32,33], thus providing the theoretical possibility for a cooperative interaction between APP and SOCCs in the cell membrane.

Subsequent analysis of the underlying transcriptional mechanisms of APP-mediated cell death potentiation by microarray analysis indicated that APP is capable of regulating transcriptional events in response to ER Ca^{2+} store depletion. Since many ER resident chaperones require high ER Ca^{2+} concentrations for their activity, ER Ca^{2+} store depletion leads to accumulation of misfolded proteins in the ER and activation of the unfolded protein response (UPR) [34,35]. Our GeneChip analysis of transcriptional changes after induction of ER stress with thapsigargin revealed that many UPR target genes were significantly upregulated in both PC12 vector and PC12 APP cells. In comparison to the PC12 vector control, most UPR target genes

Fig. 5. APP potentiates SOC entry of Ca^{2+} after ER store depletion. (A) Analysis of cytosolic calcium levels by fura-2 calcium imaging after thapsigargin-induced store depletion in the presence or absence of the SOC inhibitors SK & F96365 and 2-APB. PC12 vector and PC12 APP cells were incubated for 120 s with 10 μM SK & F96365 and 30 μM 2-APB prior to stimulation with 1 μM thapsigargin. Representative fura-2 ratio time traces of PC12 vector cells (A, left panel) and PC12 APP cells (A, right panel) treated solely with thapsigargin (black curves) are shown in comparison to PC12 vector and PC12 APP cultures pretreated with SK & F96365 (dark grey curves) and 2-APB (light grey curves), respectively. (B and C) SOC inhibitors significantly downmodulate cytoplasmic Ca^{2+} transients in response to thapsigargin (B and C, left panels). Quantitative differences of thapsigargin-induced cytoplasmic Ca^{2+} levels in the presence or absence of SK & F96365 (B, left panel) or 2-APB (C, left panel) in PC12 vector and PC12 APP cells are shown in relation to the baseline values (prior to any treatment) of the respective cell lines (nmol/l). Data represent peaks of Ca^{2+} levels after stimulation with thapsigargin; ❖$p<0.05$: Difference from respective cell line in the absence of SK & F96365 (B, left panel) and 2-APB (C, left panel). SOC inhibitors abrogate the potentiating effect of APP on intracellular Ca^{2+} levels after store depletion (B and C, right panels). Data are indicated as the difference between cytoplasmic Ca^{2+} levels (nmol/l) after treatment with thapsigargin +/− SK & F96365 (B, right panel), or thapsigargin +/− 2-APB (C, right panel) in PC12 vector and PC12 APP cells, respectively. *$p<0,05$: Difference from control cell line PC12 vector. (D) Analysis of effector caspase activation after thapsigargin treatment. PC12 vector (vector) and PC12 APP (APP) cells were preincubated with 5 μM BAPTA-AM or vehicle for 30 min and then treated with 1 μM thapsigargin in the presence or absence of 10 μM SK & F96365 (left panel) or 10 μM 2-APB (right panel) for 12 h. Controls were treated with vehicle (DMSO) in the presence or absence SK & F96365 or 2-APB and BAPTA-AM for 12 h. Caspase 3-like activity was monitored over 1 h. Data are means±SEM from $n=3$ cultures per treatment. A.U.=arbitrary fluorescence units. *$p<0.05$ vs. untreated control (PC12 vector); ❖$p<0.05$: difference from respective control cell line PC12 vector. n.s.: not significant.

implicated in alleviation of ER stress (molecular chaperones such as BiP, grp58, grp94, Erp70, calreticulin and calnexin) were induced to a comparable extent in PC12 APP cells.

In contrast to chaperones, expression of the stress-responsive pro-apoptotic kinase NIPK [36] and the transcription factor CHOP [28,37] was notably enhanced by overexpressed APP. RT-PCR analysis of the kinetics of CHOP expression confirmed that thapsigargin elicited an enhanced induction of the CHOP gene in PC12 APP cells. Interestingly, both CHOP and NIPK are downstream targets of the transcription factor ATF4 [36,38]. However, NIPK may not be directly activated by ATF4, since it has been described as a downstream target of CHOP in a recent study [39]. CHOP is ubiquitously expressed and exerts pro-apoptotic effects in various cell types. However, there is compelling evidence that CHOP plays a particular role in regulation of neuronal apoptosis in acute and chronic CNS diseases, including Alzheimer's disease [28,40]. It has been shown that CHOP mRNA stability is significantly increased in



the presence of elevated cytoplasmic Ca^{2+} levels [29], suggesting that the potentiation of CHOP expression observed in PC12 APP cells might occur on the posttranscriptional level.

Interestingly, our data reveal that buffering of cytosolic Ca^{2+} with BAPTA-AM was able to downmodulate APP-dependent potentiation of CHOP expression and cell death. In line with these observations, transcriptional upregulation of CHOP after treatment with the Ca^{2+} ionophore A23187 was also inhibitable by BAPTA-AM [29]. In contrast to the effect of Ca^{2+} chelation on CHOP expression, BAPTA-AM has been shown to have no inhibitory effect on thapsigargin-triggered expression of the ER chaperone Erp72 [41]. Collectively, these observations suggest that increases in cytosolic Ca^{2+} levels selectively enhance CHOP expression in a UPR-independent manner after Ca^{2+} store depletion with thapsigargin. From these data we conclude that APP-mediated potentiation of CHOP expression reflects a cumulative effect of increased cytosolic Ca^{2+} levels and induction of ER stress after ER store depletion. Further evidence for this hypothesis is provided by the fact that APP overexpression is not associated with transcriptional alterations in CHOP expression after induction of ER stress with tunicamycin, an ER stressor that does not affect ER Ca^{2+} levels [25]. Interestingly, silencing of CHOP expression by RNA interference was capable of completely abolishing the potentiating effect of APP on apoptosis triggered by Ca^{2+} store depletion, emphasizing the fundamental role of CHOP in this type of stress-induced, Ca^{2+}-dependent cell death. Our data indicate that by amplification of Ca^{2+} entry through SOCCs, overexpressed APP potentiates cytoplasmic Ca2+ levels and subsequent CHOP expression, culminating in enhanced cell death after Ca^{2+} store depletion.

Overexpression of wild type APP in neural cells has been previously shown to either diminish or enhance cell death by various apoptotic triggers, suggesting that APP affects the cellular sensitivity to apoptosis in a stimulus-specific manner [14,25,42,43]. Since overexpression of APP has been implicated in AD and Down syndrome [7–11], the amplified APP-dependent SOC entry of Ca^{2+} observed in this study might selectively sensitize neurons to insults triggering pathophysiological store depletion. Of note, our data suggest that CHOP seems to play a role only in the presence of high Ca^{2+} levels, since a potent downmodulating effect of CHOP siRNA could be observed only in thapsigargin-treated PC12 APP cells, but not in PC12 vector cells. Thus, Ca^{2+} levels enhanced by APP might induce CHOP-dependent apoptosis both on the transcriptional and post-transcriptional level after store depletion.

In conclusion, this study establishes that by potentiating SOC entry of Ca^{2+}, overexpression of APP triggers enhanced expression levels of the death-promoting gene *CHOP* and potently increases neuronal apoptosis after Ca^{2+} store depletion.

Acknowledgements

The authors thank Petra Mech, Christiane Schettler and Hildegard Schweers for excellent technical assistance. This work was supported by grants from the Alzheimer Forschung Initiative e.V. to D.K. (03809). and the Health Research Board and Higher Education Authority PRTLI Program of Human Genomics to J.H.M.P.

References

[1] B.A. Yankner, Mechanisms of neuronal degeneration in Alzheimer's disease, Neuron 16 (1996) 921–932.
[2] M.P. Mattson, Pathways towards and away from Alzheimer's disease, Nature 430 (2004) 631–639.
[3] D.J. Selkoe, Alzheimer's disease: genes, proteins, and therapy, Physiol. Rev. 81 (2001) 741–766.
[4] J. Walter, C. Kaether, H. Steiner, C. Haass, The cell biology of Alzheimer's disease: uncovering the secrets of secretases, Curr. Opin. Neurobiol. 11 (2001) 585–590.
[5] S.S. Sisodia, P.H. St George-Hyslop, gamma-Secretase, Notch, Abeta and Alzheimer's disease: where do the presenilins fit in? Nat. Rev., Neurosci. 3 (2002) 281–290.
[6] D.J. Selkoe, M.B. Podlisny, Deciphering the genetic basis of Alzheimer's disease, Annu. Rev. Genomics Hum. Genet. 3 (2002) 67–99.
[7] E.H. Koo, S.S. Sisodia, L.C. Cork, A. Unterbeck, R.M. Bayney, D.L. Price, Differential expression of amyloid precursor protein mRNAs in cases of Alzheimer's disease and in aged nonhuman primates, Neuron 4 (1990) 97–104.
[8] M.R. Palmert, T.E. Golde, M.L. Cohen, D.M. Kovacs, R.E. Tanzi, J.F. Gusella, M.F. Usiak, L.H. Younkin, S.G. Younkin, Amyloid protein precursor messenger RNAs: differential expression in Alzheimer's disease, Science 241 (1988) 1080–1084.
[9] R.L. Neve, D.L. McPhie, Y. Chen, Alzheimer's disease: a dysfunction of the amyloid precursor protein(1), Brain Res. 886 (2000) 54–66.
[10] J. Theuns, N. Brouwers, S. Engelborghs, K. Sleegers, V. Bogaerts, E. Corsmit, T. De Pooter, C.M. van Duijn, P.P. De Deyn, C. Van Broeckhoven, Promoter mutations that increase amyloid precursor-protein expression are associated with Alzheimer disease, Am. J. Hum. Genet. 78 (2006) 936–946.
[11] A. Rovelet-Lecrux, D. Hannequin, G. Raux, N. Le Meur, A. Laquerriere, A. Vital, C. Dumanchin, S. Feuillette, A. Brice, M. Vercelletto, F. Dubas, T. Frebourg, D. Campion, APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy, Nat. Genet. 38 (2006) 24–26.
[12] C. Reinhard, S.S. Hebert, B. De Strooper, The amyloid-beta precursor protein: integrating structure with biological function, EMBO J. 24 (2005) 3996–4006.
[13] X. Cao, T.C. Sudhof, A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60, Science 293 (2001) 115–120.
[14] D. Kögel, R. Schomburg, E. Copanaki, J.H. Prehn, Regulation of gene expression by the amyloid precursor protein: inhibition of the JNK/c-Jun pathway, Cell Death Differ. 12 (2005) 1–9.
[15] Y. Gao, S.W. Pimplikar, The gamma-secretase-cleaved C-terminal fragment of amyloid precursor protein mediates signaling to the nucleus, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 14979–14984.
[16] S.W. Barger, M.P. Mattson, Induction of neuroprotective kappa B-dependent transcription by secreted forms of the Alzheimer's beta-amyloid precursor, Brain Res. Mol. Brain Res. 40 (1996) 116–126.
[17] E.C. Toescu, A. Verkhratsky, P.W. Landfield, Ca2+ regulation and gene expression in normal brain aging, Trends Neurosci. 27 (2004) 614–620.
[18] F.M. LaFerla, Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease, Nat. Rev., Neurosci. 3 (2002) 862–872.
[19] M.P. Mattson, S.L. Chan, Neuronal and glial calcium signaling in Alzheimer's disease, Cell Calcium 34 (2003) 385–397.
[20] A. Verkhratsky, Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons, Physiol. Rev. 85 (2005) 201–279.
[21] M.A. Leissring, M.P. Murphy, T.R. Mead, Y. Akbari, M.C. Sugarman, M. Jannatipour, B. Anliker, U. Muller, P. Saftig, B. De Strooper, M.S. Wolfe, T.E. Golde, F.M. LaFerla, A physiologic signaling role for the gamma-secretase-derived intracellular fragment of APP, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 4697–4702.

[22] I.F. Smith, K.N. Green, F.M. LaFerla, Calcium dysregulation in Alzheimer's disease: recent advances gained from genetically modified animals, Cell Calcium 38 (2005) 427–437.
[23] F. Wuytack, L. Raeymaekers, L. Missiaen, Molecular physiology of the SERCA and SPCA pumps, Cell Calcium 32 (2002) 279–305.
[24] A.B. Parekh, J.W. Putney Jr., Store-operated calcium channels, Physiol. Rev. 85 (2005) 757–810.
[25] D. Kögel, R. Schomburg, T. Schürmann, C. Reimertz, H.G. König, M. Poppe, A. Eckert, W.E. Müller, J.H. Prehn, The amyloid precursor protein protects PC12 cells against endoplasmic reticulum stress-induced apoptosis, J. Neurochem. 87 (2003) 248–256.
[26] C. Lu, W. Fu, M.P. Mattson, Caspase-mediated suppression of glutamate (AMPA) receptor channel activity in hippocampal neurons in response to DNA damage promotes apoptosis and prevents necrosis: implications for neurological side effects of cancer therapy and neurodegenerative disorders, Neurobiol. Dis. 8 (2001) 194–206.
[27] K. Gulow, D. Bienert, I.G. Haas, BiP is feed-back regulated by control of protein translation efficiency, J. Cell. Sci. 115 (2002) 2443–2452.
[28] S. Oyadomari, M. Mori, Roles of CHOP/GADD153 in endoplasmic reticulum stress, Cell Death Differ. 11 (2004) 381–389.
[29] J.D. Bartlett, J.D. Luethy, S.G. Carlson, S.J. Sollott, N.J. Holbrook, Calcium ionophore A23187 induces expression of the growth arrest and DNA damage inducible CCAAT/enhancer-binding protein (C/EBP)-related gene, gadd153. Ca2+ increases transcriptional activity and mRNA stability, J. Biol. Chem. 267 (1992) 20465–20470.
[30] M.D. Bootman, T.J. Collins, L. Mackenzie, H.L. Roderick, M.J. Berridge, C.M. Peppiatt, 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry but an inconsistent inhibitor of InsP3-induced Ca2+ release, FASEB J. 16 (2002) 1145–1150.
[31] J.E. Merritt, W.P. Armstrong, C.D. Benham, T.J. Hallam, R. Jacob, A. Jaxa-Chamiec, B.K. Leigh, S.A. McCarthy, K.E. Moores, T.J. Rink, SK & F 96365, a novel inhibitor of receptor-mediated calcium entry, Biochem. J. 271 (1990) 515–522.
[32] T. Nilsson, K. Malkiewicz, M. Gabrielsson, R. Folkesson, B. Winblad, E. Benedikz, Antibody-bound amyloid precursor protein upregulates ornithine decarboxylase expression, Biochem. Biophys. Res. Commun. 341 (2006) 1294–1299.
[33] M. Kawasumi, Y. Hashimoto, T. Chiba, K. Kanekura, Y. Yamagishi, M. Ishizaka, H. Tajima, T. Niikura, I. Nishimoto, Molecular mechanisms for neuronal cell death by Alzheimer's amyloid precursor protein-relevant insults, NeuroSignals 11 (2002) 236–250.
[34] W. Paschen, T. Mengesdorf, Endoplasmic reticulum stress response and neurodegeneration, Cell Calcium 38 (2005) 409–415.
[35] M. Schroder, R.J. Kaufman, The Mammalian unfolded protein response, Annu. Rev. Biochem. 74 (2005) 739–789.
[36] D. Ord, T. Ord, Characterization of human NIPK (TRB3, SKIP3) gene activation in stressful conditions, Biochem. Biophys. Res. Commun. 330 (2005) 210–218.
[37] S.J. Marciniak, C.Y. Yun, S. Oyadomari, I. Novoa, Y. Zhang, R. Jungreis, K. Nagata, H.P. Harding, D. Ron, CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum, Genes Dev. 18 (2004) 3066–3077.
[38] T.W. Fawcett, J.L. Martindale, K.Z. Guyton, T. Hai, N.J. Holbrook, Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response, Biochem. J. 339 (Pt. 1) (1999) 135–141.
[39] N. Ohoka, S. Yoshii, T. Hattori, K. Onozaki, H. Hayashi, TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death, EMBO J. 24 (2005) 1243–1255.
[40] M. Paz Gavilan, J. Vela, A. Castano, B. Ramos, J.C. Del Rio, J. Vitorica, D. Ruano, Cellular environment facilitates protein accumulation in aged rat hippocampus, Neurobiol. Aging 27 (7) (2005) 973–982.
[41] W. Paschen, C. Gissel, T. Linden, J. Doutheil, Erp72 expression activated by transient cerebral ischemia or disturbance of neuronal endoplasmic reticulum calcium stores, Metab. Brain Dis. 13 (1998) 55–68.
[42] M. Recuero, E. Serrano, M.J. Bullido, F. Valdivieso, Abeta production as consequence of cellular death of a human neuroblastoma overexpressing APP, FEBS Lett. 570 (2004) 114–118.
[43] L. Esposito, L. Gan, G.Q. Yu, C. Essrich, L. Mucke, Intracellularly generated amyloid-beta peptide counteracts the antiapoptotic function of its precursor protein and primes proapoptotic pathways for activation by other insults in neuroblastoma cells, J. Neurochem. 91 (2004) 1260–1274.