Endoplasmic reticulum stress during amyloid β peptide-induced cell death: role of mitochondria and glutamatergic N-methyl-D-aspartate receptors

Stress do retículo endoplasmático durante a morte celular induzida pelo peptídeo β-amilóide: o papel da mitocôndria e dos recetores glutamatérgicos N-metil-D-aspartato

Rui Miguel Oliveira da Costa

Universidade de Coimbra

2011

Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra para prestação de provas de Doutoramento em Biologia, na especialidade de Biologia Celular.

Este trabalho foi realizado no Centro de Neurociências e Biologia Celular da Universidade de Coimbra e no *Cognitive Neurology and Alzheimer's Disease Center, Institute for Neuroscience, Northwestern University,* Evanston, Estados Unidos da América. A sua realização foi suportada pela bolsa de doutoramento SFRH/BD/28403/2006 atribuída pela Fundação para a Ciência e a Tecnologia.

“Todas as verdades são fáceis de entender, uma vez que são descobertas.

A questão é descobri-las."

(Galileo Galilei, 1564-1642)

Agradecimentos/Acknowledgements

"Sem esforço nada se consegue!"
Sempre me orientei por este lema, mas nunca ele fez tanto sentido como agora. Contudo, o alcançar de mais uma meta e a sensação de dever cumprido supera em muito todo o esforço, as noites sem dormir e os dias intermináveis que conduziram à dissertação aqui apresentada.
Os momentos de esforço são ultrapassados com maior facilidade se estivermos rodeados de pessoas que nos apoiem. Felizmente sempre tive esse privilégio. Gostaria por isso de agradecer a algumas pessoas que direta ou indiretamente contribuíram para a elaboração desta tese, em particular:

À Doutora Cláudia Pereira, minha orientadora científica. O meu mais sincero obrigado por todo o apoio, dedicação, paciência e sobretudo por ter confiado nas minhas capacidades e me ter dado a oportunidade de realizar este trabalho que em muito me fez crescer tanto a nível científico como pessoal.

Ao Doutor Carlos Palmeira, meu co-orientador científico. Os meus agradecimentos pela disponibilidade e co-orientação desta tese.

À Professora Doutora Catarina Oliveira, por me ter recebido no centro de Neurociências e Biologia Celular e pela disponibilidade e apoio que facilitaram o desenvolvimento do trabalho experimental aqui apresentado.

To Doctor William L. Klein, for the opportunity to develop part of my PhD work in his lab, and the willingness to share his scientific knowledge and expertise. I would also like to thank to Pascale (un grand merci pour tout), Jason, Billy, Garry, Ruchi, Mary, Pauline, Kirsten, Yan and all other members of the Klein' lab for their help, scientific advising and sympathy which made me feel at home during the period at Northwestern University.

À Fundação para a Ciência e a Tecnologia, pelo financiamento da presente tese, incluído o suporte das despesas de deslocações a reuniões científicas e o apoio à realização de trabalho experimental na *Northwestern University, Evanston/Chicago, USA*.

À Doutora Isabel Santana, por ter tornado possível a realizaçãode alguns dos estudos aqui apresentados em doentes de Alzheimer.

À Doutora Sandra Cardoso, Doutora Paula Agostinho e Doutora Paula Moreira, pela disponibilidade, pelo apoio e pelas críticas sempre construtivas que em muito ajudaram no desenvolver da tese.

Ao grupo de "sinalização e disfunção mitocondrial na neurodegenerescência" em particular à Doutora Cristina Rego, por todos os conselhos e apoio, e à Luísa Ferreira pela boa disposição e pelas construtivas discussões científicas que ajudaram no desenvolvimento de parte desta tese.

À Elisabete Ferreiro, por ter me ter ajudado/ensinado sobretudo durante o meu período de adaptação ao laboratório, e também por ter contribuído significativamente para o desenvolvimento dos capítulos 4 e 5 desta tese.

Ao João Pedro, Pedro Garção e Bruno Silva, Raquel Esteves, Rosa, Sueli, Isaura, Alessandro, Sílvia, Catarina, Daniela e aos restantes amigos e colegas do Centro de neurociências e biologia celular pela amizade dentro e fora do laboratório e por contribuírem para um excelente ambiente de trabalho.

Aos amigos da "bissaya" por partilharem comigo alegrias/frustrações durante o decorrer da elaboração desta tese. Um obrigado a todos, em especial ao Bernardo, ao Gil, ao Pápa, ao Sam e ao Hélio.

Aos meus amigos do Ju-Jitsu, os que mais "sofreram" com as minhas frustrações do trabalho, mas também os que mais facilmente durante o treino as faziam esquecer. O meu sincero "arigato" Ricardo e Zé.

A toda a minha família, por todo o vosso apoio, compreensão e carinho.

A Miri per essere quello che sei e come sei... Per essere presente tutti i giorni e per il supporto che mi hai dato contribuendo notevolmente alla realizazione di questo tesi. Grazie mille

Aos meus pais... um grande Bem-Haja! Tudo isto se deve a vós e portanto tudo será pouco para vos agradecer a confiança sempre depositada em mim e nas minhas escolhas. Obrigado por todo o carinho e apoio. Obrigado pelos ensinamentos e valores que me incutiram enquanto miúdo porque esses foram os que mais contribuíram para aqui chegar!

INDEX

PUBLICATIONS

The present thesis was mostly based on work that has been published or submitted for publication in international peer-reviewed journals.

Rui O. Costa, Pascale N. Lacor, Ildete L. Ferreira, Yves P. Auberson, William L. Klein, Catarina R. Oliveira, Ana C. Rego and Cláudia F. Pereira. "Endoplasmic reticulum stress occurs downstream of NR2B subunit of N-methyl-D-aspartate receptor in mature hippocampal cultures treated with amyloid-β oligomers"
(Submitted for publication)

Rui O. Costa, Catarina R. Oliveira and Cláudia M.F. Pereira. "Inhibition of mitochondrial cytochrome c oxidase potentiates endoplasmic reticulum Ca^{2+} deregulation induced by amyloid β-peptide."
(Submitted for publication)

Rui O. Costa*, Elisabete Ferreiro*, Sandra M. Cardoso, Catarina R. Oliveira and Cláudia M.F. Pereira. (in press) "Amyloid β-induced ER stress is enhanced under mitochondrial dysfunction conditions"
(Neurobiology of of Aging, in press)
(Impact factor: 5.937)
* Both authors contributed equally to this work.

Rui O. Costa*, Elisabete Ferreiro*, Sandra M. Cardoso, Catarina R. Oliveira and Cláudia M.F. Pereira. (2010) "ER stress-mediated apoptotic pathway induced by $A\beta_{1-40}$ peptide requires the presence of functional mitochondria."
Journal of Alzheimer's Disease 2010; 20(2):625-36. PMID: 20182029
(Impact factor: 3.830)
* Both authors contributed equally to this work.

ABBREVIATIONS

Ab, antibody

ABAD, Aβ-binding alcohol dehydrogenase

Aβ, amyloid-beta

$A\beta_{1\text{-}40}$, amyloid-beta 1-40 fibrils

$A\beta_{1\text{-}42}$, amyloid-beta 1-42 fibrils

AβO, amyloid-beta peptide oligomers

AD, Alzheimer's disease

ADDLs, amyloid-derived diffusible ligands

ADP, adenosine diphosphate

AICD, APP intracellular domain

Apo E, apolipoprotein E

APP, amyloid precursor protein

ATF6, activating transcription factor 6

ATP, adenosine triphosphate

BACE, beta-site APP cleaving enzyme

Bax, Bcl-2-associated X protein

Bak, Bcl-2-homologous antagonist/killer

BCA, bicinchoninic acid assays

Bcl-2, B-cell lymphoma 2

BiP, binding immunoglobulin protein

Bref. A, brefeldin A

BSA, bovine serum albumine

Ca^{2+}, calcium ion

$[Ca^{2+}]_i$, cytosolic calcium concentration

$CaCl_2$, calcium chloride

Cdk, cyclin-dependent kinases

cDNA, complementary DNA

CDR, clinical dementia rating

C/EBP Ccat-enhancer binding protein

CICR, Ca^{2+}-induced Ca^{2+} release

CNS, central nervous system

CHOP, C/EBP homologous protein

CO_2, carbon dioxide

COX, cytochrome c oxidase

CSF, cerebrospinal fluid

Cyp D, cyclophilin D

Dant., dantrolene

$DCFH_2$-DA, 2´,7´-dichlorodihydrofluorescein diacetate

DHE, dihydroethidium

DSM-IV, diagnostic and statistical manual of mental disorders

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

DNAse, deoxyribonuclease

DOC, deoxycholic acid

DTT, 1,4-Dithiothreitol

ECF, enhanced chemifluorescence

EDTA, ethylenediaminetetraacetic acid

EEG, electroencephalogram

EGTA, ethylene glycol-bis (2-aminoethylether)-N,N,N´,N´-tetraacetic acid

eIF2α, eucaryotic initiation factor-2-alpha

EOR, ER overload response

ER, endoplasmic reticulum

ERAD, ER-associated degradation

ETC, electron transport chain

FAD, familial Alzheimer's disease

FBS, fetal bovine serum

FCS, fetal calf serum

FDG-PET, fludeoxyglucose (^{18}F)-PET

Fura-2/AM, Fura-2 acetoxymethyl ester

Fyn, proto-oncogenic tyrosine kinase

GADD153, growth arrest and DNA damage 153 gene

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

GFAP, glial fibrillary acidic protein

CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate

GRP, glucose-regulated protein

GSH, glutathione (reduced form)

HBSS, Hank´s balanced salt solution

HCl, hydrogen chloride

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFIP, 1,1,1,3,3,3-hexafluro-2-propanol

ICC, immunocytochemistry

Ifen., ifenprodil

IgG, immunoglobulin G

Indo-1/AM, Indo-1-acetoxymethyl ester

IP3, inositol-1,4,5-triphosphate

IP3R, IP3 receptor

IRE-1, inositol requiring element-1

JNK, jun NH_2 kinase

K^+, potassium ion

KCl, potassium chloride

KCN, potassium cyanide

KH_2PO_4, potassium dihydrogen phosphate

LTP, long-term potentiation

MAM, mitochondrial-associated membranes

MCI, mild cognitive impairment

Mg^{2+}, magnesium ion

$MgCl_2$**,** magnesium chloride

mGluR5, metabotropic glutamate receptor 5

MIM, mitochondrial inner membrane

Min, minute

MMSE, minimental-state evaluation

$MnCl_2$, manganese chloride

mRNA, messenger RNA

mtDNA, mitochondrial DNA

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

N_2, nitrogen

Na^+, sodium ion

NaCl, sodium cloride

NADH, nicotinamide adenine dinucleotide

NADPH, nicotinamide adenine dinucleotide phosphate

NaF, sodium fluoride

$NaHCO_3$, sodium bicarbonate

$Na_2HPO_4 \cdot 2H_2O$, disodium phosphate 2-hydrate

NF-kB, nuclear factor-kB

NFTs, neurofibrillary tangles

NINCDS-ADRDA, national institute of neurological and communicative disorders and stroke-Alzheimer's disease and related disorders association

NRF-2, nuclear factor erythroid-2

NT2 ρ+, teratocarcinome cell line

NT2 ρ0, mtDNA-depleted teratocarcinome cell line

NMDA, N-methyl-D-aspartate

NMDARr, N-methyl-D-aspartate receptors

non-Tg, non-transgenic

NOX, NADPH oxidase

NR1, NMDA receptor 1 subunit

NR2A and NR2B, NMDA receptor 2A and 2B subunits

NVP, NVP-(R)-[(S)-1-(4 Bromophenyl)-ethylamino]-(2,3 dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl] phosphonic acid

$O_2^{\cdot -}$, superoxide anion

OptiMEM, Eagles minimum essential medium

PAGE, polyacrylamide gel electrophoresis

PARP, poly (ADP-ribose) polymerase

PBS, phosphate-buffered saline

PDI, protein disulfide isomerase

PERK, protein kinase RNA-like ER-kinase

PET, positron emission tomography

PMBCs, peripheral blood mononuclear cells

PMSF, phenylmethylsulfonyl fluoride

pNA, p-nitroaniline

p-PERK, phosphorylated RNA-dependent protein kinase-like ER-membrane

PrP, prion protein

PS, presenilin

PTP, permeability transition pore

PVDF, polyvinylidene difluoride

RIPA, radio-immunoprecipitation assay lysis buffer

RNA, ribonucleic acid

ROS, reactive oxygen species

RT, room temperature

RLU, luminescence units

RyR, ryanodine receptor

SAD, sporadic AD

Src, proto-oncogenic tyrosine kinase

SDS, sodium dodecyl sulfate

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel

Ser, serine

SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase

SEM, standart error of the mean

Thap., thapsigargin

TBS-T, tris-buffered saline with tween

TIM, transporter inner membrane

TOM, transporter outer membrane

Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol

TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

Tyr, tyrosine

Tween, polyethylene glycol sorbitan monolaurate

UPR, unfolded protein response

UPS, ubiquitin/proteasome system

UV, ultraviolet

XBP-1, X-box binding protein-1

WB, western blot

β-NADPH, β-nicotinamide adenine dinucluotide phosphate reduced form

ΔΨm, mitochondrial membrane potential

3xTg-AD, triple transgenic-AD mice

RESUMO

A doença de Alzheimer é a patologia neurodegenerativa mais prevalente em idosos, afetando quase 35 milhões de pessoas em todo o Mundo. Trata-se de uma doença crónica e progressiva caracterizada por perda de memória e declínio cognitivo devido à perda sináptica e neuronal no hipocampo e córtex cerebral. Nos últimos anos fizeram-se progressos consideráveis com o intuito de compreender melhor a patogénese da doença de Alzheimer, contudo este conhecimento não foi ainda traduzido em fármacos novos e eficazes. Atualmente, apenas duas classes de medicamentos estão disponíveis para o tratamento sintomático da doença de Alzheimer, mas estes apenas apresentam benefícios clínicos modestos em termos de melhoria das capacidades cognitivas e infelizmente não previnem a progressão da doença.

A deposição anormal do peptídeo beta-amilóide (Aβ) no cérebro de doentes de Alzheimer sugere que este desempenha um papel crucial na patogénese da doença. Apesar das espécies fibrilares serem há muito tempo consideradas como mediadoras dos seus efeitos neurotóxicos, foi demonstrado recentemente que oligómeros solúveis do Aβ (AβO) são as principais espécies responsáveis pelas alterações a nível sináptico e neuronal na doença de Alzheimer. De acordo com estas descobertas, a "hipótese da cascata de amilóide" previamente proposta foi reformulada e considera agora que os AβO são responsáveis pelas alterações cerebrais que ocorrem durante a fase inicial da doença, conduzindo à demência. Assim, prevenir a formação ou a toxicidade destes AβO poderá ser a chave definitiva para travar a progressão da doença de Alzheimer.

Com este estudo pretendeu-se ampliar os conhecimentos sobre os mecanismos moleculares subjacentes aos efeitos lesivos desencadeados pelo Aβ, focando-nos no seu papel como indutor de "stress" do retículo endoplasmático (RE). Especificamente, foi investigado: i) a comunicação entre

o RE e a mitocôndria durante a morte celular induzida por Aβ e ii) o papel dos recetores glutamatérgicos N-metil-D-aspartato (NMDARs), em particular das suas subunidades NR2A e NR2B, como um mecanismo desencadeador de "stress" do RE induzido pelo Aβ.

Na primeira parte deste trabalho (capítulo 4), usámos uma linha celular ρ0 depletada do seu ADN mitocondrial e caracterizada pela ausência de mitocôndrias funcionais, para demonstrar que a morte celular por apoptose mediada pelo "stress" do RE induzido pelo Aβ ocorre através de um mecanismo dependente da mitocôndria. Mostrámos que a isoforma $A\beta_{1-40}$ do peptídeo Aβ aumenta os níveis do chaperone GRP78 e ativa a caspase-4, dois marcadores do "stress" do RE, depletando também as reservas de Ca^{2+} neste organelo, não apenas em células ρ0 mas também nas células parentais ρ+. No entanto, observámos que somente em células ρ+ tratadas com $A\beta_{1-40}$ ocorre um aumento dos níveis de GADD153/CHOP, um fator de transcrição ativado sob condições de "stress" do RE, ativação de caspase-9 e -3 e aumento do número de células apoptóticas marcadas positivamente para TUNEL.

Com o objetivo de investigar melhor a interação mitocôndria/RE na patogénese da doença de Alzheimer, usámos células cíbridas que recriam o defeito na actividade da citocromo c oxidase (COX) mitocondrial detetado em plaquetas de doentes de Alzheimer esporádicos (capítulo 5). Mostrámos que a disfunção mitocondrial resultante da inibição da COX afeta a resposta ao "stress" do RE e subsequente morte celular desencadeada pelo Aβ. Provou-se que o aumento dos níveis de GRP78 e da atividade da caspase-4 observados em consequência do tratamento com o $A\beta_{1-40}$ é mais pronunciado em cíbridos de Alzheimer do que em cíbridos preparados a partir de plaquetas de indivíduos saudáveis. Além disso, o decréscimo da sobrevivência celular e

também o aumento na atividade da caspase-3, dos níveis da forma clivada da poli-ADP-ribose-polimerase (PARP), assim como no número de células apoptóticas observados em cíbridos de Alzheimer foi mais proeminente do que em cíbridos controlo tratados com o $A\beta_{1-40}$. A morte celular por apoptose induzida pelo $A\beta_{1-40}$ em ambas as linhas celulares mostrou envolver a libertação de Ca^{2+} do RE visto que a ativação da caspase-3 efetora de apoptose foi prevenida por dantroleno, um antagonista dos canais de Ca^{2+} associados aos reeptores de rianodina do RE.

A interação mitocôndria/RE durante a morte celular induzida pelo Aβ foi ainda avaliada usando um modelo neuronal (capítulo 6). Em culturas primárias de neurónios corticais de rato, determinou-sede que modo o dano na atividade da COX afecta a perda da homeostasia de Ca^{2+} no RE e no citosol e promove a ativação da via de morte celular por apoptose mediada pela mitocôndria. Em neurónios corticais tratados com concentrações tóxicas de $A\beta_{1-40}$, na presença de cianeto de potássio (KCN), um inibidor da COX, verificou-se uma acentuada libertação de Ca^{2+} do RE acompanhada pelo aumento dos seus níveis citosólicos, decréscimo da viabilidade celular e ainda ativação das caspases -9 e -3.

No capítulo 7, investigámos a possibilidade dos AβO desencadearem "stress" do RE e disfunção neuronal através de um mecanismo dependente dos NMDARs, analisando a contribuição das subunidades NR2A e NR2B deste recetor glutamatérgicos. Em culturas primárias de hipocampo de rato tratadas com AβO mostrámos que a produção de superóxido mediada pela NADPH oxidase ocorre após a interação AβO-NR2B desregulando a homeostasia de Ca^{2+}. Estes eventos precederam alterações na viabilidade celular e na ativação da via apoptótica mediada pelo "stress" do RE como é demonstrado pelo

aumento dos níveis de GRP78, XBP-1 e GADD153/CHOP. O papel da subunidade NR2B no "stress" do RE, induzido por AβO, e na disfunção neuronal no hipocampo foi demonstrado usando ifenprodil, um antagonista desta subunidade dos NMDARs. Por outro lado, um antagonista das subunidades NR2A, apenas atenuou ligeiramente a neurotoxicidade induzida por AβO, negligenciando o envolvimento desta subunidade.

Finalmente, estudámos o papel do "stress" do RE *in vivo* usando um modelo animal da doença de Alzheimer, osratinhos triplo transgénicos (3xTg-AD) (capítulo 8). Em comparação com a estirpe não-transgénica (non-Tg), observaram-se alterações nos marcadores de "stress" do RE em ratinhos 3xTg-AD que foram mais pronunciadas ao nível do hipocampo do que no córtex cerebral tanto em fêmeas como em machos, sendo as fêmeas aparentemente mais suscetíveis ao "stress" do RE. Além disso, os resultados obtidos neste modelo animal mostram que os níveis de marcadores de "stress" do RE estão alterados em animais jovens sugerindo o seu envolvimento nas fases precoces da patologia. Estes resultados foram corroborados por dados obtidos num modelo periférico da doença de Alzheimer. Em colaboração com a Prof. Isabel Santana dos Hospitais da Universidade de Coimbra e com o grupo 'Sinalização e disfunção mitocondrial na neurodegenerescência' do Centro de Neurociências e Biologia Celular (CNC), foi-nos possível utilizar células mononucleadas de sangue periférico humano (PBMCs), nomeadamente linfócitos, isolados de doentes em diferentes fases da doença de Alzheimer (ligeira, moderada e severa) e indivíduos com defeito cognitivo ligeiro (DCL) *versus* indivíduos não dementes (capítulo 8). Neste modelo periférico, pretendeu-se relacionar a disfunção cognitiva durante as diferentes fases da doença com marcadores de dano celular. Com este propósito investigámos as

alterações nos níveis de Ca^{2+} do RE e no citosol e analisámos diferenças na expressão de marcadores do "stress" do RE e de proteínas apoptóticas associadas ao RE. Um decréscimo no conteúdo de Ca^{2+} deste organelo intracelular, acompanhado por aumento dos seus níveis no citoplasma, foi observado em PBMCs de indivíduos com DCL e doentes na fase ligeira que apresentaram também aumento nos marcadores do "stress" do RE, nomeadamente de GRP78 e XBP-1. Em PBMCs de doentesna fase moderada-severa, os níveis destas proteínas assemelharam-se aos obtidos em controlos. Contudo, nesta fase a via apoptótica mediada pelo "stress" do RE está já activada, como demonstrado pelo aumento dos níveis de GADD153/CHOP.

Os resultados obtidos recorrendo a modelos humanos e não humanos de diferente complexidade, mostram que a morte celular apoptótica induzida pelo Aβ envolve a cooperação entre o RE e a mitocôndria, e que é desencadeado pela interação da Aβ com recetores glutamatérgicos NMDA, contribuindo assim para uma melhor compreensão dos mecanismos associados à patogénese desta doença.

(Nota: este texto foi escrito ao abrigo do novo acordo ortográfico.)

SUMMARY

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder in the elderly that currently affects almost 35 million people worldwide. It is a chronic and progressive neurodegenerative illness characterized by memory deficits and cognitive decline due to synaptic and neuronal loss in the hippocampus and cerebral cortex. Considerable progress has been made in recent years towards better understanding the pathogenesis of AD but this knowledge has not yet been successfully translated into new and effective disease-modifying drugs. Only two classes of medication are currently available for the symptomatic treatment of AD but these drugs have only modest clinical benefits in terms of improved cognition and ultimately do not prevent disease progression.

The abnormal deposition of amyloid-beta (Aβ) peptide in the brain of AD patients has suggested that it plays an essential role in AD pathogenesis. Although fibrillar species have long been considered to account for the neurotoxic effects of Aβ, it was recently demonstrated that soluble Aβ oligomers (AβO) are the main species responsible for synaptic and neuronal changes in AD. Accordingly to these findings, the previously proposed "amyloid cascade hypothesis" has been reformulated and it now considers that AβO are responsible for the brain alterations occurring during the early stages of the disease, leading to dementia. Therefore, preventing the formation or toxicity of these AβO may be the ultimate key to halting the progression of AD.

This study was aimed to better understand the molecular mechanisms underlying the deleterious effects triggered by Aβ, focusing on its role in the induction of endoplasmic reticulum (ER) stress. Specifically, it was

investigated: i) the ER/mitochondria cross-talk during Aβ-induced cell death and ii) the role of the glutamatergic N-methyl-D-aspartate receptor (NMDAR), in particular of its NR2A and NR2B subunits, as an upstream mechanism leading to Aβ-induced ER stress.

In the first part of this work (chapter 4), we used mitochondrial DNA (mtDNA)-depleted ρ0 cells, characterized by the absence of functional mitochondria to demonstrate that ER stress-mediated apoptotic cell death induced by Aβ occurs through a mitochondria-dependent mechanism. We showed that the $A\beta_{1-40}$ isoform of Aβ increases the levels of the chaperone GRP78 and activates caspase-4, two ER stress markers, and also depletes ER Ca^{2+} stores in both ρ0 cells and parental ρ+ cells. However, we found that only in $A\beta_{1-40}$-treated ρ+ cells occur an increase in the levels of GADD153/CHOP, a pro-apoptotic transcription factor activated under ER stress conditions, activation of caspase-9 and -3 and increase in the number of TUNEL-positive apoptotic cells.

In order to further investigate the mitochondria/ER interplay in AD pathogenesis, we used cybrid cells that recreate the defect in mitochondrial cytochrome c oxidase (COX) activity detected in platelets of sporadic AD patients (chapter 5). We showed that mitochondrial dysfunction arising from COX inhibition affects the ER stress response and subsequent cell death triggered by Aβ. Indeed, the increase in GRP78 levels and caspase-4-like activity observed upon $A\beta_{1-40}$ treatment was more pronounced in AD cybrids than in cybrids prepared from platelets of non-demented controls. Furthermore, the decrease in cell survival as well as the increase in caspase-3-like activity, levels of cleaved poli-ADP-ribose-polymerase (PARP), a caspase-3 substrate, and TUNEL-positive apoptotic cells observed in $A\beta_{1-40}$-treated AD cybrids

was more prominent than in control cybrids. Apoptotic cell death induced by Aβ in both cell lines was shown to involve ER Ca^{2+} release since activation of the apoptosis effector caspase-3 was prevented by dantrolene, an inhibithor of Ca^{2+} channels associated with ER ryanodine receptors.

The mitochondria/ER cross-talk during Aβ-induced cell death was further evaluated using a neuronal model (chapter 6). In primary cultures of rat brain cortical neurons it was analyzed how the impairment of COX activity affects the loss of ER and cytosolic Ca^{2+} homeostasis and promotes the activation of the mitochondria-mediated apoptotic cell death pathway. In cortical neurons treated with toxic concentrations of $A\beta_{1-40}$ in the presence of potassium cyanide (KCN), a COX inhibitor, it was observed that the increase in ER Ca^{2+} release and subsequent rise of cytosolic Ca^{2+} levels, the decrease in cell survival and activation of apoptosis-related caspase-9 and -3 were potentiated in comparison with neurons treated with $A\beta_{1-40}$ in the absence of KCN.

In chapter 7, we investigated whether Aβ oligomers (AβO) trigger ER stress by an NMDAR-dependent mechanism leading to neuronal dysfunction and analyzed the contribution of NR2A and NR2B subunits of this glutamate receptor. In primary cultures of rat brain hippocampal neurons treated with AβO we showed that NADPH oxidase-mediated superoxide production occurs downstream of AβO-NR2B interaction and impairs Ca^{2+} homeostasis. These events precede changes in cell viability and activation of the ER stress-mediated apoptotic pathway as demonstrated by the increase in levels of GRP78, XBP-1 and GADD153/CHOP. The role of NR2B subunit, but not of NR2A, on AβO-induced ER stress and hippocampal neuronal dysfunction was demonstrated using ifenprodil, an antagonist of NR2B subunits and NVP-

AAM077, an NR2A antagonist that only slightly attenuated AβO-induced neurotoxicity.

Finally, we studied the role of ER stress *in vivo* using an AD animal model, the triple transgenic mice (3xTg-AD) (chapter 8). In comparison with non-transgenic (non-Tg) mice, there were alterations in ER stress markers in 3xTg-AD mice that were more pronounced in the hippocampus than in the cerebral cortex both in male and female mice, and females were shown to be more susceptible to ER stress. Furthermore, data obtained in this animal model show that the levels of ER stress markers are changed in young mice suggesting its role in early disease stages. These results were corroborated by studies in a peripheral model of AD. In collaboration with Prof. Isabel Santana from the "Hospitais da Universidade de Coimbra" and the group "Mitochondrial dysfunction and signalling in neurodegeneration" from the Center for Neuroscience and Cell Biology (CNC) we were able to use human peripheral blood mononuclear cells (PBMCs), namely lymphocytes, isolated from patients in different stages of the disease (mild, moderate and severe) and individuals with mild cognitive impairment (MCI) *versus* non-demented age-matched subjects (chapter 8). This peripheral model was used in order to identify the relationship between cognitive impairment occurring during the progression of the disease and markers of cell injury. A decrease in ER Ca^{2+} levels, followed by an increase in its cytosolic levels, was observed in PBMCs from individuals with MCI and mild AD patients that also presented increased levels of ER stress markers, namely GRP78 and XBP-1. In PBMCs from moderate-severe AD patients, the levels of these ER stress markers were similar to that measured in controls. However, on the early stages the ER stress-mediated

apoptotic pathway was already triggered, as demonstrated by an increase in GADD153/CHOP levels.

The results obtained using human and non-human models with different complexity, show that apoptotic cell death induced by Aβ involves the cooperation between ER and mitochondria and that it is triggered by the interaction of Aβ with the NMDA subtype of glutamatergic receptors for glutamate, thus increasing our knowledge about the mechanisms implicated in AD pathogenesis.

CHAPTER 1 – Introduction

1.1. Endoplasmic reticulum structure and function: brief overview.

The endoplasmic reticulum (ER), first described by (Porter *et al.* 1945), is an eukaryotic organelle that forms an interconnected network of tubules, vesicles and cisternae. It is delimited by a phospholipid membrane, which separates the cytosol from the lumen, also called cisternal space, continuous with the perinuclear space (Fig. 1.1). Being an extensive membrane network of cisternae (sac-like structures) that are held together by the cytoskeleton (Fig. 1.2), the presence of this organelle is very diffuse across the cell, having a large surface area that allows the storage of several proteins (Baumann & Walz 2001). In neuronal cells, ER is also widely distributed, not only in the cellular body, but also across distal parts such as dendrites and dendritic spines (Park *et al.* 2008), axonal shaft (Aihara *et al.* 2001), growth cones and synaptic terminals (Mattson *et al.* 2000). As an essential intracellular organelle, it ensures protein translation as well as the correct folding/assembly, glycosylation and sorting of proteins into the secretory system (Aridor & Balch 1999) and is involved in intracellular Ca^{2+} homeostasis (Paschen 2001).

Three types of this crucial organelle are recognized: rough ER, smooth ER and sarcoplasmic reticulum. Depending on the cell where it resides, or the kind of ER, this cellular component may assume different and specialized functions. Rough ER is distinguishable from smooth ER by the association with ribosomes that confers a rough aspect to the organelle. For this reason, rough ER is responsible for most of protein synthesis, including the production and storage of glycogen and other macro-molecules (Baumann & Walz 2001) that can then be transported to the Golgi complex for protein sorting, labeling and transport for it final destination. Smooth ER is responsible for lipids and steroids synthesis, carbohydrates and steroids metabolism (Maxfield &

Wustner 2002), regulation of Ca^{2+} concentration, drug detoxification and attachment of receptors to cell membrane proteins (Mattson *et al.* 2000). By last, sarcoplasmic reticulum, found in smooth and striated muscles, is a subtype of smooth ER which unique function is the regulation of Ca^{2+} levels by acting as a large Ca^{2+} storage facility, pumping Ca^{2+} ions upon muscle cell stimulation (Toyoshima *et al.* 2000).

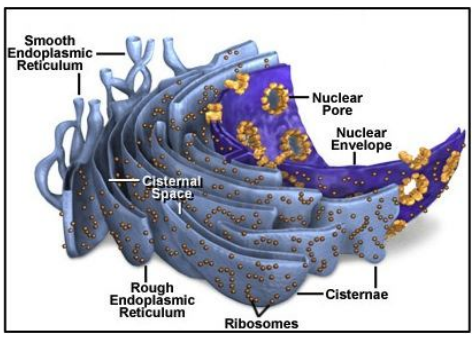


Figure 1.1 *Endoplasmic reticulum, a schematic model*.
From: http://micro.magnet.fsu.edu/cells/endoplasmicreticulum/endoplasmicreticulum.html

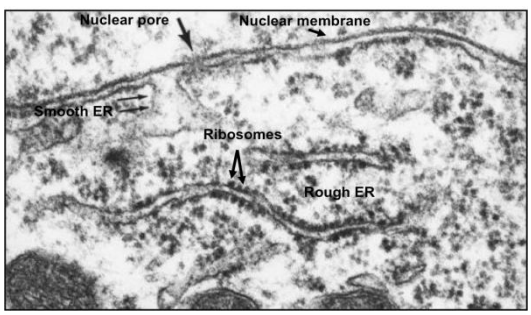


Figure 1.2 *Electron micrograph of a neuronal nuclear envelope detail, showing nuclear membrane, nuclear pore and both smooth ER (double arrows) and rough ER with the associated ribossomes.*
(Adapted from: Siegel GJ, Agranoff BW, Albers RW, et al., editors. "Basic Neurochemistry: Molecular, Cellular and Medical Aspects". 6th edition. Philadelphia: Lippincott-Raven; 1999. http://www.ncbi.nlm.nih.gov/books/NBK28209/)

1.2. Endoplasmic reticulum stress: causes and consequences.

1.2.1. Adaptive mechanisms involved in endoplasmic reticulum (ER) homeostasis: ER associated degradation, ER overload response, unfolded protein response.

Healthy cells require normal ER functioning, including the maintenance of the Ca^{2+} homeostatic balance and the correct protein folding, which are provided by a stringent quality-control system. By this mechanism, ER is capable of selectively export correctly folded proteins or to retrotranslocate misfolded proteins for proteosomal degradation in the cytosol, a process known as ER-associated protein degradation (ERAD) (Bonifacino & Weissman 1998; Chapman *et al.* 1998; Travers *et al.* 2000; Vembar & Brodsky 2008).

Due to the accumulation of unfolded or misfolded proteins in the ER lumen or deregulation of ER Ca^{2+} homeostasis caused by pathological stimuli (Cnop *et al.* 2010), ER performance can be affected leading to ER stress. Under these conditions, several adaptive mechanisms are activated in order to restore ER functioning and to preserve cell survival (Xu *et al.* 2005). The two main signalling pathways that can be activated as a self-protective mechanism are the unfolded protein response (UPR) and the ER overload response (EOR). Both processes facilitate the protein folding process in the ER and reduce overall translation (Harding *et al.* 1999; Kaufman 2002; Forman *et al.* 2003).

The EOR (reviewed in Chevet *et al.* 2001) is generated as a result of the abnormal accumulation of normal or misfolded proteins in the ER, leading to the transcriptional activation ofthe nuclear factor-kB (NF-kB) (Pahl & Baeuerle 1997), involved in cellular responses to stimuli such as stress, cytokines or free radicals (reviewed in Gilmore 2006) but also in mechanisms of learning and memory (Albensi & Mattson 2000). NF-kB is activated by

several drugs that can either act on the N-glycosylation or protein transport between ER-Golgi complex, such as tunicamycin or brefeldin A, respectively (Pahl & Baeuerle 1995). This transcription factor is activated upon ER over-retention of several membranar proteins (Pahl *et al.* 1996), a process called ER overload, but it was also proved to be triggered by the excessive acummulation of non-membranar proteins in the ER. Although certain stimuli could trigger both pathways, EOR is distinct from UPR, supporting the hypothesis that NF-κB works in a novel signal transduction pathway between the ER and the nucleus (Pahl & Baeuerle 1995), requiring the release of Ca^{2+}, followed by the formation of reactive oxygen intermediates species (ROS) during ER overload (Pahl & Baeuerle 1996). Even though, both mechanisms are able to provide the cell with the capacity to respond rapidly to the accumulation of incorrectly folded proteins within the ER, increasing the folding capacity or target them for the degradation machinery.

UPR, found to be well conserved and spread in several species from yeast to mammals, is a good example of how ER manages to restore cellular homeostasis. Since it was first described twenty years ago, several molecules have been found to be involved in the mechanisms responsible for UPR. ER possesses molecular chaperones and protein-modifying enzymes within the lumen specialized in protein folding (Merksamer & Papa 2010). Being a dynamic organelle, the capacity of the ER to fold proteins can be adjusted in response to changes in cellular protein folding requirements through UPR (Ron & Walter 2007). This adaptive response try to compensate this deregulation decreasing the influx of proteins into the ER lumen by attenuating the translation of proteins of the secretory pathway (Trusina *et al.* 2008) and

increasing *de novo* mRNA and protein synthesis of ER chaperones to increase the folding capacity (Wang *et al.* 1996; Chapman *et al.* 1998).

Glucose-regulated protein 78 (GRP78), also called binding immunoglobulin protein (BiP), is an important ER-resident chaperone that is upregulated under ER stress conditions. GRP78 has been considered a valuable ER stress marker (Liu *et al.* 2010) that acts as a defense mechanism against ER stress (Imaizumi *et al.* 2001; Naidoo 2009; Yoshida 2009). GRP78 promotes the correct folding of proteins that have been accumulated within the ER lumen and is considered to be an important molecule in the protein quality control, preventing unfolded proteins from being transported to the Golgi apparatus and further downstream (Kohno *et al.* 1993).

The binding to misfolded/unfolded proteins to GRP78 results in its detachment from the three main components of the UPR: inositol requiring element-1 (IRE-1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Naidoo 2009) (resumed in Fig. 1.3). This event allows phosphorylation and dimerization of IRE-1 and PERK, and also the translocation of ATF6 to the Golgi, leading to the activation of these transducers located across the ER membrane.

After dimerization, and due to a selective mRNA splicing mechanism, IRE-1α stimulates the production of X-box binding protein-1 (XBP-1), that induces the expression of chaperones and other proteins involved in protein folding and/or degradation (Yoshida 2007). Upon GRP78 relief, PERK homodimerizes and auto-phosphorylates. Activated PERK phosphorylates the eukaryotic initiation factor eIF2a, which results in the attenuation of protein translation. Additionally, an anti-oxidant response is up-regulated through activation of the nuclear factor erythroid-2 (NRF-2) (Harding *et al.* 2000). ATF6, the third ER

stress sensor, is cleaved in the Golgi after the detachment from BiP and becomes activated. Cleaved ATF6 fragment migrates to the nucleus inducing the expression of genes that encode molecular chaperones such as GRP78, protein disulfide isomerase (PDI) or GRP94, thus increasing the cellular folding capacity (Zhang & Kaufman 2006). Nonetheless, ATF6 is also responsible for XBP-1 regulation (Schroder 2006).

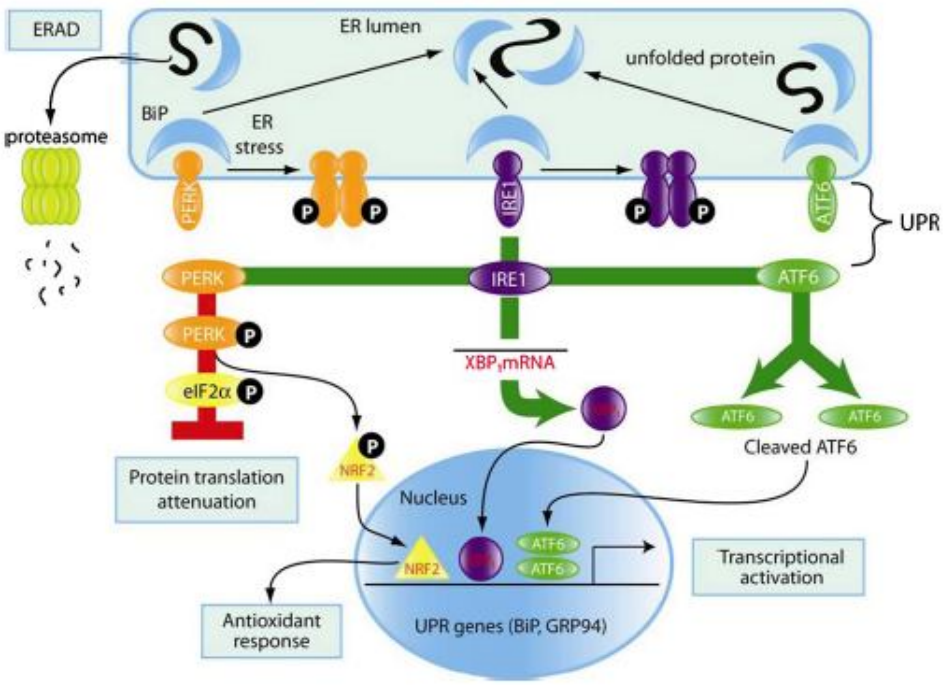


Figure 1.3 The *three main components of the unfolded protein response*.
Upon GRP78/BiP detachment from the 3 transducers of ER stress, induced by the accumulation of misfolded or unfolded proteins in ER lumen, PERK, IRE-1 and ATF6 become activated. Then, PERK phosphorylates eIF2a to attenuate protein translation and phosphorylates NRF-2 to up-regulate an anti-oxidant response. Cleaved ATF6 translocates to the nucleus and leads to induction of molecular chaperones such as GRP78/BiP or GRP94 to increase ER folding capacity. IRE-1 activation leads to XBP-1 splicing, transcriptional activation of chaperones and stimulation of protein degradation. Adapted from Naidoo 2009.

1.2.2. Endoplasmic reticulum stress-mediated apoptotic pathway.

Through the mechanisms described in the previous section (1.2.1.), cells are usually able to manage ER stress and recover normal functioning. However, prolonged or extensive ER stress results in the failure of these adaptive signaling pathways. Under these conditions, ER stress-mediated apoptotic cell death is activated (resumed in Fig. 1.4) (Imaizumi *et al.* 2001; Kim *et al.* 2008a). Cell death signalling is regulated by the same ER stress sensors involved in UPR. Active IRE-1 is responsible for the activation of c-Jun NH_2 terminal kinase (JNK) and caspases pathway (Merksamer & Papa 2010). On the other hand, ATF6 is able to regulate pro-apoptotic transcription factors like the (C/EBP) homologous protein (CHOP), also known as growth arrest and DNA damage 153 gene (GADD153) (Schapansky *et al.* 2007; Pino *et al.* 2009). The transcription of this factor is also dependent on ATF4 (Naidoo 2009) which is up-regulated downstream of PERK activation. One consequence of CHOP activation is the inhibition of anti-apoptotic proteins, such as Bcl-2, leading to ER Ca^{2+} release and subsequent activation of Ca^{2+}-dependent pathological mechanisms (Oyadomari & Mori 2004; Schroder & Kaufman 2005). The mitochondrial involvement during ER stress-mediated apoptotic cell death is now well accepted (discussed in section 1.6.). Indeed, CHOP and JNK promote the translocation of Bax to the mitochondria where it facilitates the release of pro-apoptotic factors required for caspase cascade activation (Naidoo 2009).

Caspase-12, and its homologous caspase-4 in humans (Hitomi *et al.* 2004), has been considered as one of the central mechanisms leading to apoptosis in cells experiencing ER stress (Niederer *et al.* 2005; Cheung *et al.* 2006; Kim *et*

al. 2009). The activation of this caspase results from the conversion of the ER membrane-localized pro-caspase-12 into its cytosolic active form (Morishima *et al.* 2002). Recently, it was shown to be modulated via the ubiquitin/proteasome system (UPS) (Song *et al.* 2008) and it has been implicated in ER stress-specific apoptosis contributing to amyloid-beta (Aβ) neurotoxicity in Alzheimer's disease (AD) (Cheung *et al.* 2006). It has been reported that caspase-4 is activated under ER stress conditions increasing caspase-3 activity (Kim *et al.* 2009). However, other groups reported that ER stress only activates caspase-9 leading also to apoptotic cell death, by a mechanism that does not require caspase-4 or -12 (Obeng & Boise 2005).

Another mechanism that could be activated in parallel with UPR, to avoid accumulation of misfolded proteins, involves targeting of ER parts to lysosomes through macroautophagy as a last effort to relief cellular stress and to prevent the occurance of apoptotic mechanisms (Kincaid & Cooper 2007; Heath-Engel *et al.* 2008).

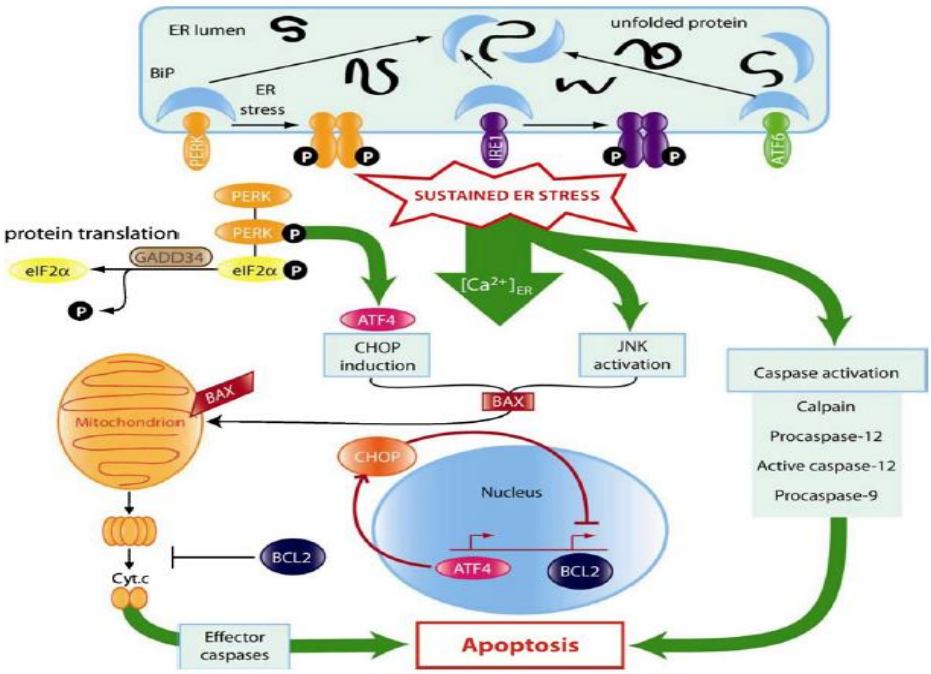


Figure 1.4 *Sustained ER stress leads to pro-apoptotic signaling.*
Prolonged UPR activation leads to ER Ca^{2+} release and to cell death signaling. Activated IRE-1 acting on downstream factors activates JNK and caspases. ATF4-dependent transcription, induced by activated PERK, leads to augmentation of the pro-apoptotic transcription factor CHOP. CHOP inhibits Bcl-2 leading to Ca^{2+} release; higher Ca^{2+} levels sensitize mitochondria to other insults inducing cell death. Bcl-2 exerts an anti-apoptotic function in the ER. CHOP and JNK also promote the translocation of Bax to the mitochondria where it facilitates the release of cytochrome c required for caspase activation. ER specific caspases, such as the caspase-12, are thought to directly induce cell death through activation of caspases 9 and 3. Adapted from Naidoo 2009.

In conclusion, upon activation of processes such as UPR (Imaizumi *et al.* 2001; Yoshida 2009), EOR (Pahl & Baeuerle 1997), ERAD (Travers *et al.* 2000) or even macro-autophagy (Hoyer-Hansen & Jaattela 2007; Heath-Engel *et al.* 2008), ER is able to manage with different kinds of stress and to restore cellular homeostasis, preserving cell survival (Yoshida 2009). However, under conditions of severe or prolonged ER stress, these adaptive signalling pathways

fail and apoptotic cell death is activated contributing to the pathology of several important human diseases, including diabetes, cancer and neurodegenerative disorders, such as AD (Kim *et al.* 2008a).

1.2.3. Deregulation of endoplasmic reticulum Ca^{2+} homeostasis.

Ca^{2+} ion impact nearly every aspect of cellular life (Clapham 2007). It plays an important role in signal transduction pathways, where it acts as a crucial second messenger, in neurotransmitters release from neurons, contraction of all muscle cell types or even in fertilization. ER is one of the most important Ca^{2+} stores and it is estimated that 1-3 mM of this ion is kept in ER lumen, mostly associated to Ca^{2+}-binding proteins (Koch 1990; Meldolesi & Pozzan 1998; Michalak *et al.* 2002). Therefore, loss of Ca^{2+} balance leads to cellular malfunction. In fact, disturbance of ER Ca^{2+} homeostasis is commonly associated with ER stress (Szegezdi *et al.* 2009) and subsequent activation of the mitochondrial-mediated apoptotic cell death pathway (Chami *et al.* 2008; Costa *et al.* 2010; Gonzalez *et al.* 2010). Ca^{2+} released from ER under ER stress conditions is taken up by mitochondria and is required to induce the release of pro-apoptotic factors, such as cytochrome c (Niederer *et al.* 2005; Pizzo & Pozzan 2007; Deniaud *et al.* 2008).

ER Ca^{2+} deregulation has been described to be involved in several neurodegenerative diseases, namely in AD. Toxic Aβ fibrils and oligomers, both implicated in AD ethiopathogenesis (Sakono & Zako 2010; Tomiyama 2010), have been described to be able to deplete ER Ca^{2+} content in neurons, increasing cytosolic Ca^{2+} levels (Bhatia *et al.* 2000; Ferreiro *et al.* 2004). ER Ca^{2+} release culminates in apoptotic cell death, due to the close communication

between ER and mitochondria (Scorziello *et al.* 1996; Huang *et al.* 2000; Niederer *et al.* 2005; Ferreiro *et al.* 2008b; Resende *et al.* 2008b).

ER Ca^{2+} homeostasis is regulated through a subset of channels, namely inositol-1,4,5-triphosphate (IP3R) and ryanodine receptor (RyR) families (Berridge 1995; Lanner *et al.* 2010) which could be activated both by electrical or chemical stimulation (Verkhratsky 2002). However, Ca^{2+}-induced Ca^{2+} release (CICR), first described by (Endo 1977), corresponds to a mechanism through which cytosolic Ca^{2+} is able to sensitize the ER Ca^{2+} release channels IP3R or RyR (Berridge *et al.* 2000). CICR occurring through RyR can also be triggered and amplified by Ca^{2+} released through IP3R (Finch *et al.* 1991; Friel & Tsien 1992; Yao & Parker 1992). Non-least important, ER possesses mechanisms to facilitate Ca^{2+} entrance to this compartment even against the concentration gradient. This is accomplished through Ca^{2+} pumps dependent on energy, such as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA Ca^{2+}-ATPase). It promotes the transference of Ca^{2+} from the cell cytosol to the lumen of the sarcoplasmic reticulum through ATP hydrolysis (Satoh *et al.* 2011).

1.3. Pathological role of endoplasmic reticulum stress in several human diseases.

In the past few years, ER stress has been largely implicated in several human diseases (reviewed in Zhao & Ackerman 2006; Lin *et al.* 2008), in part due to its role in the accumulation of misfolded/unfolded proteins and in the activation of cell death pathways (Zhao & Ackerman 2006). ER stress has been demonstrated to be involved in several pathological conditions, such as heart (Minamino & Kitakaze 2010), kidney (Inagi 2010) and liver diseases (Tilg & Moschen 2010). Furthermore, atherosclerosis (Bai *et al.* 2010), inflammatory diseases (Hotamisligil 2010; McGuckin *et al.* 2010) and the widely spread diabetes/obesity (Hummasti & Hotamisligil 2010) have also been reported to involve ER stress. Moreover, brain associated diseases such as ischemia (Tajiri *et al.* 2006; Oida *et al.* 2008) and several neurodegenerative disorders have been correlated with ER dysfunction (Lindholm *et al.* 2006). Among them, several authors consider that the origin of neuronal death occurring in diseases such as Parkinson's disease (Imai *et al.* 2000; Imai *et al.* 2001), Huntington´s disease (Reijonen *et al.* 2008), amyotrophic lateral sclerosis (Wootz *et al.* 2004; Nishitoh *et al.* 2008), prion-related disorders (Hetz & Soto 2006; Ferreiro *et al.* 2007) and finally with AD (Sato *et al.* 2001; LaFerla 2002; Katayama *et al.* 2004) are also related with induction of ER stress.

1.4. Brief overview of Alzheimer's disease: the role of amyloid β peptide.

In 1907 Alois Alzheimer, a German psychiatrist and neuropathologist, described for the first time a pathology considered nowadays as the most common age-related neurodegenerative disease and cause of dementia in the elderly. Affecting presently almost 35 million people worldwide (http://www.alz.org), the prevalence of AD tends to further increase in the next decades due to increased life expectancy in developed countries and it is estimated to reach over 100 million people in 2050 (Brookmeyer *et al.* 1998). In Portugal, more than 70,000 individuals suffer from AD (http://www.Alzheimerportugal.org).

This chronic neurodegenerative disorder that affects the central nervous system (CNS) is characterized by progressive memory loss and deterioration of cognitive functions due to synaptic and neuronal loss in the hippocampus and cerebral cortex. Typical hallmarks of AD also include intracellular neurofibrillary tangles consisting of abnormally hyperphosphorylated tau protein and extracellular senile plaques, mainly composed of fibrillar Aβ and usually surrounded by reactive astrocytes, activated microglia and dystrophic neuritis (Selkoe 2001; Golde 2003) (Fig. 1.5).

Sporadic AD (SAD) represents the majority of AD cases. The main risk factor for this late-onset (after 65 years of age) form of AD is aging but some genetic and environmental factors are associated with increased susceptibility to develop SAD, such as the presence of the ε4 allele of apolipoprotein E (ApoE), low educational level, or cardiovascular disease (Pereira *et al.* 2005; Blennow *et al.* 2006).

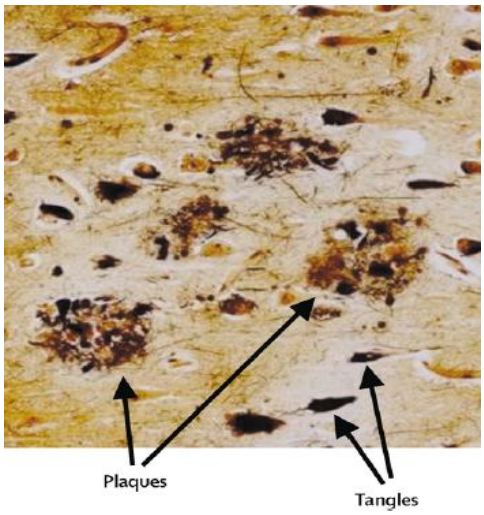


Figure 1.5 *Presence of amyloid senile plaques and neurofibrillary tangles in the cerebral cortex of an AD patient.* From Blennow *et al.* 2006.

The familiar AD (FAD) form is caused by autosomal dominant mutations in genes encoding the amyloid precursor protein (APP), presenilin 1 or presenilin 2 that were shown to increase the production of an Aβ isoform with 42 amino acids ($A\beta_{1-42}$). Although the molecular mechanisms underlying SAD are unknown, it is likely to also involve $A\beta_{1-42}$ accumulation. In fact, SAD *post-mortem* brains present elevated protein levels and activity of the β-secretase enzyme (Yang *et al.* 2003), which belong to a group of enzymes responsible for Aβ generation from APP (Nathalie & Jean-Noel 2008) (Fig. 1.6).

Aβ is generated via an amyloidogenic pathway involving an alternative two-step proteolytic cleavage of the integral membrane glycoprotein APP (Newman *et al.* 2007; Thinakaran & Koo 2008) which encloses an Aβ domain (Nathalie & Jean-Noel 2008). APP processing is accomplished by membrane-associated metalloproteinases named secretases (Esler & Wolfe 2001).

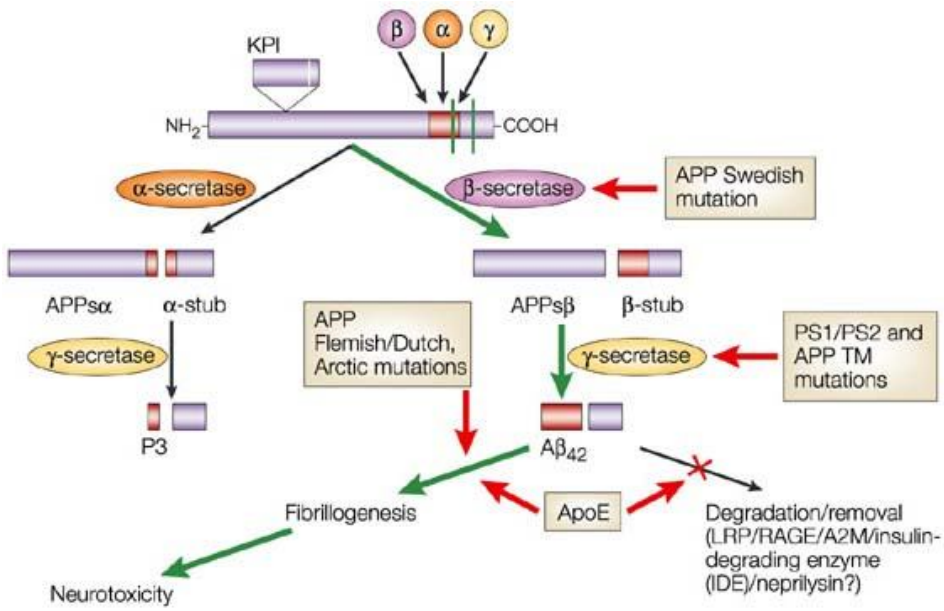


Figure 1.6 *APP processing mediated by α-, β- and γ-secretases*.
APP is susceptible to endoproteolytic cleavage through two main pathways involving different secretases. These sequential cleavages can occur by α-secretase and then by γ-secretase (non-amyloidogenic pathway) or by β-secretase and then by γ-secretase (amyloidogenic pathway), which preclude or cause the formation of the amyloidogenic Aβ peptide, respectively. FAD-associated mutations in presenilins (PS1/PS2) or in APP favour this amyloidogenic processing, potentiating Aβ formation. Polymorphisms in ApoE, which increase the risk to develop SAD, can promote Aβ fibrillization and/or inhibit Aβ clearance mechanisms leading to Aβ accumulation. From Sisodia & St George-Hyslop 2002.

When the first APP cleavage is made by α-secretase the liberation of amyloidogenic species is precluded. Instead, the result of this fragmentation is a large soluble extracellular amino-terminal portion of APP (APPsα) and a carboxy-terminal fragment that consists of 83 residues (C83) which, upon γ-secretase cleavage, originates the non-amyloidogenic P3 peptide (Dulin *et al.* 2008). In alternative, APP could be processed through an amyloidogenic pathway that generates Aβ species. In this case, APP undergoes two sequential

endoproteolytic steps that are mediated by distinct enzymatic activities known as β- and γ-secretases. β-secretase, also called BACE-1 (beta-site APP-cleaving enzyme) is the main responsible for Aβ production and is currently being targeted in order to stop AD development (Boddapati *et al.* 2011). When APP is cleaved by BACE-1, a short soluble amino-terminus (APPsβ) fragment and a carboxy-terminal fragment (C99) are obtained. The membrane-associated C99 fragment is then cleaved by γ-secretase. The cleavage occurs at the ε-site within the transmembranar domain, liberating the APP intracellular domain (AICD). This is followed by cleavage of the Aβ-containing fragment at the γ-secretase site after residue 40 or 42 of Aβ (LaFerla 2002). The two most abundant forms are $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, with 40 and 42 amino acids, respectively (Selkoe 1993). Even though, APP amyloidogenic processing was shown to produce other fragments of different lengths (Czirr *et al.* 2008). Furthermore, this Aβ peptide can assume different assembly states (Selkoe 2001; Parihar & Hemnani 2004). Soluble Aβ is thought to undergo a conformational change to high β-sheet content, rendering it prone to aggregate into soluble oligomers and larger insoluble fibrils that deposit in plaques. Aβ can aggregate into smaller molecular weight forms, described for the first time as small, globular structures of Aβ which could be secreted as dimers or trimers, called Aβ oligomers (AβO) or ADDLs (amyloid β-derived diffusible ligands) (Lambert *et al.* 1998) (Fig. 1.7).

Since it is the main component of senile plaques (Masters *et al.* 1985), Aβ has been largely studied in last years and is now considered by many as the key molecule in the pathogenesis of AD (Hardy & Selkoe 2002). Based on several evidences, the "amyloid cascade hypothesis" has been proposed to explain the neurodegenerative process in AD (Hardy & Selkoe 2002). Support for this

hypothesis also includes the finding that the FAD mutations are present in the genes for both the substrate (APP) and the key enzyme presenilin (PS) for Aβ generation. Most *APP* mutations also cluster around the secretase sites, and both the *APP* and *PS* mutations increase $A\beta_{1\text{-}42}$ production. Furthermore, patients with Down's syndrome, who possess an extra copy of the *APP* gene, develop Aβ plaques early in life and develop AD later in life, and the recent finding of a duplication of the *APP* locus in families with FAD support to the notion that life-long APP overexpression triggers Aβ deposition.

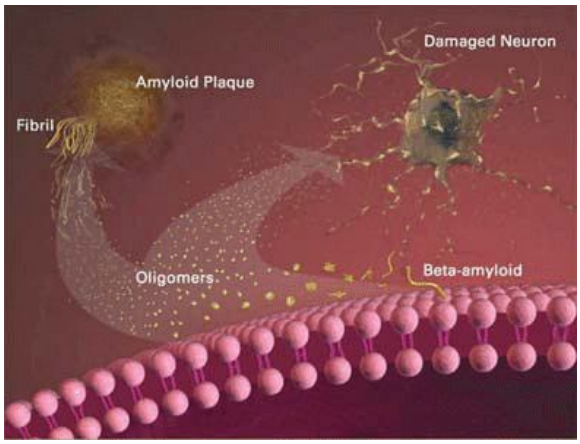


Figure 1.7 *Different amyloid species, different forms of toxicity*.
Different Aβ species are associated with neuronal damage and plaque formation in AD. Recently discovered soluble oligomeric Aβ species have been pointed out as the most neurotoxic forms of Aβ, activating several toxic pathways that contribute to neurodegeneration. Adapted from http://www.kleinlab.org/our_research.htm.

According to the "amyloid cascade hypothesis", the initiating event in AD is the unbalance between the mechanisms of Aβ production and Aβ clearance, with increased Aβ production in FAD and decreased Aβ clearance in SAD, ultimately leading to neuronal degeneration and dementia. As a result of Aβ accumulation, soluble oligomers are formed that have a negative impact in synaptic function, impairing hippocampal long-term potentiation (LTP) and synaptic plasticity. Furthermore, Aβ triggers an inflammatory response upon microglia and astrocytes activation, oxidative stress and ionic dyshomeostasis, which result in neuronal dysfunction and neurotransmitters deficit, finally leading to cognitive alterations and dementia. On the other hand, tau pathology with neurofibrillary tangle formation is regarded by many as a downstream event of Aβ pathology as a result of the unbalance between tau kinases and phosphatases that contributes to neuronal dysfunction and cognitive symptoms (Hardy & Selkoe 2002; Citron 2004), as depicted in Fig. 1.8.

However, even though this theory is supported by numerous findings, some controversy still remains in the recognition of Aβ peptide as the trigger of this brain pathology. Instead it is by opposition some times regarded as consequence of cell death (Recuero *et al.* 2004).

Only Aβ deposited in plaques was initially assumed to be neurotoxic (Nathalie & Jean-Noel 2008) but recent findings suggest that soluble Aβ oligomers might be the culprits in AD pathology (Sakono and Zako, 2010). Rather than Aβ fibrils, AβO are thought to be the main species implicated in synaptic dysfunction and loss in AD (Golde 2003; Heinitz *et al.* 2006; Lesne *et al.* 2006). In fact, density of fibrillar amyloid plaques does not correlate well with the severity of dementia (Masliah *et al.* 1990). On the other hand, AβO levels and evidences of synaptic targeting by AβO in mature hippocampal

neurons were proved to be strongly correlated with the extent of synaptic damage, memory loss and cognitive impairment related to AD (Klein *et al.* 2001; Lacor *et al.* 2004; Klein 2006; Lacor *et al.* 2007). Also, while aggregated Aβ is the most frequent form of Aβ found in AD brains, in peripheral blood and in cerebral spinal fluid from AD patients only soluble Aβ can be found (Cirrito & Holtzman 2003; Gong *et al.* 2003; Kokubo *et al.* 2005)

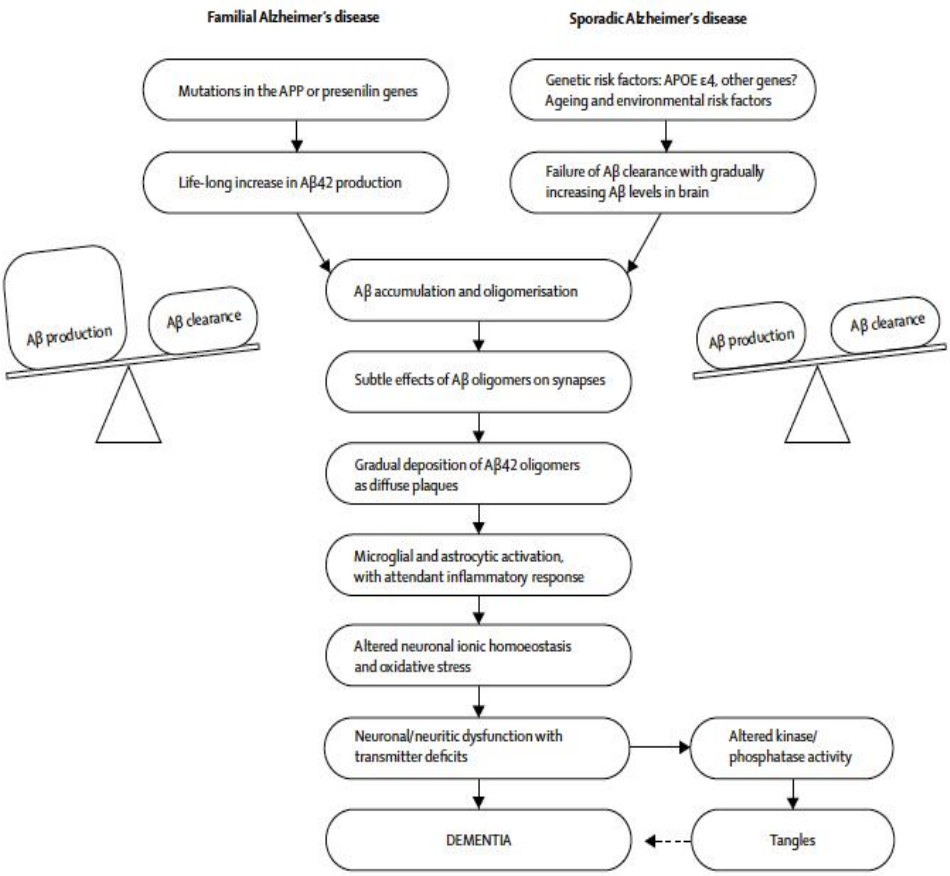


Figure 1.8 *Amyloid cascade hypothesis*.

Deregulation in the balance between Aβ clearance and Aβ production initiates a cascade of events that culminates in synaptic dysfunction and neuronal death, leading to dementia. Aβ overproduction is a consequence of pathogenic mutations in early-onset FAD. In late-onset SAD, the synergistic action between aging and several environmental and/or genetic risk factors compromises Aβ clearance mechanisms. Increased levels of Aβ, specially $Aβ_{1-42}$, promotes the formation of soluble oligomers and aggregates which impair synaptic and neuronal function resulting in neurotransmitter deficits and cognitive symptoms. Aβ also triggers tau pathology with tangle formation that could contribute to neuronal dysfunction and cognitive symptoms. Adapted from Blennow *et al.* 2006.

1.4.1. Endoplasmic reticulum stress in Alzheimer's disease.

The role of the ER stress response in AD is presently being investigated. Based on both *in vitro* and *in vivo* studies, several authors argue that it is one of the main players in synaptic dysfunction and neuronal death occurring in this neurodegenerative disorder (Mattson *et al.* 2001; Nishitsuji *et al.* 2009). It has been reported that the levels of ER stress markers are increased in AD post-mortem brain tissues suggesting that the prolonged activation of the ER stress response is involved in neurodegeneration (Hoozemans *et al.* 2005; 2009).

In transgenic mice modeling AD, the ER-resident caspase-12 is strongly up-regulated (Song *et al.* 2008), as well as BiP/Grp78 and CHOP/GADD153 (Ling *et al.* 2009). Additionally, a global molecular profile of hippocampal and cortical gene expression revealed that ER stress-related genes are differentially regulated during the initial and intermediate stages of Aβ deposition (Selwood *et al.* 2009). Several evidences obtained in cultured cells suggest that Aβ is implicated in ER stress occurring in AD (Chafekar *et al.* 2008; Nishitsuji *et al.* 2009; Takahashi *et al.* 2009). In primary cultured neurons, GRP78 has been shown to be up-regulated upon treatment with both fibrillar or oligomeric Aβ (Ferreiro *et al.* 2006; Resende *et al.* 2008b) and Aβ was shown to activate the ER stress-mediated apoptotic cell death pathway (Ferreiro *et al.* 2006; Ferreiro *et al.* 2008b; Resende *et al.* 2008b).

Perturbation of ER Ca^{2+} homeostasis, a trigger for the accumulation of unfolded or misfolded proteins and activation of the ER stress response, is also an important step in the beginning or progress of neuronal dysfunction in AD. A markedly decrease of calreticulin immunoreactivity (ER Ca^{2+} binding

protein) was described in AD postmortem brain (Lai *et al.* 2009). Recent studies in AD transgenic mice have shown that enhanced Ca^{2+} response is associated with increased levels of RyR and alters synaptic transmission and plasticity mechanisms before the onset of histopathology and cognitive deficits (Smith *et al.* 2005; Stutzmann *et al.* 2006; Chakroborty *et al.* 2009). Moreover, mutant PS interacts with the IP3R-Ca^{2+} release channel, resulting in Ca^{2+} signaling abnormalities (Stutzmann *et al.* 2003; 2004) that have been suggested to be an early pathogenic event in AD involved in presynaptic dysfunction (Zhang *et al.* 2009). Several findings implicate Aβ as a trigger of ER Ca^{2+} dyshomeostasis. Overexpression of APP was shown to potently enhance cytosolic Ca^{2+} levels and cell death after ER Ca^{2+} store depletion and under these conditions GADD153/CHOP is significantly upregulated (Copanaki *et al.* 2007). In cultured cortical neurons, it was previously demonstrated that Aβ depletes ER Ca^{2+} stores, promoting Ca^{2+} release through IP3R and RyR, thus increasing intracellular Ca^{2+} levels and compromising cell survival (Ferreiro *et al.* 2004; Resende *et al.* 2008b). Additionally, Aβ was described to mediate changes in intracellular Ca^{2+} homeostasis in neurons through a direct increase of the RyR3 isoform expression and function (Supnet *et al.* 2006).

1.4.2. Amyloid-β peptide: a cause or consequence of mitochondrial dysfunction?

Mitochondria are the main organelles responsible for the supply of energy to cells. Mitochondria are almost independent organelles that contain their own DNA, essential for both respiration and ATP synthesis. Despite human mitochondrial DNA (mtDNA) encodes several subunits of the five complexes of the electron transport chain (ETC) (Fig. 1.9) required for ATP production,

nuclear DNA also encodes subunits of the ETC complexes and enzymes responsible for replication, repair, transcription and translation (Anderson *et al.* 1981; Wallace 1994) which enter the mitochondria through the transporter inner membrane (TIM) and transporter outer membrane (TOM) machinery (Hansson Petersen *et al.* 2008).

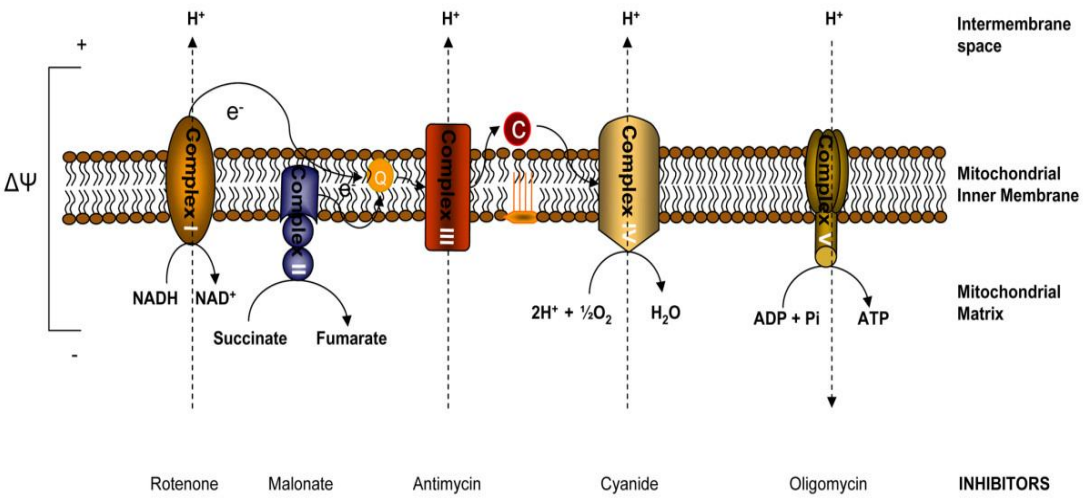


Figure 1.9 *Mitochondrial electron transport chain*.
Mitochondrial energy production is accomplished by the electron transport chain located at the mitochondrial inner membrane (MIM). It is composed of five multimeric complexes. Electron transport between complexes I to IV is coupled to proton extrusion into the intermembrane space, creating an electrochemical gradient (ΔΨ), also called mitochondrial membrane potential (ΔΨm), across the MIM. Energy created by protons flow through complex V (ATP synthase), is utilized to synthesize ATP from ADP. Mitochondrial respiratory chain can be chemically inhibited, as shown. C, cytochrome c; Q, ubiquinone. From Bayir & Kagan 2008.

Imaging studies by positron emission tomography (PET) revealed that glucose utilization is lower in AD brains than in age-matched controls. Furthermore, reduced brain glucose metabolism was observed in subjects with mild cognitive impairment (MCI), suggesting that insufficient energy production may be a crucial factor in preclinical AD (Mosconi 2005) (Fig. 1.10). Hypometabolism in AD is believed to result from decreases in the activity of mitochondrial cytochrome c oxidase (COX), pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase complexes (Blass 2001). Furthermore, evidences for mitochondrial abnormalities such as mitochondria fragmentation, morphological changes and density reduction were found in the brain of AD patients (Small *et al.* 1995; Ferrer 2009; Ferreira *et al.* 2010).

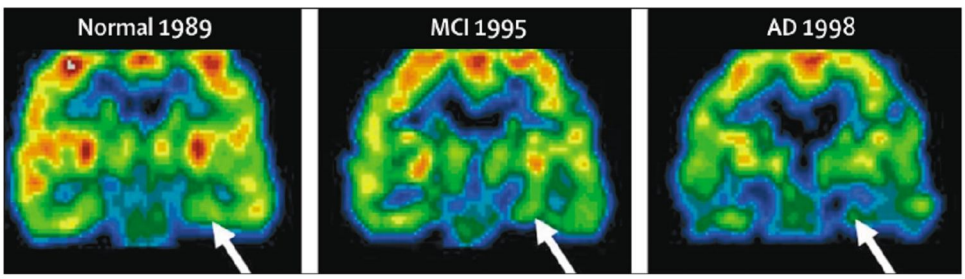


Figure 1.10 *Mitochondrial Longitudinal metabolic reductions in Mild Cognitive Impairment (MCI) and AD.*
FDG-PET scans in a 71-year-old cognitively normal woman at baseline (1989) and over 9 years. During this observation period the patient declined to MCI and later was diagnosed with AD, which was confirmed at autopsy. For each observation a coronal PET scan is depicted at the level of the entorhinal cortex and anterior hippocampus. Arrows point to the inferior surface of the entorhinal cortex with progressively darker colours on the PET scans, which indicates progressive reductions in glucose metabolism. From Blennow *et al.* 2006.

Variations in human mtDNA may contribute to individual differences in mitochondrial function and also to the increased susceptibility to various diseases, such as AD (Shoffner *et al.* 1993; Hutchin & Cortopassi 1995; van der Walt *et al.* 2004). In fact, AD brains exhibit an increase in mtDNA control region mutations that can account for the sporadic appearance of mitochondrial defects seen in late-onset AD (Coskun *et al.* 2003). The accumulation of mtDNA mutations could be at the origin of the ETC malfunction observed in AD brains (Lin *et al.* 2002; Coskun *et al.* 2003; Lin & Beal 2006; Kruger *et al.* 2010), in particular in COX activity (Kish *et al.* 1992; Bosetti *et al.* 2002; Cardoso *et al.* 2004b; Pickrell *et al.* 2009), leading to ATP depletion, mitochondria-mediated oxidative stress and apoptotic cell death (Kwong *et al.* 2007). In studies conducted in AD cytoplasmic hybrids (cybrids), cell lines carrying the mitochondrial complex IV (COX) defect from AD patients that arise from their mtDNA (Ojaimi *et al.* 1999; Petrozzi *et al.* 2007), a significant decrease in mitochondrial membrane potential ($\Delta\Psi m$), cytochrome c release and caspases activation were detected and were shown to increase cellular susceptibility to Aβ toxicity (Cardoso *et al.* 2004b). Moreover, it was demonstrated that Aβ can potentiate mitochondrial dysfunction and cell death in AD cybrids (Khan *et al.* 2000).

Mitochondria dysfunction is considered by many authors as a pathological trigger for AD (Ienco *et al.* 2011). Accordingly to the "mitochondrial cascade hypothesis", schematically represented in Fig. 1.11, it is postulated that an accumulation of mitochondrial damages induced by Aβ could lead to impaired energy generation by mitochondria, accumulation of ROS and activation of cell death pathways (reviewed in Mancuso *et al.* 2007; Swerdlow & Khan 2009; Chen & Yan 2010; Swerdlow *et al.* 2010). This hypothesis is supported by

studies conducted in mtDNA-depleted Rho0 (ρ0) cells, which proved that Aβ requires functional mitochondria to induce toxicity (Cardoso *et al.* 2001; Cardoso *et al.* 2002).

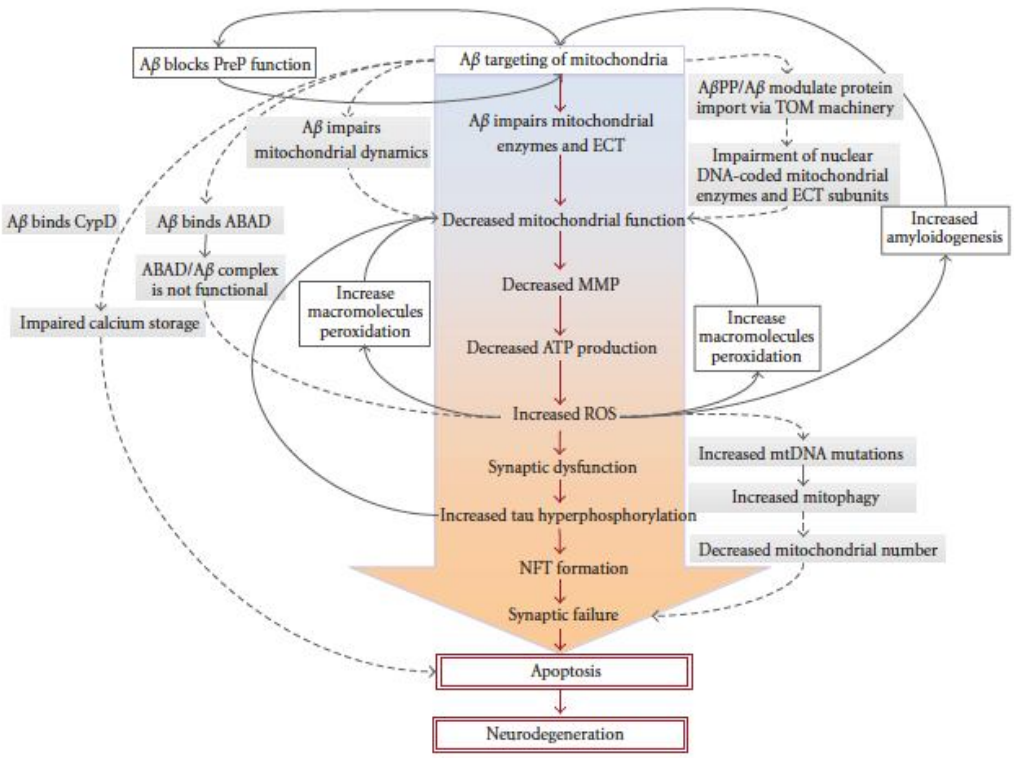


Figure 1.11 *Mitochondrial cascade hypothesis for AD.*
This cascade of events represents a hypothetical explanation for the role of Aβ in mitochondrial dysfunction. In this model, increased ROS production, neurofibrillary tangles (NFTs) formation, synaptic failure and neurodegeneration are the main events. Several other pathways are involved in this cascade via feedback or forward revealing several vicious cycles within a larger vicious cycle. All of them, once set in motion, amplify their own processes, thus accelerating the development of AD. Adapted from Pagani & Eckert 2011.

It is presently believed that Aβ enters the mitochondria, induces ROS formation, disrupts the ETC and ultimately causes the release of pro-apoptotic factors. In fact, Aβ was proved to interact with the Aβ-binding alcohol dehydrogenase (ABAD) in brain mitochondria of AD patients and transgenic mice (Lustbader *et al.* 2004), promoting leakage of ROS, inhibition of mitochondrial activity and induction of cell death (Takuma *et al.* 2005). Casperson and collegues (Caspersen *et al.* 2005) also reported that Aβ accumulation in the mitochondriais associated with diminished enzymatic activity of ETC complexes, namely with COX, and with a reduction in the rate of oxygen consumption. More recent evidences demonstrated the ability of Aβ to enter mitochondria causing mitochondrial dysfunction (Manczak *et al.* 2006; Reddy 2006) and the TOM machinery was shown to be a via of Aβ transport into the mitochondria (Hansson Petersen *et al.* 2008). Furthermore, RAGE-mediated signaling was shown to contribute to intraneuronal Aβ accumulationand promotion of neuronal degeneration through perturbation of mitochondrial function (Takuma *et al.* 2009). Aβ was also demonstrated to interact with Cyclophilin D (Cyp D), an integral part of the mitochondrial permeability transition pore (PTP) whose opening leads to cell death (Du *et al.* 2009), corroborating the hypothesis of the direct effect of Aβ on mitochondria. In an AD mouse model, Cyp D deficiency was shown to attenuate mitochondrial dysfunction induced by Aβ, to improve synaptic function and alleviate Aβ-mediated reduction of LTP, finally recovering learning and memory deficits (Du *et al.* 2009). Recently, mitochondrial associated secretases were demonstrated to be involved in APP processing showing that Aβ can be produced locally in mitochondria contributing to mitochondrial

dysfunction in AD (Pavlov *et al.* 2010). These findings corroborate the previously reported accumulation of full-length APP in the mitochondria (Anandatheerthavarada *et al.* 2003) that correlates with mitochondrial dysfunction and impairment of energy metabolism. By last, in AD transgenic mice, mitochondrial Aβ levels have been associated with the extent of mitochondrial dysfunction in different brain regions and also with the degree of cognitive impairment (Dragicevic *et al.* 2010).

Collectivelly, the above studies demonstrate that mitochondrial dysfunction is one of the mechanisms underlying Aβ-induced neurodegeneration (reviewed in Moreira *et al.* 2006; Chen & Yan 2010; Ferreira *et al.* 2010).

1.5. Cross-talk between endoplasmic reticulum and mitochondria.

The ER is a site for the synthesis of proteins and lipids that will be subsequently secreted or used in several other organelles. In addition to the luminal connection of ER with the nuclear envelope, it also establishes close contacts with many intracellular organelles such as mitochondria, Golgi apparatus, secretory granules, lysosymes and endosomes, as represented in Fig. 1.12 (Park *et al.* 2008), facilitating the exchange of ER-derived products.

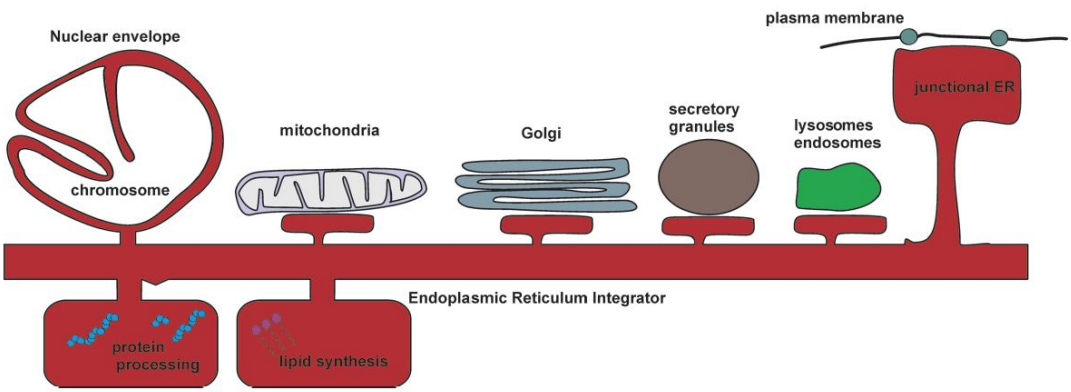


Figure 1.12 *The endoplasmic reticulum (ER) is in close contact with several organelles and with the plasmatic cell membrane.* Adapted from Park *et al.* 2008.

Several groups point to the interaction between ER and mitochondria as an important process in normal cellular functioning, as well as during programmed cell death triggered by ER stress (Kim *et al.* 2006; Zhang & Armstrong 2007; Lei *et al.* 2008; Arduino *et al.* 2009; Klee *et al.* 2009; Sharaf El Dein *et al.* 2009; Vitiello *et al.* 2009).

Numerous studies indicate that ER stress-induced apoptotic cell death requires a mitochondrial component, highlighting the close cooperation between these two organelles (reviewed in Giorgi *et al.* 2009). ER directly communicates with mitochondria through close contacts referred as mitochondria-associated membranes (MAM) that support Ca^{2+} transfer from ER to mitochondria. In fact, apoptotic stimuli known to act through Ca^{2+} release from the ER induce a prolonged increase in the mitochondrial Ca^{2+} concentration (Chami *et al.* 2008; Deniaud *et al.* 2008; Arduino *et al.* 2009). Bcl-2-associated X protein/Bcl-2-homologous antagonist/killer (Bax/Bak) translocation and oligomerization in the outer mitochondrial membrane, cytochrome c release, loss of $\Delta\Psi m$ and caspase-9 activation are common hallmarks of ER stress-induced apoptosis (Hacki *et al.* 2000; Zhang & Armstrong 2007). Further, caspase-3 and -9 were proven to be activated through ER Ca^{2+} release in human cells (Gonzalez *et al.* 2010). Besides having a role in the mitochondrial-mediated apoptosis, various proteins of the Bcl-2 family, including the anti-apoptotic protein Bcl-2, modulate Ca^{2+} content in both mitochondria and ER (Foyouzi-Youssefi *et al.* 2000). Conversely, overexpression of the pro-apoptotic Bax/Bak proteins favours the transfer of Ca^{2+} from ER to mitochondria and induces cell death (Nutt *et al.* 2002; Scorrano *et al.* 2003). Transmission of a Ca^{2+} signal from IP3R to mitochondria was demonstrated to be associated with IP3-induced opening of the permeability transition pore and, in turn, cytochrome c release (Szalai *et al.* 1999). Similarly, phosphorylation of IP3R by Akt reduces cellular sensitity to apoptotic stimuli through a mechanism that involves diminished Ca^{2+} flux from the ER to the mitochondria (Szado *et al.* 2008). Cytochrome c released from mitochondria can also bind to the ER IP3R and promotes Ca^{2+} release (Boehning *et al.* 2003). Released ER Ca^{2+} triggers the extrusion of a

large amount of cytochrome c from all the mitochondria in the cell, amplifying the death signal (Boehning *et al.* 2004). Despite the evidence that demonstrate the involvement of mitochondrial and ER dysfunction in AD pathogenesis, information regarding the role of ER-mitochondria cross-talk in this neurodegenerative disorder is sparse although supporting data is now beginning to emerge. It was recently shown that PS1 and PS2 are highly enriched in a subcompartment of the ER that is associated with MAM that forms a physical bridge between the two organelles (Area-Gomez *et al.* 2009). Moreover, the association of hyperphosphorylated tau with ER membranes was detected in AD brains and also in the brain of asymptomatic mice that overexpress mutant tau. Interestingly, these mice exhibited more contacts between ER membranes and mitochondria (Perreault *et al.* 2009), suggesting that accumulation of tau at the surface of ER membranes might contribute to tau-induced neurodegeneration through mitochondria. Previous results demonstrated that Ca^{2+} released from ER through IP3R and RyR receptors in cortical neurons treated with Aβ are responsible for the depolarization of mitochondrial membrane, release of cytochrome c upon translocation of Bax to mitochondria and activation of caspase-9 (Ferreiro *et al.* 2006; 2008b), thus implicating the ER/mitochondria cross-talk in neurodegeneration occurring upon Aβ-induced ER dysfunction.

ROS play an important role in the cooperation between ER and mitochondria during apoptosis (reviewed in Csordas & Hajnoczky 2009). First, ROS are able to promote accumulation of misfolded proteins within the ER lumen (He *et al.* 2008; Yan *et al.* 2008) by reducing the activity of chaperones such as BiP/GRP78 (Nuss *et al.* 2008). Second, many of the proteins involved in the regulation of Ca^{2+} in the ER are also susceptible to oxidants (Huang *et*

al. 2004) and thus oxidative stress conditions lead to perturbations of the ER Ca^{2+} homeostasis (Racay *et al.* 1995). Third, GADD153/CHOP can potentiate electron transfer to molecular oxygen, forming ROS (Marciniak *et al.* 2004). Fourth, Ca^{2+} that results from the depletion of ER Ca^{2+} stores can be taken up by juxtaposed mitochondria as described before, inducing ROS formation (Tardif *et al.* 2005). Recently, it was demonstrated that the release of Ca^{2+} from ER in cortical neurons treated with Aβ is responsible for the depletion of the antioxidant glutathione (GSH) and accumulation of ROS that are involved in loss of $\Delta\Psi m$ (Ferreiro *et al.* 2008b), thus highlighting ROS as potential mediators of ER-mitochondria cross-talk in AD.

1.6. Glutamatergic N-methyl-D-aspartate receptors: sites of action for amyloid-β oligomers?

Glutamate is the major excitatory neurotransmitter in the CNS and a well-known agonist of several receptors, including the ionotropic N-methyl-D-aspartate receptors (NMDARs). These glutamate receptors are the predominant molecular device for controlling synaptic plasticity and memory function (Li & Tsien 2009). However, overstimulation of NMDARs and excessive Ca^{2+} entry can initiate several downstream events leading to neuronal cell death, a process known as excitotoxicity (Lipton 2006).

NMDARs are multimeric ionotropic receptors composed of four subunits, being NR1 subunit essential for the function of the channel. The other subunits could be NR2A–NR2D with long extensions of their C-terminals into the cytoplasm, or NR3A-B that assemble together generating several receptors with different pharmacological and physiological properties (Salter & Kalia 2004). The correct functioning of this receptor requires the presence of glutamate and glicine and also that their allosteric/modulatory sites are not occupied. A feature of NMDARs is their voltage–dependent activation. Under physiological conditions, Mg^{2+} blocks the channeland the receptor is inactive. However, upon membrane depolarization, Mg^{2+} blockage is removed, the channel opens and the receptor becomes activated. Therefore, when all these requisites are fulfilled, NMDARs become permeable to Ca^{2+}, Na^{+} and K^{+}, as show in Fig. 1.13.

Since NMDARs could be formed by different combinations of different NR1, NR2 and/or NR3 subunits, it is probable that its function and modulation may be distinct depending on the arrangement of their subunits. For instance, the C-terminal extensions of NR2 subunits serve as domains for interaction with kinases, supporting signal transduction pathways in the neurons. In the case of

NR2A, these subunits interact with the Ser/Thr cyclin-dependent kinase-5 (Cdk-5) (Li *et al.* 2001), and both NR2A and NR2B subunits are phosphorylated by the Tyr kinases, Fyn and Src, previously described to potentiate the activity of the NMDARs (Yu *et al.* 1997).

Kumar and colleagues (Kumar *et al.* 2002) described that the expression of NMDAR subunits is tightly regulated during development. It was demonstrated that the NR2A/NR2B ratio increases during development, being NR2A and NR2B subunits highly expressed in the cortex and hippocampus of adult mammalian brain (Kumar *et al.* 2002). However, NR2B and also NR2D mRNAs were reported to be detected during the prenatal period, having higher expression at embryonic stages and then decreasing after birth, whereas NR2A and NR2C mRNAs were only detected near birth (Monyer *et al.* 1994).

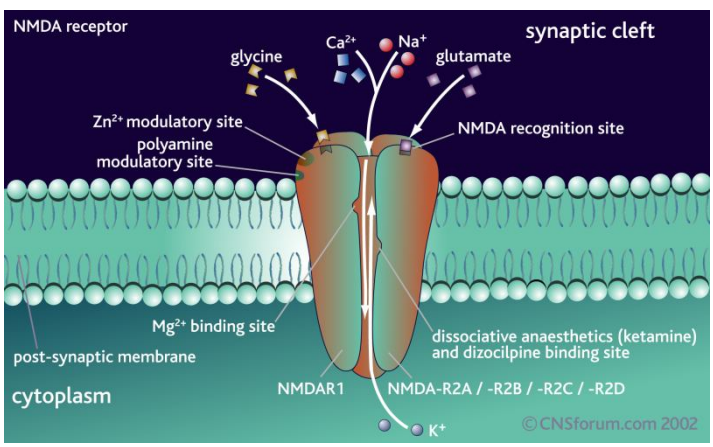


Figure 1.13 *NMDA receptors for glutamate.*
This ionotropic receptor, composed by 4 subunits, allows Ca^{2+}, Na^{+} and K^{+} exchange between cytoplasm and synaptic cleft. NMDARs have several alosterical modulatory sites and thus their activation is a very well regulated process. From: http://www.cnsforum.com/imagebank/item/hrl_rcpt_sys_NMDA/default.aspx

NMDARs have been implicated in the aetiology of several diseases. Since NMDARs function is involved in synaptic plasticity, a cellular mechanism for learning and memory, their role in AD ethiopathogenesis has been extensively studied (Lipton 2006). Glutamatergic synaptic loss has been correlated with the onset and severity of memory impairment that precedes late neurodegeneration (Myhrer 1998; Parameshwaran et al. 2008; Shankar & Walsh 2009). Reduced synaptic density and selective neuronal dysfunction that occurs in the disease seems to be partially due to the overactivation of NMDARs and consequent increase of intracellular Ca^{2+} (Mattson et al. 1992; Harkany et al. 2000; Kelly & Ferreira 2006; Fan & Raymond 2007). Although it was not possible to prove the physical interaction between Aβ and NMDARs, Aβ, including soluble oligomers, was shown to activate NMDARs (Cowburn et al. 1997; Dinamarca et al. 2008; Alberdi et al. 2010). Furthermore, Aβ is able to modify the sub-cellular localization of NMDAR subunits, namely of NR1 (Snyder et al. 2005; Lacor et al. 2007) in both cortical and hippocampal neurons, changing its cell surface expression levels (Goto et al. 2006; Johansson et al. 2006). In hippocampal neuronal cultures, NR1 subunit was detected in the AβO-pulled protein complex, together with many other proteins, suggesting its interaction with AβO (De Felice et al. 2007; Lacor et al. 2007) that was proved to be responsible for synapse damage and consequent neuronal loss (Lacor et al. 2004; reviewed in Klein 2006; Lacor et al. 2007). These damaging effects were suggested to be, in part, due to NADPH oxidase (NOX)-mediated ROS overproduction upon AβO-induced NMDARs activation (Shelat et al. 2008). Recently, it was demonstrated that NOX is the main source of superoxide radicals generation that occurs upon NMDAR activation (Brennan et al. 2009).

Despite the evidences suggesting an interaction between AβO and NR1 subunits of NMDARs, so far it has been difficult to determine which synaptic receptor could serve as a binding site for AβO since neither antibody competition or selective knock-out of receptors (e.g. PrP or metabotropic glutamate receptor 5 (mGluR5)) have fully eliminated AβO binding (Laurén J. *et al.* 2010; Renner *et al.* 2010). It was recently shown that excitatory synapses containing the NR2B NMDAR subunit, predominantly expressed at the pre-synaptic sites (Zhang & Diamond 2006), appear to be the principal sites of AβO accumulation, being this effect counteracted by NMDAR antagonists (Deshpande *et al.* 2009). Similar results were obtained by Hu and colleagues (Hu *et al.* 2009) in an *in vivo* study were $A\beta_{1\text{-}42}$-mediated inhibition of hippocampal plasticity was prevented byselective antagonists of NMDARs, including of the NR2B subunit.

Several pharmacological approaches targeting NMDARs have been made in order to develop effective disease-modifying therapies for AD. One of the current therapeutical approaches to slow disease progression in moderate-severe AD stages involves the use of memantine, an uncompetitive antagonist of NMDARs (reviewed in Lipton 2006; Herrmann *et al.* 2011). Memantine, which has been shown to improve cognitive function in AD patients (Reisberg *et al.* 2003; Rainer *et al.* 2011), is able to re-establish glutamatergic homeostasis (reviewed in Parsons et al., 2007; Robinson et al., 2006) and to prevent NMDAR-mediated Ca^{2+} influx and oxidative stress in AβO-treated hippocampal neuronal cultures (De Felice et al., 2007), as well as Aβ-induced synaptotoxicity (Lacor et al, 2007) and neurodegeneration (Miguel-Hidalgo et al., 2002). *In vivo*, memantine was also showed to reduce the neurodegenerative process in rat hippocampus (Szegedi et al., 2010), revealing

the therapeutical importance of blocking the NMDAR. Since activation of these receptors is associated with synaptic dysfunction preceding neurodegeneration (Kelly and Ferreira, 2006), it can play a major role during early disease stages, when AβO assumes a great importance.

CHAPTER 2 – Objectives

AD is a chronic and progressive neurodegenerative disorder that affects millions of individuals in the world and the number of persons with AD is expected to triple by mid-century. If steps are not taken to delay the onset or slow the progression of AD, the economic and personal tolls will be immense. Considerable progress has been made in recent years towards better understanding the pathogenesis of AD but this knowledge has not yet been successfully translated into new and truly effective disease-modifying drugs. Nevertheless, a wide array of anti-amyloid and neuroprotective therapeutic approaches are under investigation on the basis of the "amyloid cascade hypothesis", the leading mechanistic theory of AD which states that an imbalance in production or clearance of Aβ results in protein accumulation and triggers a cascade of events including oxidation, inflammation and excitotoxicity, tau hyperphosphorylation and neurofibrillary tangle formation, leading to neurodegeneration and dementia. Therefore, interventions in these processes might block the cascade of events comprising AD pathogenesis.

This study was aimed to better understand the molecular mechanisms underlying the deleterious effects triggered by Aβ peptide, focusing on its role in the induction of ER stress. Specifically, it was investigated: i) the role of the glutamatergic NMDAR) for glutamate, in particular of its NR2A and NR2B subunits, as an upstream mechanism leading to Aβ-induced ER stress and ii) mitochondrial dysfunctionas an ER stress downstream mechanism involved in Aβ-induced cell death. To accomplish these purposes, different models were used as depicted in Fig. 2.1.

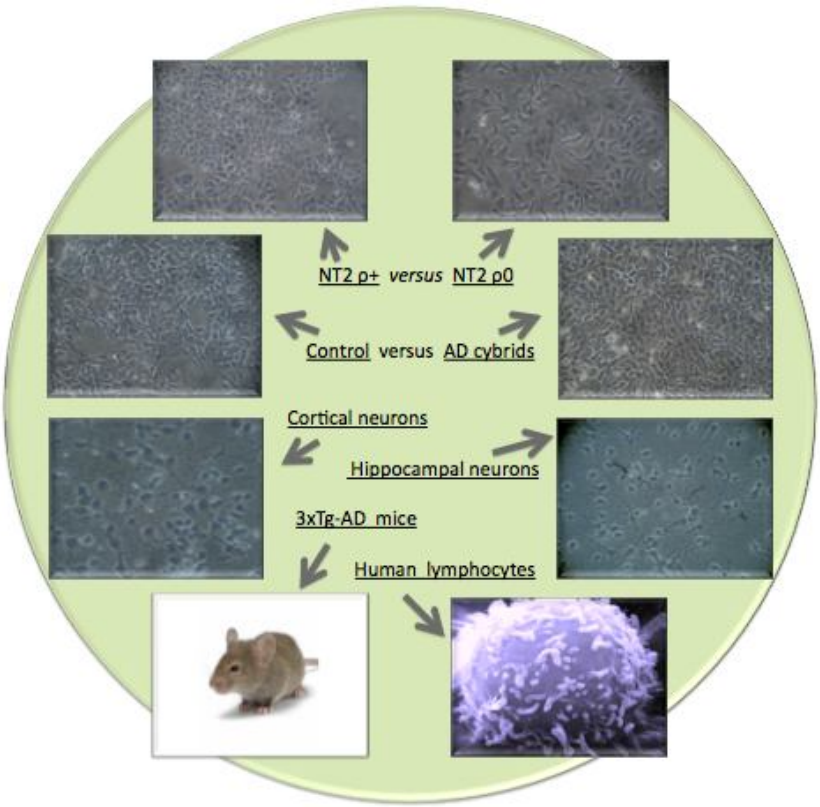


Figure 2.1 *Models used in this study to investigate the molecular mechanisms implicated in AD pathogenesis*.
As *in vitro* cellular models, Aβ-treated mtDNA-depleted NT2 ρ0 cells *versus* parental NT2 ρ+ cells, primary cultures of rat brain cortical or hippocampal neurons and AD *versus* control cybrids, were used (images were taken under 200x magnification). Furthermore, studies were conducted in brain extracts obtained from the cerebral cortex and hippocampus of triple transgenic AD mice (3xTg-AD) or wild-type littermates as well as in peripheral blood mononuclear cells (PBMCs) obtained from non-demented controls, subjects with mild cognitive impairment or mild, moderate/severe AD patients. The human lymphocyte representation is a scanning electron microscope (SEM) image.

The mtDNA-depleted NT2 cell line (NT2 ρ0 cells), characterized by the absence of functional mitochondria, allowed us to discriminate the role of mitochondria in the ER stress-induced apoptotic cell death pathway triggered

by Aβ, as described in Chapter 4. In Chapter 5, we took advantage of a technique that allowed recreating in a cell line (AD cybrids) the mitochondrial defect present in platelets from AD patients (inhibition of cytochrome c oxidase, COX) to explore how mitochondrial dysfunction affects the cellular susceptibility to ER stress induced in response to Aβ stimulus. The effect of impaired COX activity on Aβ-induced ER Ca^{2+} dyshomeostasis was further investigated in Chapter 6 using primary cultures of rat brain cortical neurons challenged with Aβ upon treatment with KCN, a chemical COX inhibitor. In Chapter 7, using primary cultured rat brain hippocampal neurons, it was investigated whether oligomeric AβO triggers ER stress by an NMDAR-dependent mechanism and the contribution of NR2A and NR2B subunits was analyzed using antagonists of this glutamate receptor. Furthermore, the NOX-mediated superoxide production downstream of NR2A or NR2B and its role in ER and cytosolic Ca^{2+} dyshomeostasis was analyzed in AβO-treated hippocampal cultures.

Finally, the Chapter 8 was aimed to evaluate the extension of ER stress in an *in vivo* model of AD, the triple transgenic-AD mice (3xTg-AD) mice model, which develops plaques, tangles and cognitive deficits in an age-dependent manner and thus mimics AD progression in humans. Also in this chapter, ER Ca^{2+} deregulation and several ER stress markers were accessed in peripheral blood mononuclear cells (PBMCs) from non-demented controls, individuals with MCI and probable AD patients (mild and moderate/severe disease stages) in an attempt to establish a relation between ER dysfunction and the severity of the disease.

CHAPTER 3 - Materials and Methods

3.1. Materials.

Table 3.1.1. Information relative to the companies from which the materials and equipment, used in this study were purchased.

Antibodies:	
Alexa Fluor 488 goat anti-rabbit IgG conjugate	Molecular Probes (Leiden, Netherlands)
Alexa Fluor 594 goat anti-mouse IgG conjugate	Molecular Probes (Leiden, Netherlands)
Alexa Fluor 633 goat anti-rabbit IgG conjugate	Molecular Probes (Leiden, Netherlands)
Goat alkaline phosphatase-linked anti-mouse secundary antibody	Amersham Pharmacia Biotech (Buckinghamshire, UK)
Goat alkaline phosphatase-linked anti-rabbit secundary antibody	Amersham Pharmacia Biotech (Buckinghamshire, UK)
Mouse monoclonal anti-β-actin	Biovision inc. (mountain view, CA, USA)
Mouse monoclonal anti-GADD153/CHOP	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Mouse monoclonal anti-GAPDH	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Mouse monoclonal anti-GRP78	BD Biosciences (San Diego, CA, USA)
Mouse monoclonal anti-NR2B subunit	BD Biosciences (San Diego, CA, USA)
Mouse monoclonal anti-PARP	Cell Signaling (Denvers, MA, USA)
NU-1 (Northwestern University-1) (anti-AβO)	Produced in W.L Klein's Lab (Chicago, Illinois, USA)
Rabbit polyclonal anti-NR1 subunit (anti-ct-terminal)	Chemicon International, Inc. (Temecula, CA, USA)
Rabbit polyclonal anti-NR1 subunit (NMDAζ1)	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Rabbit polyclonal anti-NR2A subunit (anti-ct-terminal)	Chemicon International, Inc. (Temecula, CA, USA)
Rabbit polyclonal anti-NR2A subunit (NMDAε1)	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Rabbit polyclonal anti-NR2B subunit	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Rabbit polyclonal anti-NR2B subunit (anti-ct-terminal)	Chemicon International, Inc. (Temecula, CA, USA)
Rabbit polyclonal anti-pro-caspase 12	BD Biosciences (San Diego, CA, USA)
Rabbit polyclonal anti-XBP-1	Abcam (Cambridge, UK)
Aβ peptides:	
$A\beta_{1-40}$	Bachem (Bubendorf, Switzerland)
$A\beta_{1-42}$	Bachem (Bubendorf, Switzerland) American peptide (Sunnyvale, CA,USA)

Cellular lines:

Human teratocarcinoma NT2 cell line (NT2 ρ+)	Statagene (La Jolla, CA, USA)
mtDNA-depleted NT2 cell line (NT2 ρ0)	A generous gift from Professor R. Swerdlow (University of Kansas, KS, USA)

Cell culture reagents:

B27 supplement	GIBCO BRL, Life Technologies (Paisley, UK)
Fatty acid-free BSA	Sigma Chemical Co. (St. Louis, MO, USA)
Bovine serum albumin (BSA)	Sigma Chemical Co. (St. Louis, MO, USA)
Neurobasal medium	GIBCO BRL, Life Technologies (Paisley, UK)
Optimem Medium	GIBCO BRL, Life Technologies (Paisley, UK)
Penicillin	GIBCO BRL, Life Technologies (Paisley, UK)
Streptomycin	GIBCO BRL, Life Technologies (Paisley, UK)

Enzymes:

Deoxiribonuclease I (DNase I)	Sigma Chemical Co. (St. Louis, MO, USA)
Trypsin	Sigma Chemical Co. (St. Louis, MO, USA)

Enzyme inhibitors:

Phenylmethylsulfonyl (PMSF)	Sigma Chemical Co. (St. Louis, MO, USA)
Protease inhibitor cocktail (leupeptin, pepstatin A, chymostatin and antipain)	Sigma Chemical Co. (St. Louis, MO, USA)
Trypsin inhibitor type type-II-soybean	Sigma Chemical Co. (St. Louis, MO, USA)

Enzyme substrates:

ac-DEVD-pNA (Caspase-3)	Calbiochem (Damastadt, Germany)
ac-LEVD-pNA (Caspase-4)	MBL International CO. (Wobum, MA, USA)
ac-LEDH-pNA (Caspase-9)	Biosource international (Nivelles, Belgium)

Fluorescent dyes:

2´,7´-Dichlorodihydrofluorescein diacetate ($DCFH_2$-DA)	Molecular Probes (Leiden, Netherlands)
Dihydroethidium (DHE)	Molecular Probes (Leiden, Netherlands)
Fura-2-acetoxymethyl Ester (Fura-2/AM)	Molecular Probes (Leiden, Netherlands)
Hoechst 33342	Molecular Probes (Leiden, Netherlands)
in situ Cell Death Detection Kit, Fluorescein	Roche Applied Sciences (Manhein, Germany)

Indo-1-acetoxymethyl ester (Indo-1/AM)	Molecular Probes (Leiden, Netherlands)
9,9′-Bis (N-methylacridinium nitrate), Lucigenin	Sigma Chemical Co. (St. Louis, MO, USA)

Laboratory equipments:

Axiovert 200 fluorescence inverted microscope	Zeiss (Jena, Germany)
Axiovert 200M fluorescence microscope	Zeiss (Thornwood, NY, USA)
Coll SNAP digital camera	Roper Scientific (Trenton, NJ, USA)
Lambda DG4 apparatus	Sutter Instruments company (Nocato, CA, USA
Leica TCS SP2 Laser confocal	Leica Microsystems Inc. (Buffalo Grove, Illinois, USA)
Lmax II 384 microplate reader	Molecular Devices Inc. (Sunnyvale, CA, USA)
SpectraMax Gemini microplate reader	Molecular Devices Inc. (Sunnyvale, CA, USA)
Spectra Max Plus 384 microplate reader	Molecular Devices Inc. (Sunnyvale, CA, USA)

Other chemicals:

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma Chemical Co. (St. Louis, MO, USA)
β-nicotinamide adenine dinucluotide phosphate reduced form (ß-NADPH)	Sigma Chemical Co. (St. Louis, MO, USA)
BCA Protein Assay Reagent	Thermo Scientific, Pierce protein research products (Rockford, IL, USA)
Brefeldin A	Sigma Chemical Co. (St. Louis, MO, USA)
Cycloheximide	Sigma Chemical Co. (St. Louis, MO, USA)
DakoCytomation fluorescent solution	DakoCytomation (Carpinteria, CA, USA)
Dantrolene	Sigma Chemical Co. (St. Louis, MO, USA)
Ficoll-Plaque Plus separating solution	GE healthcare bio-Sciences (Pittsburgh, PA, USA)
Ifenprodil	Sigma Chemical Co. (St. Louis, MO, USA)
NVP-(R)-[(S)-1-(4 Bromophenyl)-ethylamino]-(2,3 dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic Acid	A generous gift from Y.P. Auberson (Novartis Pharma AG, Basel, Switzerland)
Phenol red-free Ham's F-12 medium	Cambrex Bio Science (Walkersville, Maryland, USA)
Pluronic acid F-127	Molecular Probes (Leinden, Netherlands)
Prolong mounting medium	Molecular Probes (Leinden, Netherlands)
Thapsigargin	Sigma Chemical Co. (St. Louis, MO, USA)

Software used

GraphPad Prism	GraphPad Software, Inc. (La Jolla, CA, USA)
ImageJ	National Intitutes of Health (Bethesda, Maryland, USA)
MetaFluor	Molecular Devices Inc. (Sunnyvale, CA, USA)
Metamorph	Universal Imaging Corporation (Buckinghamshire, UK)

Western blot reagents:

Enhanced chemiluminescence (ECF) reagent	Amersham Pharmacia Biotech (Buckinghamshire, UK)
Low-Range Rainbow prestained protein standard	Amersham biosciences (Piscataway, NJ, USA
Other reagents and apparatus	Bio-rad (Hercules, CA, USA)
Polyvinylidene difluride (PVDF) membrane	Amersham Pharmacia Biotech (Buckinghamshire, UK)

Table 3.1.2. Information regarding the provinience, host species, molecular weight and dilutions of the primary antibodies used in this study.

Antibodies	Catalog number	Host specie	Molecular weight (kDa)	Dilution*
Anti-AβO (NU-1, Northwestern University-1)	—	Mouse monoclonal	Aβ oligomers (different sizes)	1:1000 (ICC)
Anti-GADD153/CHOP	SC-7351	Mouse monoclonal	30 kDa	1:500 (WB) or 1:200 (ICC)
Anti- GAPDH	SC-137179	Mouse monoclonal	37 kDa	1:2500 (WB)
Anti-GRP78	BD610979	Mouse monoclonal	78 kDa	1:250 (WB)
Anti-NR1 subunit (anti-ct-terminal)	Milipore 05432	Rabbit polyclonal	130 kDa	5 µg/mL (ICC)
Anti-NR1 subunit (NMDAζ1)	SC- 101757	Rabbit polyclonal	115 kDa	5 µg/mL (ICC)
Anti-NR2A subunit (anti-ct-terminal)	Milipore AB10531	Rabbit polyclonal	177 kDa	5 µg/mL (ICC)
Anti-NR2A subunit (NMDAε1)	SC 31539	Rabbit polyclonal	177 kDa	5 µg/mL (ICC)
Anti-NR2B subunit	SC 9057	Rabbit polyclonal	180 kDa	5 µg/mL (ICC)
Anti-NR2B subunit (anti-ct-terminal)	Milipore 06-600	Rabbit polyclonal	166 kDa	5 µg/mL (ICC)

Anti-PARP	Cell signalling 9546	Mouse monoclonal	89 kDa	1:1000 (WB)
Anti-pro-caspase 12	BD551430	Rabbit polyclonal	55 kDa	1:1000 (WB)
Anti-XBP-1	Abcam 37151	Rabbit polyclonal	54 kDa	1:1000 (WB)

*** WB, western blot; ICC, immunocytochemistry**

3.2. Experimental models and treatments.

3.2.1. Human teratocarcinoma NT2 cell lines (ρ+ and ρ0).

Human teratocarcinoma NT2 cell line were grown in 75 cm^2 tissue flasks in modified Eagles Minimum Essential Medium (OptiMEM) containing 10% (v/v) heat inactivated fetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 μg/mL). The mtDNA-depleted NT2 cell line (designed ρ0), obtained after treatment of parental cells (designed ρ+) with low concentrations of ethidium bromide (Swerdlow *et al.* 1997), was grown in OptiMEM containing 10% (v/v) heat inactivated FCS, penicillin (50 U/mL) and streptomycin (50 μg/mL) and further supplemented with uridine (150 μg/mL) and pyruvate (200 μg/mL). Both cell lines (NT2 ρ+ and ρ0 cells) were maintained at 37 ºC in a humidified incubator under an atmosphere of 95% air and 5% CO_2. Regular control measurements of mitochondrial cytochrome c oxidase (complex IV (COX)) activity were performed to guarantee the inhibition of ETC in mtDNA-depleted NT2 ρ0 cells.

Cells were plated at a density of 0.067 x 10^6 cells/cm^2 for WB and measurement of caspase-3, caspase-4, and caspase-9-like activities, or at a density of 0.12 x 10^6/cm^2 on glass coverslips, for single-cell Ca^{2+} imaging or TUNEL assay, and were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. 24 hours after plating, cells were treated with brefeldin A (2 μM), thapsigargin (2 μM) or fibrillar $A\beta_{1-40}$ (1 μM) for 3, 6 or 24 hours at 37 ºC. When used, dantrolene (10 μM) was added 1 hour before $A\beta_{1-40}$ treatment. Untreated cells were used as control.

3.2.2. Control and Alzheimer's cybrid cell lines.

3.2.2.1. Subjects.

Study sample included 3 patients (mean age was 65.7 ± 8.3), with probable AD, evaluated at the Memory Clinic of the University Hospital of Coimbra. The diagnosis of dementia was based on the guidelines of the Diagnostic and Statistical Manual of Mental Disorders-DSM-IV (American Psychiatric Association, 1994) and the diagnosis of probable AD was established according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann *et al.* 1984). In order to implement these standard criteria, all patients underwent extensive clinical evaluations including neurological, psychiatric and neuropsychological testing, routine laboratory tests to exclude other causes of dementia, as well as EEG, brain imaging, cerebrospinal fluid (CSF) analysis and ApoE genotyping. This evaluation confirmed the probable diagnosis of AD and allowed further genetic classification. One patient was homozygotic for the ApoE ε4 allele and another one for the ApoE ε3 allele. The third patient was heterozygotic with a ε4/3 genotype.

Patients were followed during several years with periodic clinical evaluations including specific staging scales, which provide objective information about the evolution of the disease and the severity of dementia in various domains. When the study was performed, one patient had a severe dementia and two subjects were staged as moderate dementia at a global level, in accordance with the Clinical Dementia Rating (CDR) (Berg 1988). Cognitive impairment was also quantified using the Minimental-State Evaluation (MMSE) (Folstein *et al.* 1975)

and the mean score of the patient group was 7.7 ± 5.4 (range 2-12). Healthy controls (mean age was 64.3 ± 8.4) had no subjective or objective evidence of cognitive impairment. This study was approved by the Ethics Commitee of the "Hospitais da Universidade de Coimbra" and subject's participation was considered after informed consent.

3.2.2.2. Preparation of cybrid cells.

AD and control cytoplasmic hybrids, or cybrids, result from the fusion of mtDNA-depleted NT2 ρ0 cells with platelets isolated from peripheral blood samples obtained from AD patients or aged-matched control subjects, as previously described (Swerdlow *et al.* 1997; Cardoso *et al.* 2004b). Each sample of blood led to the creation of one cell line of a total of 6 cell lines.

Since NT2 ρ0 cells have no detectable complex I or COX activity they are autotrophic for pyruvate and therefore need to be grown in culture medium supplemented with uridine and pyruvate (Miller *et al.* 1996; Swerdlow *et al.* 1997). After fusion with platelets, the resultant cybrid cells regain mitochondrial activity associated to the mitochondria present in AD or control platelets. This feature allowed the selection of untransformed cells through the withdrawal of uridine and pyruvate supplements from the culture medium. Therefore, cells were grown in OptiMEM containing 10% (v/v) heat inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). Both AD and control cybrids were maintained at 37 ºC in a humidified incubator under an atmosphere of 95% air and 5% CO_2. Regular control measurements of mitochondrial COX activity were performed to confirm the maintenance of the

ETC defect in AD cybrids according to the method of Warton and Tzagoloff (Warton & Tzagoloff 1967).

Cells were plated on dishes and on glass coverslips that have no cell adhesion material at a density of 0.067 x 10^6 cells/cm^2 for WB and measurement of caspase-like activity or at 0.017 x 10^6 cells/cm^2 for the MTT and TUNEL assays. Tweenty-four hours after plating, cells were treated with brefeldin A (2 μM), thapsigargin (2 μM) or fibrillar $A\beta_{1–40}$ (1 μM) for 3, 6 or 24 hours. When used, dantrolene (10 μM) was added 1 hour before $A\beta_{1-40}$ treatment. Untreated cells were used as control.

3.2.3. Primary cultures of rat brain neurons.

3.2.3.1. Primary cultured cortical neurons.

Cortical neuronal cultures we prepared from E15-E16 Wistar rat embryos according to the method described by Hertz and collaborators (Hertz *et al.* 1989) slightly modified (Agostinho & Oliveira 2003). Briefly, removed cortices were aseptically dissected and washed in Ca^{2+}- and Mg^{2+}-free Krebs (in mM): 120 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 13 glucose, 10 HEPES (pH 7.4), and supplemented with 0.001% (v/v) phenol red. Cortices were then incubated in Krebs solution supplemented with BSA (0.3 g/L), containing trypsin (0.5 g/L) and DNAse I (0.04 g/L), for 7 min at 37 ºC. Tissue digestion was stopped by addition of trypsin inhibitor (type II-S) (0.75 g/L) in Krebs buffer containing DNAse I (0.04 g/L), followed by a centrigugation at 140 x *g* for 5 min. After washing the pellet once with Krebs buffer, cells were dissociated mechanically and ressuspended in fresh Neurobasal Medium with 2 mM L-

glutamine, 2% (v/v) B27 supplement, 100 U/mL penicillin and 100 U/mL streptomycin.

Neurons were plated on poly-L-lysine (0.1 g/L)-coated multiwells or glass coverslips at the following density: 0.125 x 10^6 cells/cm^2 for the MTT assay and measurement of cytosolic calcium levels; 0.1 x 10^6 cells/cm^2 for single-cell Ca^{2+} imaging; 0.25 x 10^6 cells/cm^2 for determination of caspase-like activity. The cultures were maintained in a serum-free Neurobasal Medium supplemented with B27, at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Under these conditions, glial growth is less than 10% (Ferreiro et al., 2006). Cortical neurons differentiated for 5-7 days were treated at 37 ºC with fibrillar $A\beta_{1-40}$ (1 μM), prepared as described bellow from synthetic $A\beta_{1-40}$, in the absence or presence of KCN (1 mM) a complex IV inhibitor of the ETC, during 3, 6 or 24 hours. When used, KCN was added 1 hour before $A\beta_{1-40}$ treatment. As a positive control for ER stress, neurons were also treated with the SERCA-ATPase inhibitor thapsigargin (2 μM) for 3, 6 or 24 hours. Untreated cells were used as control.

3.2.3.2. Primary cultured hippocampal neurons.

Hippocampal cultures were prepared from hippocampi of E18-E19 Wistar rat embryos (Ambrosio *et al.* 2000). Briefly, the hippocampi were dissected under magnifying glass observation and collected in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS, in mM): 137 NaCl, 5.36 KCl, 0.44 KH_2PO_4, 0.34 $Na_2HPO_4 \cdot 2H_2O$, 4.16 $NaHCO_3$, 5 glucose, 1 sodium piruvate, 10 HEPES (pH 7.4), supplemented with 0.001% (v/v) phenol red. Tissues were then treated with 0.035% trypsin (0.4 mg/mL) for 5 min at 37 ºC, and further

washed with type II-S trypsin inhibitor (1.5 mg/mL in HBSS). Hippocampi were rapidly rinsed with HBSS and then mechanically dissociated in Neurobasal Medium supplemented with B27, 25 μM glutamate, 0.5 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin.

Neurons were plated on poly-L-lysine (0.1 g/L)-coated multiwells or glass coverslips at a density of 0.045 x 10^6 cells/cm^2 for WB, 0.075 x 10^6 cells/cm^2 for the MTT assay or at a density of 0.025 x 10^6 cells/cm^2 for single-cell Ca^{2+} imaging or immunocytochemistry. The cultures were maintained in a serum-free Neurobasal Medium supplemented with B27, at 37 °C in a humidified atmosphere containing 95% air and 5% CO2. Once a week, half medium was changed with fresh medium without added glutamate. Hippocampal cells differentiated for 17-21 days were treated with AβO (0.5 μM, prepared as described bellow from $Aβ_{1-42}$) in the absence or in the presence of the antagonists of NR2A or NR2B subunits of NMDAR NVP-[(R)-[(S)-1-(4-bromophenyl)-ethylamino]-(2,3-dioxo-1,2,3,4 tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) (Auberson *et al.* 2002) or ifenprodil at 37 ºC during 3, 6 or 24 hours. When used, NVP-AAM077 (50 nM), and ifenprodil (10 μM) were added 15 min before AβO treatment. As a positive control for ER stress, neurons were also treated with thapsigargin (2 μM) for 3, 6 or 24 hours. Untreated cells were used as control.

Pregnant female Wistar rats used to prepare primary cultures of cortical or hippocampal neurons were obtained from our local colony (Faculty of Medicine, Univ. Coimbra). Animals were kept under controlled light and humidity conditions, being anesthesized under halothane atmosphere before sacrifice by cervical displacement and decapitation (FELASA guidelines).

3.2.4. Human peripheral blood mononuclear cells.

Peripheral blood mononuclear cells (PBMCs) were obtained from 66 to 90 years-old patients diagnosed with Mild Cognitive Impairment (MCI) (n=16) or probable AD in different stages: mild AD (n=15) or moderate-severe AD (n=13). Age-matched control subjects were neurologically healthy and the mean age was 78 ± 9 (n=9). All individuals were evaluated at the Memory Clinic of the "Hospitais da Universidade de Coimbra", as described above in section 3.2.2.1.

Briefly, peripheral blood (20 mL) was withdrawn from the antecubital vein using heparin as anticoagulant. Collected blood was carefully loaded on 6 mL Ficoll-Plaque Plus separating solution, avoid mixing of blood and separation fluid, and centrifuged for 20 min at 1260 x *g* at 18 ºC in a swing-out rotor, without brake. After centrifugation, PBMCs form a distinct band at the sample/medium interface, which was collected without removing the upper layer (serum). Cells were then washed with PBS, ressuspended in Krebs medium (with Ca^{2+} and Mg^{2+}) and counted using a hemocytometer. Free-floating PBMCs were seeded on dishes that have no cell adhesion material at a density of $1x10^6$ cells/mL and were then processed to measure cytosolic *versus* ER Ca^{2+} levels. The remaining cells were lysed in RIPA buffer containing 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholic acid (DOC) and 0.1% (w/v) SDS, supplemented with 1 mM DTT, 1 mM PMSF, 1:1000 protease inhibitor cocktail (1 µg/mL leupeptin, pepstatin A, chymostatin and antipain), 2 mM sodium orthovanadate, 50 mM sodium fluoride, and stored at -80 ºC to posterior WB analysis.

3.2.5. Triple transgenic mice model (3xTg-AD).

The triple transgenic mice (3xTg-AD) were obtained through the micro-injection of human APP cDNA harboring the swedish mutation (KM670/671NL) and human four-repeat tau harboring the P301L mutation into single cell embryos of homozygous PS1m146v knock-in mice (Oddo *et al.* 2003a; Oddo *et al.* 2003b), originally generated as a hybrid 129/C57BL6 background (Guo *et al.* 1997). 3xTg-AD and non-transgenic (non-Tg) mice were obtained from Prof. LaFerla (Univ. California, Irvine, USA) and were breeded and maintained in our local colony. Animals were housed under a constant temperature, humidity and a 12 h light/dark cycle in a pathogen-free environment and all studies were performed according to the principles and procedures outlined in the EU guidelines (86/609/EEC). Brain cortices and hippocampi isolated from male and female 3-, 6- and 12-month-old 3xTg-AD mice (and age- and gender-matched non-Tg mice) were frozen and stored at -80ºC before being homogenized in RIPA buffer supplemented with 100 µM PMSF, 2 mM DTT, 2 mM sodium orthovanadate, 50 mM sodium fluoride and 1:1000 protease inhibitor cocktail (1 µg/mL leupeptin, pepstatin A, chymostatin and antipain) and processed for WB analysis. Protein content of brain homogenates was determined using the BCA Protein Assay Reagent.

Table 3.4. Summary of cell treatments performed in the different experimental protocols.

Treatments	Function	Abrev./Conc.	Incubation period
Amyloid-beta 1-42 oligomers	Central nervous system neurotoxins involved in AD.	AβO (0.5 μM)	30 min, 2, 3 or 6 hours.
Amyloid-beta 1-40 fibrils	Main component of neuritic plaques observed in AD patient brains.	$A\beta_{1-40}$ (1 μM)	3, 6 or 24 hours.
NVP-*(R)-[(S)*-1-(4 Bromophenyl)-ethylamino]-(2,3 dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid	Antagonist of NMDA-NR2A subunit.	NVP (50 nM)	15 min previous to AβO treatment, and then maintained for 3 or 6 hours.
Apocynin	Inhibitor of NADPH oxidase.	Apoc. (1 mM)	15 min previous to AβO treatment, and then maintained for 3 or 6 hours.
Brefeldin A	ER stress inducer (blocker of retrograde Golgi-derived vesicles inducing protein accumulation in ER lumen). Positive control for ER stress.	Bref. A (2 μM)	3, 6 or 24 hours.
Dantrolene	Inhibitor of Ca^{2+} channels coupled to the ER ryanodine receptor	Dant. (10 μM)	1 hour previous to $A\beta_{1-40}$ treatment, and then maintained for 24 hours.
Ifenprodil	Antagonist of NMDA-NR2B subunit.	Ifen. (10 μM)	15 min previous to AβO treatment, and then maintained for 3 or 6 hours.
KCN	Inhbitor of mitochondrial ETC (acts at complex IV)	KCN (1 mM)	1 hour previous to $A\beta_{1-40}$ treatment, and then maintained for 3, 6 or 24 hours.
Thapsigargin	ER stress inducer, (SERCA Ca^{2+} ATPase inhibitor). Positive control for ER stress.	Thap. (2 μM) 2.5 μM were used in SCCI experiments to deplete ER Ca^{2+})	3, 6 or 24hours.

3.3. Methods.

3.3.1. Preparation of $A\beta_{1-40}$ fibrils and $A\beta_{1-42}$ oligomers.

Synthetic $A\beta_{1-40}$ peptide was reconstituted according to the manufacturer's instructions. $A\beta_{1-40}$ was dissolved in sterile distilled water at a concentration of 6 mg/mL, diluted to 1 mg/mL (231.5 μM) with phosphate saline buffer (PBS) and was then "aged" for 7 days at 37 ºC. Under these conditions, $A\beta_{1-40}$ fibrils are the main Aβ species formed (O'Nuallain *et al.* 2005).

AβO were prepared from $A\beta_{1-42}$ according to the procedure first described by Lambert and colleagues (Lambert *et al.* 1998), with some modifications (Resende *et al.* 2008b). Synthetic $A\beta_{1-42}$ was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to 1 mM. HFIP was then removed in a Speed Vac and the dried HFIP film was stored at -20 ºC. The peptide film was then ressuspended to obtain a 5 mM solution in anhydrous dimethyl sulfoxide (DMSO) (Dahlgren *et al.* 2002). AβO were prepared by diluting the solution in phenol red-free Ham's F-12 medium without glutamine to a 100 μM final concentration and incubated overnight at 4 ºC (Lambert *et al.* 1998). The preparation was centrifuged at 15000 x *g* for 10 min at 4 ºC to remove insoluble aggregates, and the supernatant containing soluble oligomers was transferred to clean tubes and stored at 4 ºC. Concentration of oligomers was determined using the Bio-Rad protein assay reagent. As a protein quality control procedure to ensure the presence of soluble oligomers rather than fibrils, AβO preparations containing 10 μg of protein were diluted (1:1) with sample buffer: 40% (v/v) glycerol, 2% (w/v) SDS, 0.2 M Tris-HCl, pH 6.8 and Comassie G-250, and then separated by electrophoresis on a 4-16% Tris-Tricine SDS gel (Klafki *et al.* 1996), stained with Comassie Brilliant Blue and

identified accordingly to their molecular weight. Samples were not boiled to minimize disaggregation prior to electrophoresis (Fig. 3.1).

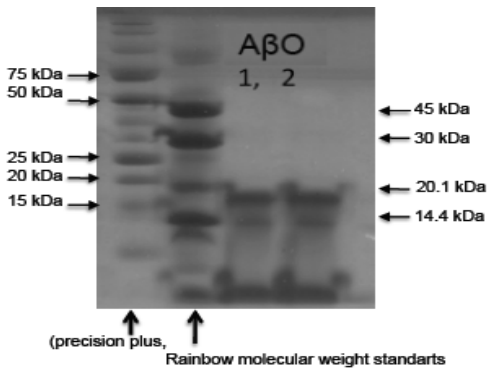


Figure 3.1 *Characterization of AβO preparations by SDS-page*.
Data analysis was performed based on the molecular weight using two protein standards (pre-stained Low-Range Rainbow and precision plus protein standard). Results show the enrichment of the AβO preparation in soluble oligomers and the absence of fibrils. Experiment was performed in duplicate (lane 1 and 2).

3.3.2. Analysis of endoplasmic reticulum stress markers by western blotting.

Total cell extracts were prepared from treated or untreated cells that were lysed in a buffer containing (in mM): 25 HEPES-Na, 2 $MgCl_2$, 1 EDTA, 1 EGTA, supplemented with 100 µM PMSF, 2 mM DTT and a protease inhibitor cocktail (1 µg/mL leupeptin, pepstatin A, chymostatin and antipain). The cellular suspension was rapidly frozen/defrosted three times in liquid N_2, and centrifuged at 14,000 x g, during 5 min. Protein concentration in the supernatant was measured using the Bio-Rad protein assay reagent. Samples were denaturated at 95 ºC for 5 min in a 6 times concentrated sample buffer:

500 mM Tris, 600 mM DTT, 10% (w/v) SDS, 30% (v/v) glycerol and 0.012% (w/v) bromophenol blue. Samples were then processed for WB analysis.

Equal amounts of each protein sample prepared as described above were separated by electrophoresis on a 10% SDS-polyacrylamide gels (SDS-PAGE) or 7% SDS-PAGE gel and electroblotted onto PVDF membranes. The identification of proteins of interest was facilitated by the usage of a prestained precision protein standard, which was run simultaneously. After the proteins were electrophoretically transferred, the membranes were blocked for 1 hour at room temperature (RT) in Tris-buffer composed of 150 mM NaCl, 25 mM Tris-HCl (pH 7.6) and 0.1% (v/v) Tween 20 (TBS-T) containing 5% (w/v) non-fat dry milk to eliminate nonspecific binding. Membranes were next incubated overnight at 4 ºC in TBS-T containing 5% (w/v) non-fat dry milk with one of the following antibodies: mouse monoclonal primary antibody anti-GRP78, mouse monoclonal anti-GADD153/CHOP, mouse monoclonal anti-PARP, rabbit polyclonal primary antibody anti-XBP-1, rabbit polyclonal primary antibody anti-pro-caspase-12 (1:1000). Mouse monoclonal primary antibody anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2500) or rabbit polyclonal anti-α-tubulin antibody (1:20000) were used as protein loading control.

Membranes were washed several times and then incubated in TBS-T with 1% (w/v) non-fat dry milk for 2 hours at RT with alkaline phosphatase-conjugated anti-mouse or anti-rabbit secondary antibody. Immunoreactive bands were detected on a Bio-Rad Versa Doc 3000 Imaging System after incubation of membranes with ECF reagent for 5-10 min. Antibody dilutions are presented in the Table 3.1.2.

3.3.3. Binding of Aβ oligomers and antibody competition assay.

Hippocampal cells were incubated for 15 min with either 0.5 µM AβO, prepared according to (Lambert *et al.* 1998; Lacor *et al.* 2004), or the same amount of vehicle solution added directly into the culture medium. Cells were rinsed twice with Neurobasal Medium supplemented with B27 and fixed for immunocytochemistry experiments with 3.7% (w/v) formaldehyde in Neurobasal Medium (1:1 volume) for 10 min followed by an additional 10 min incubation with undiluted fixative, as described previously (Lacor *et al.* 2004; Lacor *et al.* 2007). Fixed cells were blocked with 10% (v/v) normal goat serum in PBS for 45 min followed by incubation during 4 hours with NU-1 antibody to identify bound AβO (Lambert *et al.* 2007). Detection was obtained using an Alexa-488 conjugated anti-mouse secondary antibody for 1 hour. Preparations were mounted with Prolong mounting medium and imaged using a Leica TCS SP2 Laser confocal microscope. Blocking of AβO hot-spot binding was performed by incubating neurons for 15 min with 5 µg/mL antibodies directed to extracellular epitopes (nt) of NR1 (NMDAζ1), NR2A (NMDAε1) or NR2B subunits of NMDAR prior to AβO treatment for further 15 min. Controls included antibodies directed against the intracellular epitopes (ct) of these receptor subunits or antibodies pre-adsorbed with the control peptide antigen. Analysis and quantification of AβO hot-spots was made using metamorph software.

3.3.4. Measurement of Ca^{2+} levels.

3.3.4.1. Cytosolic Ca^{2+} levels.

The concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) was measured using the acetoxymethyl ester of Indo-1 (Indo-1/AM), an intracellular Ca^{2+} indicator that is ratiometric and UV light excitable. The presence of an acetoxymethyl ester group (AM) confers membrane permeability to Indo-1. Once in the cytosol, this group is cleaved by non-specific esterases trapping the probe inside the cell. The emission of Indo-1 shifts from 475 nm without Ca^{2+} to 400 nm with Ca^{2+} when excitation is performed at 350 nm.

Treated or untreated cells were rinsed once with PBS (at 37 ºC to avoid thermic shock) and incubated with Indo-1/AM (3 μM) for 45 min at 37 ºC, in the dark, in Krebs medium (in mM): 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 6 Glucose, 10 HEPES-Na and 1 $CaCl_2$, pH 7.4). Cells were further incubated in Indo-1/AM-free Krebs medium for 15 min to ensure the complete hydrolysis of the dye. The Indo-1 fluorescence was measured fluorimetrically using a SpectraMax Gemini EM microplate reader at 350 nm excitation and 410 nm emission. Calibration of cytosolic Ca^{2+} levels was performed using the Ca^{2+} ionophore ionomycin (3 μM) and $MnCl_2$ (3 mM) that were added at the end of each measurement. The free $[Ca^{2+}]_i$ was calculated as previously described by Bandeira-Duarte and colleagues (Bandeira-Duarte *et al.* 1990).

3.3.4.2. Endoplasmic reticulum Ca^{2+} content.

Ca^{2+} release from the ER was evaluated by single cell Ca^{2+} imaging using the acetoxymethyl ester of the fluorescent probe Fura-2/AM, as previously described (Ferreiro *et al.* 2008b) with some minor modifications. Fura-2/AM is a high affinity Ca^{2+} indicator that is ratiometric and UV light excitable. Due to the presence of the acetoxymethyl ester group (AM), Indo-1 crosses the plasma membrane. This group is cleaved within the cytosol by non-specific esterases trapping the probe inside the cell. Upon calcium binding, the fluorescent excitation maximum of the indicator undergoes a blue shift from 380 nm (Ca^{2+}-free) to 340 nm (Ca^{2+}-saturated), while the fluorescence emission maximum is relatively unchanged at ~510 nm. At low concentrations, the alternate excitation at 340 and 380 nm allows precise measurements of the intracellular Ca^{2+} levels, represented as F340/F380 ratio.

Treated and untreated cells, plated in glass coverslips, were washed 2 times in Krebs medium suplemented with 10 mM $NaHCO_3$, 0.05 mM EGTA and 0.1% (w/v) fatty acid-free BSA, pH 7.4. Then, cells were loaded with Fura-2/AM (5 μM) in Krebs medium supplemented with 0.2% (w/v) pluronic acid for 40 min, at 37 ºC in the dark. Afterwards, cells were washed 3 times in Ca^{2+}-free Krebs medium and the coverslip was assembled to perfusion chamber, in the same Ca^{2+}-free medium, in an inverted fluorescence microscope Axiovert 200. Cells were alternately excited at 340 and 380 nm using a Lambda DG4 apparatus, and emitted fluorescence at 510 was collected with a 40x objective and was driven to a coll SNAP digital camera. Acquired values were processed using the MetaFluor software. Experiments were run for 8 or 12 min, and complete ER Ca^{2+} depletion was accomplished by the addition of thapsigargin

(2.5 μM) after a baseline was established (which was used to determine cytosolic Ca^{2+} levels). The peak amplitude of Fura-2 fluorescence (ratio at 340/380 nm) was used to evaluate ER Ca^{2+} content. The fluorescence increase is proportional to ER Ca^{2+} content and the absence of external Ca^{2+} guarantees that the increase in the Fura-2 fluorescence ratio is due solely to the release of Ca^{2+} from the ER.

Alternatively, neurons plated in multiwells were washed 2 times in Krebs buffer and then loaded with Fura-2/AM (5 μM) in the same buffer supplemented with 0.2% (w/v) pluronic acid for 40 min, at 37 ºC in the dark. Afterwards, cells were gently washed 3 times in Ca^{2+}-free Krebs medium and then alternately excited at 340 and 380 nm and Fura-2 fluorescence was measured at 510 nm using a SpectraMax Gemini EM microplater reader. ER Ca^{2+} content was analyzed as described for single cell experiments upon addition of thapsigargin (2.5 μM). For PBMCs, the procedure was similar, but the protocol was adapted since these cells were in suspension. In this case, all the washes were done through centrifugation (1000 x *g* for 2 min at RT) and incubation with the probe was performed within an eppendorf. In order to record Fura-2 fluorescence, cells were washed and plated in multiwells in Ca^{2+}-free Krebs medium.

3.3.5. Measurement of NADPH oxidase activity.

NOX activity was evaluated using the chemiluminescent probe lucigenin, accordingly to the method described by Li and colleagues (Li *et al.* 1998), with some minor modifications. Lucigenin is reduced by the superoxide anion radical ($O_2{\cdot}^-$) produced through NOX activity, originating a lucigenin cation

radical. In the presence of NOX-derived $O_2\cdot^-$, the lucigenin cation radical yields an unstable dioxetane intermediate, which decomposes to produce two molecules of N-methylacridone. One of these molecules is in an electronically excited state and upon relaxation to the ground state emits a photon that can be detected, being its presence correlated with $O_2\cdot^-$ levels and consequently with NOX activity.

Untreated or treated cells were washed 2 times with PBS and lysed with 25 mM Tris-HCl (pH 7.4). Then, 30 µg of protein were added to a 96-well plate containing 100 µM NADPH and 5 µM lucigenin in a final volume of 200 µL (adjusted with 25 mM Tris-HCl). Luminescence readings were performed during 15 min using an Lmax II 384 microplater reader and results were calculated as relative luminescence units (RLU) per min per mg protein and were normalized to control values.

3.3.6. Measurement of superoxide anion ($O_2\cdot^-$) levels.

The levels of ROS, in particular the superoxide anion $O_2\cdot^-$ produced in large quantities by NOX, were assessed using the fluorescent DHE dye. DHE is the reduced form of the commonly used DNA intercalating dye ethidium bromide and is able to penetrate the cell membrane of live cells, staining their cytoplasm blue as well as the chromatin/nucleus red. $O_2\cdot^-$ radicals oxidize DHE to ethidium increasing red fluorescence upon DNA intercalation.

Treated or untreated cells were rinsed once with PBS at 37 ºC and incubated with DHE (10 µM) in Krebs medium for 1 hour at 37 ºC in the dark. Then, the fluorescence of DHE was measured for 1 hour at 518 nm excitation and 605 nm emission using a SpectraMax Gemini EM microplater reader. The levels of

$O_2^{\cdot -}$ were determinated calculating the difference between final and basal fluorescence values and were normalized to those determined in controls.

3.3.7. Determination of caspase-like activities.

Caspases activity was measured as described previously (Ferreiro *et al.* 2004). It was achieved through a colorimetric assay based on the cleavage of p-nitroaniline (pNA) from the terminus of specific peptide substracts for each caspase. Cleaved pNA increases its absorption at 405 nm. The free pNA can be quantified through the linear correlation between the absorption and the amount of the dye released.

Untreated or treated cells were lysed in a buffer containing (in mM): 25 HEPES-Na, 2 $MgCl_2$, 1 EDTA, 1 EGTA, supplemented with 100 μM PMSF, 2 mM DTT and a protease inhibitor cocktail (1 μg/mL leupeptin, pepstatin A, chymostatin and antipain). The cellular suspension was rapidly frozen/defrosted three times in liquid N_2, and then centrifuged for 10 min at 20,200 x g. The supernatant was collected and analyzed for protein content using the Bio-Rad protein dye assay reagent. Aliquots of cell extracts containing 25 or 40 μg of protein were incubated for 2 hours at 37 ºC, in a reaction buffer containing 25 mM HEPES-Na, 10 mM DTT, 10% (w/v) sucrose and 0.1% (w/v) CHAPS (pH 7.4) with 100 μM Ac-DEVD-pNA, ac-LEVD-pNA or ac-LEDH-pNA, chromogenic substrates for caspase-3, -4, and -9, respectively (Cregan *et al.* 1999). Caspase-like activity was determined by measuring substrate cleavage at 405 nm using a Spectra max plus 384 microplater reader. Results were expressed as arbitrary units of absorbance, and normalized to control values.

3.3.8. Immunocytochemical detection of an endoplasmic reticulum-associated pro-apoptotic factor.

Treated or untreated cells were rinsed 3 times with PBS and then fixed with 3.7% (w/v) formaldehyde in Neurobasal Medium (1:1 volume) for 10 min followed by an additional 10 min with undiluted fixative. Cells were then rinsed extensively in PBS. Coverslip-plated hippocampal cells were incubated in blocking solution containing 3% (w/v) BSA in PBS with 0.1% (v/v) Triton X-100 for 45 min at RT to prevent non-specific binding. Subsequently, neurons were incubated overnight at 4 °C with a monoclonal mouse anti-GADD153/CHOP antibody (1:200), diluted in blocking solution. Thereafter, coverslips were rinsed in PBS with 1% (w/v) BSA and incubated with the appropriated Alexa-conjugated secondary antibody anti-mouse IgG labelled with Alexa Fluor 488 (1:500) diluted in PBS with 1% (w/v) BSA for 90 min at RT. Following additional rinses in PBS neurons were stained with Hoechst 33342 (10 μg/mL, in PBS) for 5 min in the dark, at RT. Finally, the preparations were mounted using DakoCytomation fluorescent mounting medium. Images were collected using a 40x objective in an Axiovert 200M fluorescence microscope.

3.3.9. MTT reduction assay to evaluate cell viability.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is reduced to formazan by metabolic active cells (Mosmann 1983). Briefly, untreated or treated cells were incubated with 0.5 mg/mL MTT prepared in Krebs medium for 2 hours at 37°C. Then the medium was removed and the formed blue formazan crystals were dissolved with 0.04 M HCl in isopropanol

and quantified by measuring the absorbance at 570 nm in a Spectra max plus 384 microplate reader. Results were obtained comparing the average absorbance of treated cells with that measured in untreated control cells, and expressed as the percentage (%) of controls.

3.3.10. Analysis of apoptotic cell death by the TUNEL assay.

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) staining was performed using an *in Situ* Cell Death Detection Kit, Fluorescein, according to the manufacter's directions. This method is based on the detection of single- and double- stranded DNA breaks that occur at the early stages of apoptosis. DNA cleavage may yield single-stranded and double-stranded DNA breaks that can be detected by labelling of the free 3'-OH termini with modified nucleotides, like fluorescein-dUTP in an enzymatic reaction.

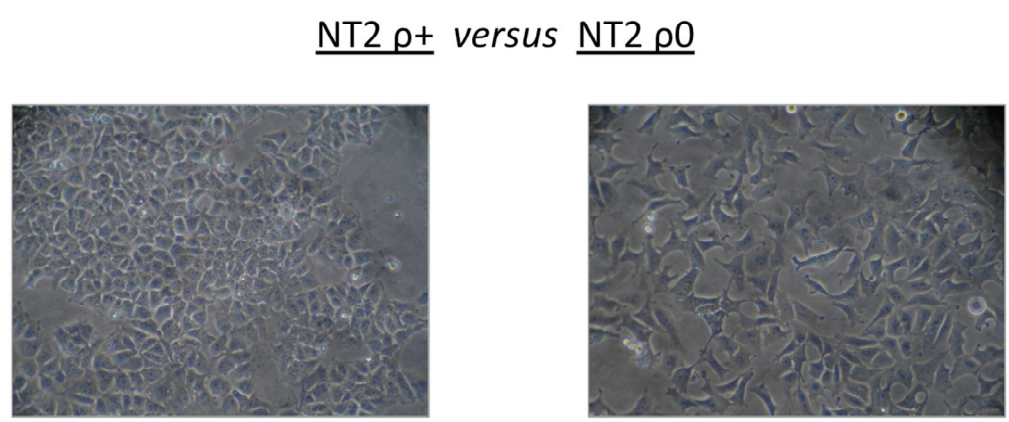
Cells were washed two times in PBS (pH 7.4) and were fixed with 4% (w/v) paraformadehyde for 30 min at RT. Then, slides were rinsed twice in PBS buffer and immersed in 0.1% (v/v) Triton X-100 supplemented with 0.1% (w/v) sodium citrate in iced PBS, for 2 min, to permeabilize the cells. Coverslips were rinsed again 3 times with PBS and incubated with TUNEL mixture for 1 hour at 37 ºC, in the dark. Finally, coverslips were rinsed 3 times with PBS and mounted with Dakocytomation Fluorescent solution onto a microscope slide for visualization in an Axiovert Microscope 200 using the 40x objective. DNAse-treated cells were used as a positive control.

3.3.11. Statistical analysis.

Results are expressed as means ± the standart error of the mean (SEM) of the number of experiments indicated in the figure captions. Statistical significance was obtained using an analysis of variance (ANOVA), followed by Dunnett's post-hoc tests for multiple comparisons or by the unpaired two-tailed Student's t-test. A $p < 0.05$ value was considered statistically significant.

CHAPTER 4

Endoplasmic reticulum stress-mediated apoptotic pathway induced by Aβ peptide requires the presence of functional mitochondria



Based on: Rui O. Costa, Elisabete Ferreiro, Sandra M. Cardoso, Catarina R. Oliveira and Cláudia M.F. Pereira. (2010) ER stress-mediated apoptotic pathway induced by $A\beta_{1-40}$ peptide requires the presence of functional mitochondria.

Published in Journal of Alzheimer´s Disease. J Alzheimers Dis. 2010;20(2):625-36.

(PMID: 20182029)

4.1. Summary.

Aβ peptide plays a significant role in the pathogenesis of AD. Previously we found that Aβ induces both mitochondrial and ER dysfunction leading to apoptosis, and now we address the relevance of ER-mitochondria cross-talk in apoptotic cell death triggered by Aβ peptide. Using mitochondrial DNA-depleted ρ0 cells derived from the human NT2-teratocarcinoma cell line, characterized by the absence of functional mitochondria, and the parental ρ+ cells, we report here that treatment with the synthetic $A\beta_{1-40}$ peptide, or the classical ER stressors thapsigargin or brefeldin A, increases GRP78 expression levels and caspase activity, two ER stress markers, and also depletes ER calcium stores. Significantly, we show that the presence of functional mitochondria is required for ER stress-mediated apoptotic cell death triggered by toxic insults such as Aβ. We found that the increase in the levels of the pro-apoptotic transcription factor GADD153/CHOP, which mediates ER stress-induced cell death, as well as caspase-9 and -3 activation and increased number of TUNEL-positive cells, occurs in treated parental ρ+ cells but is abolished in ρ0 cells. Our results strongly support the close communication between ER and mitochondria during apoptotic cell death induced by the Aβ peptide and provide insights into the molecular cascade of cell death in AD.

4.2. Introduction.

The ER is an essential intracellular organelle that ensures the correct folding/assembly, glycosylation and sorting of proteins in the secretory system (Aridor & Balch 1999) and is involved in intracellular Ca^{2+} homeostasis (Paschen 2001). When these processes are perturbed, two signaling pathways can be activated as a self-protective mechanism: the UPR, leading to the induction of ER chaperones (Wang *et al.* 1996; Chapman *et al.* 1998) and the EOR, leading to the production of cytokines (Pahl & Baeuerle 1997). Both processes facilitate the protein folding process in the ER and reduce overall translation (Harding *et al.* 1999; Kaufman 2002; Forman *et al.* 2003), or enhance proteosomal degradation of misfolded ER proteins in the cytosol by a process named ERAD (Bonifacino & Weissman 1998; Travers *et al.* 2000). Another mechanism that avoids accumulation of misfolded proteins involves targeting of parts of ER to lysosomes through autophagy (Kincaid & Cooper 2007; Heath-Engel *et al.* 2008). As a consequence, the accumulation and aggregation of misfolded proteins that would otherwise be toxic is reduced and cellular stress is relieved (Kozutsumi *et al.* 1988; Cudna & Dickson 2003). Once an imbalance between the load of proteins on the ER and its ability to process the load arises, ER stress occurs (Yoshida 2009). When ER stress is too extensive or long-termed, it results in apoptotic cell death, involving nuclear fragmentation, condensation of chromatin, and shrinkage of the cell body (Imaizumi *et al.* 2001). Switch from survival to cell death under ER stress conditions can also involve activation of autophagy that occurs in the absence of chromatin condensation (Hoyer-Hansen & Jaattela 2007). Important mediators of ER stress-associated death include the activation of the ER associated procaspase-12 (in mouse) or procaspase-4 (in human) and increased

expression of the proapoptotic transcription factor GADD153/CHOP (Cheung *et al.* 2006; Pino *et al.* 2009). Numerous studies indicate that mitochondria, as an organelle that establishes physical interactions with ER (Giorgi *et al.* 2009) and sequesters Ca^{2+} released from the ER, is a mandatory component in the induction of apoptosis by ER stress (Pizzo & Pozzan 2007). Under ER stress conditions, mitochondrial Ca^{2+} overload has been shown to be involved in mitochondrial apoptosis (Deniaud *et al.* 2008; Ferreiro *et al.* 2008a; Ferreiro *et al.* 2008b). Moreover, Bax/Bak translocation and oligomerization in the outer mitochondrial membrane, cytochrome c release, loss of $\Delta\Psi m$ and caspase-9 activation are common hallmarks of ER stress-induced apoptosis (Hacki *et al.* 2000; Zhang & Armstrong 2007). Since it is considered a cause of pathologically relevant apoptosis, ER stress has received growing attention in the last years and has been shown to be involved in acute damaging conditions such as ischemia (Tajiri *et al.* 2006; Oida *et al.* 2008) as well as in several chronic neurodegenerative disorders. Several authors consider that the origin of neuronal death occurring in diseases such as Parkinson's disease (Imai *et al.* 2000; Imai *et al.* 2001), Huntington's disease (Reijonen *et al.* 2008), amyotrophic lateral sclerosis (Nishitoh *et al.* 2008), prion-related disorders (Hetz & Soto 2006; Ferreiro *et al.* 2007), and Alzheimer's disease (Sato *et al.* 2001; LaFerla 2002; Katayama *et al.* 2004) is related with ER dysfunction. Protein levels of the ER stress marker BiP/GRP78 are increased in the brain of AD patients (Hoozemans *et al.* 2005). Moreover, fibrillar Aβ, and especially soluble oligomeric Aβ, that play an essential role in AD pathogenesis (Lambert *et al.* 1998; Heinitz *et al.* 2006), trigger neuronal ER stress and ER Ca^{2+} store depletion (Ferreiro *et al.* 2006; Resende *et al.* 2008a; Resende *et al.* 2008b; Nishitsuji *et al.* 2009). We have previously shown that, in primary cultures of

cortical neurons, Aβ leads to ER stress and ER Ca^{2+} release and activates an ERmediated apoptotic pathway that involves the translocation of Bax to mitochondria, loss of ΔΨm, cytochrome c release and activation of caspases-9 and -3, finally leading to apoptotic cell death (Ferreiro *et al.* 2004; Ferreiro *et al.* 2006). In our laboratory, Cardoso and colleagues (Cardoso *et al.* 2001; Cardoso *et al.* 2002) demonstrated that Aβ toxicity is abolished in mtDNA depleted cells, suggesting an important role of mitochondria in Aβ- induced cell death. After several experiments relied on the measurement of known ER stress and apoptotic markers in mtDNA-depleted cells, we report here that the presence of a functional mitochondria is required for ER stress-mediated apoptosis triggered by several stimuli, namely by Aβ peptide. These findings contribute to further elucidate the molecular mechanisms underlying Aβ-induced cell death and may uncover novel therapeutic strategies to prevent or ameliorate AD progression.

4.3. Results.

4.3.1. $A\beta_{1-40}$ peptide induces endoplasmic reticulum stress in both NT2 ρ+ and ρ0 cells.

Our previous results established that Aβ peptide induces neuronal ER stress leading to the activation of the mitochondrial apoptotic pathway (Ferreiro *et al.* 2008a). In primary cultures of cortical neurons, we observed that Aβ increases ER stress markers and ER Ca^{2+} release leading to the translocation of Bax to mitochondria, loss of $\Delta\Psi m$, cytochrome c release and activation of caspase-9 and -3 (Ferreiro *et al.* 2004). We have also shown that Aβ toxicity is abolished in mtDNA-depleted NT2 ρ0 cells (Cardoso *et al.* 2001; Cardoso *et al.* 2002), suggesting an important role of mitochondria in Aβ-induced cell death. In order to further investigate ER/mitochondria cross-talk in cell death triggered by Aβ, we took advantage of NT2 cells depleted of mtDNA (ρ0 cells), characterized by the absence of functional mitochondria (Swerdlow *et al.* 1997). The differential effect of Aβ in ρ0 cells and in parental ρ+ cells was compared with that of brefeldin A and thapsigargin, classical ER stressors (Klausner *et al.* 1992; Rogers *et al.* 1995). First, ER stress was evaluated in NT2 ρ+ and ρ0 cells after incubation with $A\beta_{1-40}$, brefeldin A or thapsigargin. In both cell lines, it was observed a significant increment in the levels of the ER chaperone GRP78 after 6 hours treatment (Fig. 4.1).

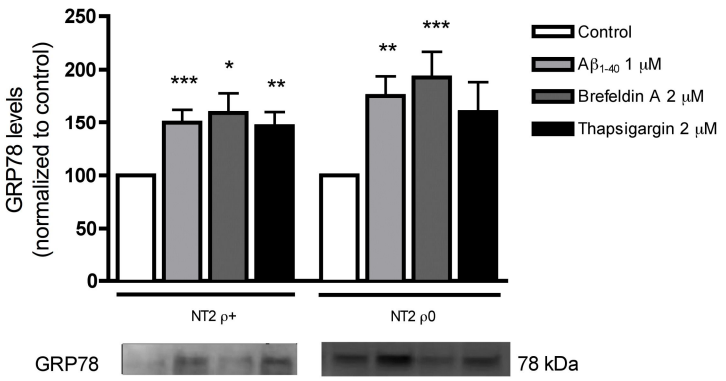


Figure 4.1 *Aβ1-40 peptide, brefeldin A and thapsigargin increase the levels of the ER chaperone GRP78 in NT2 ρ+ and ρ0 cells.*
NT2 cells were mantained for 6 hours at 37 ºC in the presence of $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM). Total protein lysates were prepared and analysed by immunoblotting using an anti-GRP78 antibody. Values are the means ± SEM of at least 3 independent experiments. *p<0.05; **p <0.01; ***p <0.001 (ANOVA with Dunnett post-hoc tests) compared with vehicle-treated cells.

Increased GRP78 expression occurred in $A\beta_{1-40}$-, brefeldin A-, or thapsigargin-treated ρ+ and ρ0 cells concomitantly with caspase-4 activation (Fig. 4.2). Human caspase-4 is a member of caspase-1 superfamily that includes caspase-12, which has been implicated in ER stressinduced apoptosis in rodents (Hitomi *et al.* 2004). This ER-resident caspase is cleaved during ER stress conditions (Nakagawa *et al.* 2000; Rao *et al.* 2001) and also upon Aβ administration (Hitomi *et al.* 2004), suggesting that caspase-4 activation plays a key role in ER stress induced by Aβ (Katayama *et al.* 2004). Several lines of evidence demonstrate that Aβ-induced apoptosis is due, at least in part, to the perturbation of intracellular Ca^{2+} homeostasis (Scorziello *et al.* 1996; Huang *et al.* 2000; Ferreiro *et al.* 2008b; Resende *et al.* 2008b). We have further demonstrated that the release of Ca^{2+} through channels associated with ER receptors (IP3R and RyR) contribute to the rise of cytosolic Ca^{2+} levels in

neurons exposed to the Aβ peptide (Ferreiro *et al.* 2004; 2006). Having this in consideration, the other aspect that we monitored was the disturbance of ER Ca^{2+} homeostasis, one of the mechanisms associated to ER stress conditions (Xu *et al.* 2005). Using single cell Ca^{2+} imaging, we were able to assess changes in ER Ca^{2+} content in ρ+ and ρ0 cells upon treatment with $A\beta_{1-40}$, brefeldin A or thapsigargin. A significant depletion of Ca^{2+} in ER stores was detected when ρ+ and ρ0 cells were treated for 3 hours with Aβ or ER stress inducers (Figures 4.3 A–C), an effect that persisted after 24 hours incubation (Figures 4.3 D–F). As shown in Fig. 4.3 C, the ER Ca^{2+} content is lower in ρ0 cells than in parental ρ+ cells in the absence of Aβ, brefeldin A, or thapsigargin treatment suggesting that mitochondrial impairment affects ER Ca^{2+} homeostasis.

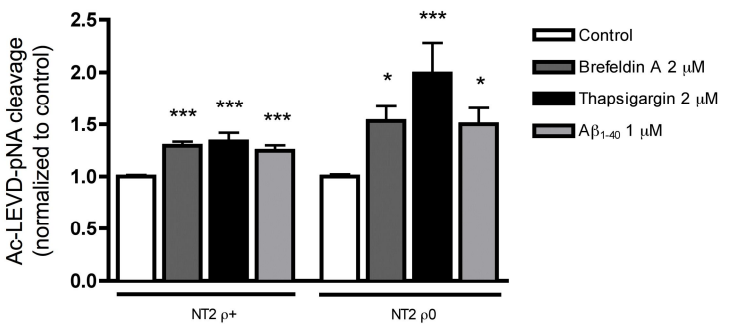


Figure 4.2. *The ER-resident caspase-4 is activated in NT2 ρ+ and ρ0 cells in response to $A\beta_{1-40}$, brefeldin A or thapsigargin treatment.*
NT2 cells were incubated for 6 hours at 37 ºC with $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM). Caspase-4-like activity was determined in cell lysates by measuring the cleavage of the chromogenic substrate Ac-LEVD-pNA at 405 nm. Results are the means ± SEM of values corresponding at least to 3 independent experiments, performed in duplicate. *p<0.05; ***p <0.001, significantly different compared with respect to control values.

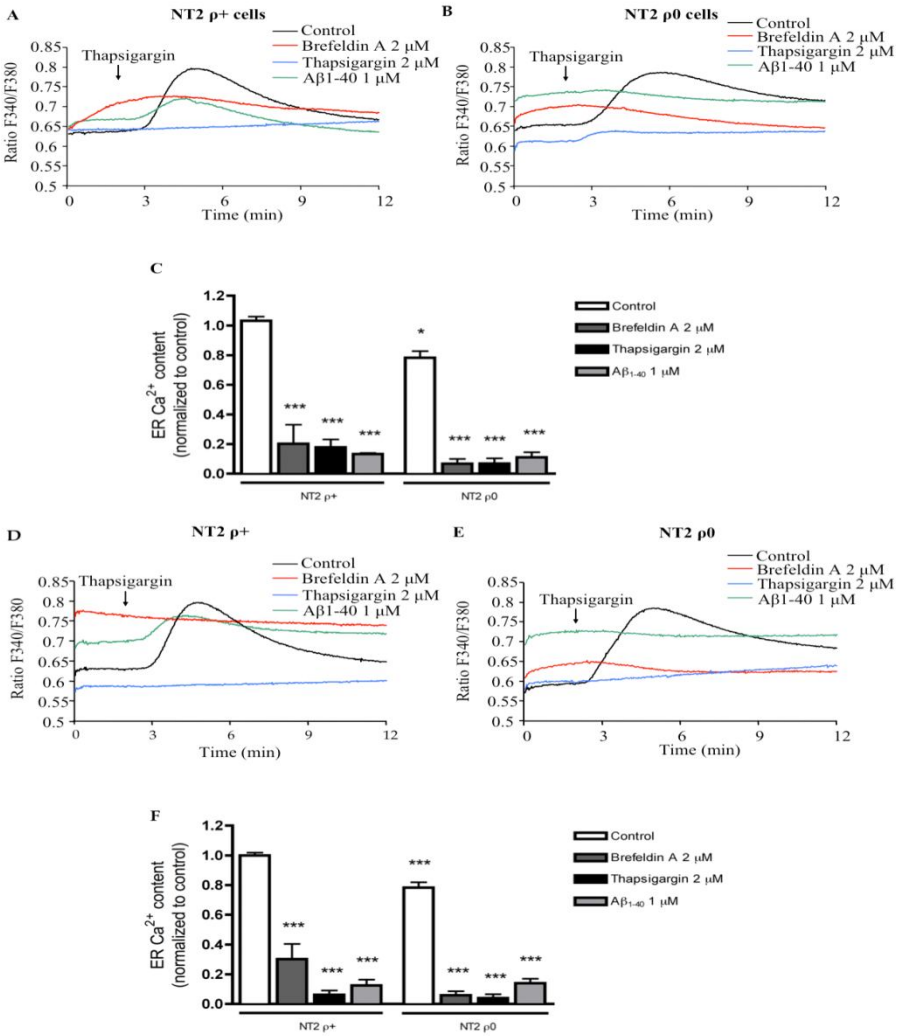


Figure 4.3 *ER Ca^{2+} content is depleted in NT2 ρ+ and ρ0 cells after $Aβ_{1-40}$, brefeldin A or thapsigargin exposure.*

NT2 cells were maintained for 3 hours (A, B, C) or 24 hours (D, E, F) at 37 ºC in the presence of $Aβ_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM). Then, levels of Ca^{2+} in ER stores were evaluated by monitorizing the fluorescence of Fura-2 in the absence of external Ca^{2+}. The difference between Fura-2 fluorescence ratio 340/380 nm before and after the addition (at t=2 min) of thapsigargin (2.5 μM), that irreversibly inhibits the ER Ca^{2+}-ATPase, was used to evaluate ER Ca^{2+} content. At least three different experiments were used to determine the average ER Ca^{2+} content for each condition. Data was expressed as the mean ± SEM. *** p <0.001, significantly different compared with respect to control values.

4.3.2. $A\beta_{1-40}$ induces apoptotic cell death due to endoplasmic reticulum stress in NT2 ρ+ but not in ρ0 cells.

So far, we were able to show that ER stress occurs in the presence of Aβ in NT2 parental cells as well as in mtDNA-depleted NT2 ρ0 cells without functional mitochondria. To clarify the role of mitochondria in ER stress-mediated cell death, we measured several apoptotic features in both ρ+ and ρ0 cell lines after exposure to $A\beta_{1-40}$ and also to the classical ER stress inducers brefeldin A and thapsigargin. A significant increase in the levels of the pro-apoptotic transcription factor GADD153/CHOP was observed after 6 hours in treated ρ cells. However, despite the increase in ER stress markers triggered by Aβ, brefeldin A, or thapsigargin in ρ0 cells (Figures 4.1–4.3), the levels of GADD153/CHOP, an important mediator of ER stress-induced cell death (Cheung *et al.* 2006), were not significantly changed in these cells lacking functional mitochondria (Fig. 4.4).

In parental ρ+ cells incubated with $A\beta_{1-40}$, the increase in GADD153/CHOP levels was followed by the activation of caspase-9 (Fig. 4.5 A) and caspase-3 (Fig. 4.6 B), finally leading to apoptosis that was assessed by a fluorescent TUNEL assay (Fig. 4.7). In contrast, when Aβ was added to mtDNA-depleted ρ0 cells, it was not able to activate caspases-9 and -3 (Figs 4.5 A and 4.5 B) or to increase the number of TUNEL-positive apoptotic cells (Fig. 4.7). The increase in caspase-9-like activity and caspase-3-like activity observed in ρ+ cellsincubated with $A\beta_{1-40}$ during 24 hours, was preventedin the presence of dantrolene, an ER Ca^{2+}channelblocker (Figures 4.5 B and 4.6 B), demonstrating that the releaseof $Ca^{2+}$from ER is involved in the activation ofthe mitochondria-mediated apoptotic pathway in Aβ-treated cells. Activation of the effector caspase-3 that underlies neuronal death in AD (Li *et al.* 1997)

and morphological features of apoptotic cell death were also evaluated in both ρ+ and ρ0 cells 24 hours after incubation with brefeldin A or thapsigargin. Results were similar to that obtained in $A\beta_{1-40}$-treated cells (Figures 4.5–4.7).

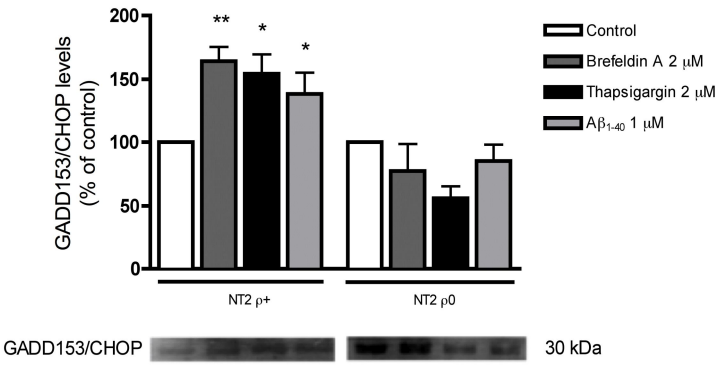


Figure4.4 *$A\beta_{1-40}$ peptide, brefeldin A and thapsigargin increase the levels of the pro-apoptotic transcription factor GADD153/CHOP in NT2 ρ+ and ρ0 cells.*
NT2 cells were mantained for 6 hours at 37 ºC in the presence of $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM). Total cell lysates were prepared and analysed by immunoblotting using an anti-GADD153/CHOP antibody. Values are the means ± SEM of at least 3 independent experiments. *p<0.05; **p <0.01, significantly different compared with vehicle-treated cells

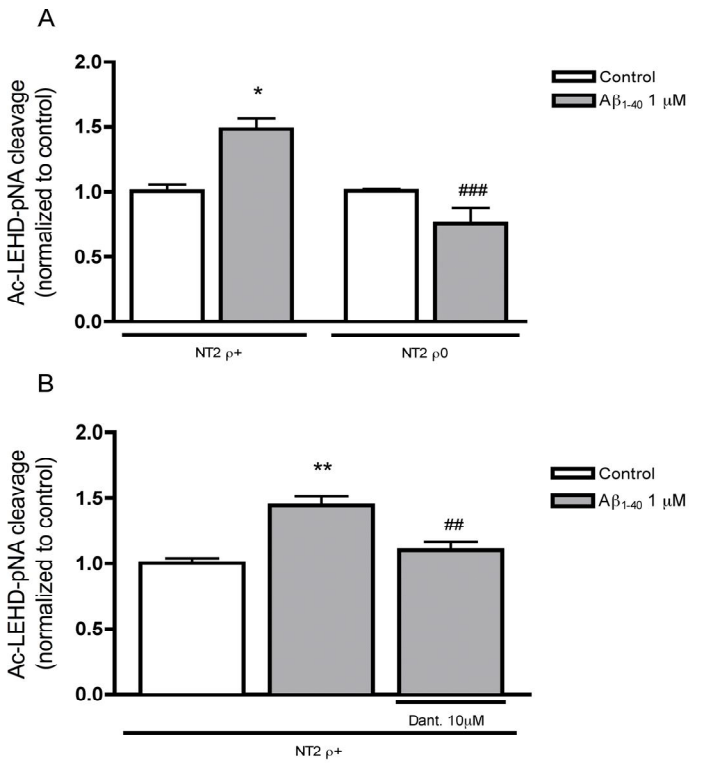


Figure 4.5 *$A\beta_{1\text{-}40}$ activate caspase-9 in NT2 ρ+ cells, due to ER Ca^{2+} release, but not in ρ0 cells.*

NT2 cells were maintained 24 hours at 37 ºC in the presence of $A\beta_{1\text{-}40}$ (1 μM) (A) Dantrolene (10 μM) was added to ρ+ cells 1 hour before $A\beta_{1\text{-}40}$ treatment (B). The results, determined by measuring the cleavage of the chromogenic substrate Ac-LEDH-pNA at 405 nm, are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, significantly different with respect to control values. $^{\#\#}p<0.001$, significantly different when compared to $A\beta_{1\text{-}40}$-treated NT2 ρ+ cells.

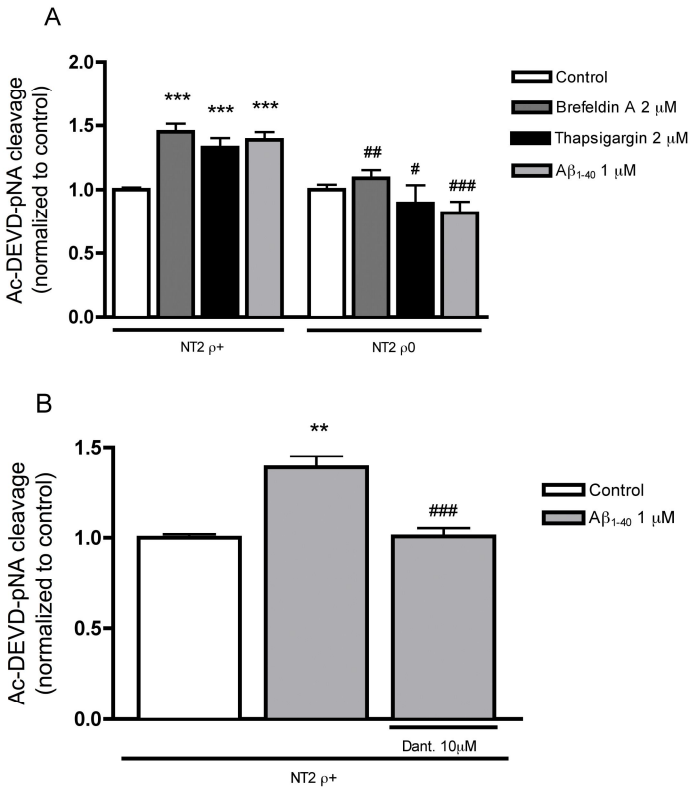


Figure 4.6 *Caspase-3 is activated by $A\beta_{1-40}$, brefeldin A and thapsigargin in NT2 ρ+ cells due to ER Ca^{2+} release, but not in ρ0 cells.*

NT2 cells were incubated during 24 hours in the presence of $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM) (A). When tested, dantrolene (10 μM) was added 1 hour before $A\beta_{1-40}$ incubation (A). Caspase-3-like activity was determined by measuring the cleavage of the chromogenic substrate Ac-DEVD-pNA at 405 nm. Results are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays. $^{**}p<0.01$; $^{***}p<0.001$, significantly different with respect to control values.

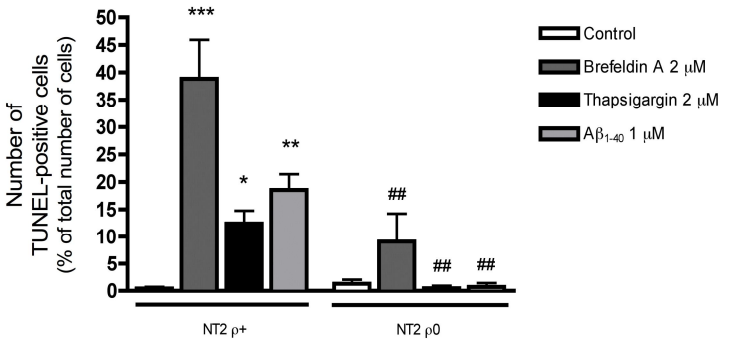


Figure 4.7 *The number of TUNEL-positive apoptotic cells is increased in the NT2 ρ+ cell line upon treatment with $A\beta_{1\text{-}40}$, brefeldin A or thapsigargin but not in ρ0 cells.*
TUNEL assay was performed, as described in material and methods section, in NT2 ρ+ and ρ0 cells incubated during 24 hours in the absence or in the presence of $A\beta_{1\text{-}40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM). Total number of TUNEL-positive cells in both cell lines was quantified and is represented in graph. The results are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays. $*p<0.05$; $**p<0.01$; $***p<0.001$, significantly different with respect to control values.

4.4. Discussion

The results here presented highlight the importance of mitochondria during ER stress-mediated apoptotic cell death induced by the Aβ peptide ($A\beta_{1-40}$ isoform) and support the ER/mitochondria cross-talk upon exposure to this AD-associated peptide. Our previous studies have shown that Aβ peptide induces ER stress in primary cultures of cortical neurons (Ferreiro *et al.* 2004; Ferreiro *et al.* 2006) and activates an ER stress-mediated apoptotic pathway that involves the translocation of Bax to mitochondria, release of cytochrome c, and subsequent activation of caspase-9 (Ferreiro *et al.* 2008b). Mitochondrial-related apoptotic features were demonstrated to occur upon Aβ-induced ER Ca^{2+} release (Hoozemans *et al.* 2005). These results were in line with studies reporting the role of impaired Ca^{2+} homeostasis and mitochondrial dysfunction in Aβ-induced cell death (Huang *et al.* 2000).

To further confirm the mitochondrial involvement inER stress-mediated apoptotic pathway triggered by the Aβ peptide, we used mtDNA-depleted NT2 human teratocarcinoma ρ0 cells which were reportedto be an excellent model to study the role of mitochondriain aging and age-related neurodegenerative diseasessuch as AD (Hu *et al.* 2000). This cell line is characterized by loss of mitochondrial function since 13 protein subunits of mitochondrial respiratory chain are encoded by mtDNA (Wallace 1994; DiMauro 2006). In parallel, we used the parental NT2 ρ+ cell line with normal mitochondria. In NT2 ρ0 cells, mitochondrial membrane potential remainsintact, probably due to the transport of protons out of the mitochondrial matrix, caused by the reversal of ATP synthase activity (Nijtmans *et al.* 1995), as observed in other ρ0 cell types (Buchet & Godinot 1998; Appleby *et al.* 1999). Despite the absence of functional electron transport chain, NT2 ρ0 cells can still undergo into

apoptosis after exposure to staurosporine, an activator of the mitochondrial apoptotic pathway (Jiang *et al.* 1999). However, we have previously shown that the decrease incell survival triggered by Aβ is significantly lower in mtDNA-depleted ρ0 cells than in the parental cell line, providing evidence for the relevance of mitochondria in Aβ-induced toxicity (Cardoso *et al.* 2001; Cardoso *et al.* 2002). Here, we extended these studies to investigate the involvement of mitochondria in ER stress-mediated apoptosis induced by the Aβ peptide.

First, we evaluated the potential stressful effect of $A\beta_{1-40}$ peptide on ER. As positive controls for ER stress in our study, we used two known ER stress inducers: thapsigargin, an inhibitor of SERCA/Ca^{2+}-ATPase, which disrupts ER Ca^{2+} homeostasis (Kuo *et al.* 1998), and brefeldin A, which leads to the accumulation of proteins in the ER lumen due to the blockage of retrograde Golgi-derived vesicles (Chardin & McCormick 1999). In NT2 ρ+ and ρ0 cells, an increment in GRP78 expression levels was observed upon treatment with $A\beta_{1-40}$ peptide (1 μM), brefeldinA (2 μM), or thapsigargin (2 μM), independently of the presence or absence of functional mitochondria (Fig. 4.1). Under ER stress conditions, cells tend to increase the transcription of genes encoding ER-resident chaperones such as GRP78 as part of a defense mechanism termed unfolded protein response (Imaizumi *et al.* 2001). GRP78 promotes the correct folding of proteins that have been accumulated within the ER lumen and is considered to be an important molecule in the quality control of proteins, preventing unfolded proteins from being transported to the Golgi apparatus and further downstream (Kohno *et al.* 1993) and is thus considered a good marker for ER stress. The disturbance of ER Ca^{2+} homeostasis is also considered an evidence of ER stress (Baumgartner *et al.* 2009). Previous

reports demonstrated that Aβ peptide depletes ER neuronal Ca^{2+} content, increasing cytosolic Ca^{2+} levels (Bhatia *et al.* 2000; Ferreiro *et al.* 2004).

Here, we describe the early disturbance of ER Ca^{2+} homeostasis in NT2 $\rho+$ and ρ0 cells after treatment with Aβ, thapsigargin, or brefeldin A (Fig. 4.3). Although the absence of functional mitochondria did not affect the depletion of Ca^{2+} stores induced by Aβ or the ER stressors, it was possible to notice that basal levels of cytosolic Ca^{2+} were shown to be higher in ρ0 cells than in ρ+ cells (Figures 4.3-4.3 D). Nakagawa and colleagues (Nakagawa *et al.* 2000) reported that caspase-12 knock-out mice not only showresistance to ER stress but also to death caused by Aβ, demonstrating that caspase-12 mediates an ER stress-specific apoptosis pathway and contributes to Aβ neurotoxicity, recently shown to be modulated via the ubiquitine/proteosome system (Song *et al.* 2008). Despite this caspase is not present in humans, several studies suggested that caspase-4 is an ER stress response caspase in humans (Hitomi *et al.* 2004). Under ER stress conditions, caspase-4 is activated and can be associated with the activation of caspase-3 (Kim *et al.* 2008b). However, other groups reported that ER stress only activates caspase-9 leading to apoptotic cell death by a mechanism that does not require caspase-4 (Obeng & Boise 2005). In our study, we measured caspase-4-like activity in ρ+ and ρ0 cells treated with the ER stress inducers thapsigargin and brefeldin A, as well as with the $A\beta_{1-40}$ peptide, and we observed significant activation of this ER-resident caspase in both cell lines, comparatively to control conditions (Fig. 4.2) that was correlated with increased GRP78 levels (Fig. 4.1) and depletion of ER Ca^{2+} content (Fig. 4.3). We were able to prove the induction of ER stress by the $A\beta_{1-40}$ peptide in parental cells, and also in cells lacking functional

mitochondria, and next we examined whether it could lead to apoptotic cell death.

To better understand the role played by mitochondria in ER stress-mediated apoptotic pathway, we took advantage of our chosen model, the mtDNA-depleted ρ0 cells. According to the literature, several groups point to the interaction between ER and mitochondria as an important process in normal cellular functioning, as well as during programmed cell death triggered by ER stress (Kim *et al.* 2006; Lei *et al.* 2008; Zhang *et al.* 2008; Arduíno *et al.* 2009; Klee *et al.* 2009; Sharaf El Dein *et al.* 2009; Vitiello *et al.* 2009). Our previous work supported the involvement of ER/mitochondria in the Aβ-induced neuronal death (Ferreiro *et al.* 2006; Ferreiro *et al.* 2008b). We now demonstrate that the expression levels of GADD153/CHOP, a transcription factor involved in the apoptotic pathway mediated by ER stress (Oyadomari & Mori 2004; Schapansky *et al.* 2007), are increased in ρ+ cells but not in mtDNA-depleted ρ0 cells after addition of Aβ, thapsigargin, or brefeldin A (Fig. 4.4), suggesting that ER stress occurs in the absence of functional mitochondria but the ER stress-mediated apoptotic pathway is not able to proceed. As previously described, activation of caspases, a family of cysteine proteases that cleave substrates at specific aspartate residues, is a central mechanism in the apoptotic cell death process (Thornberry & Lazebnik 1998). Caspase-9 activation is a mitochondria-mediated cell death marker. Upon cytochrome c release from mitochondria, apoptosome is formed, and caspase-9 is activated initiating the caspase cascade leading to caspase-3 activation and consequent cell death (Li *et al.* 1997). In the present study, we demonstrated the activation of caspase-9 and caspase-3 (Figures 4.5-4.6) and a significantly increase in TUNEL-positive apoptotic cells (Fig. 4.7) on NT2 ρ+ treated cells,

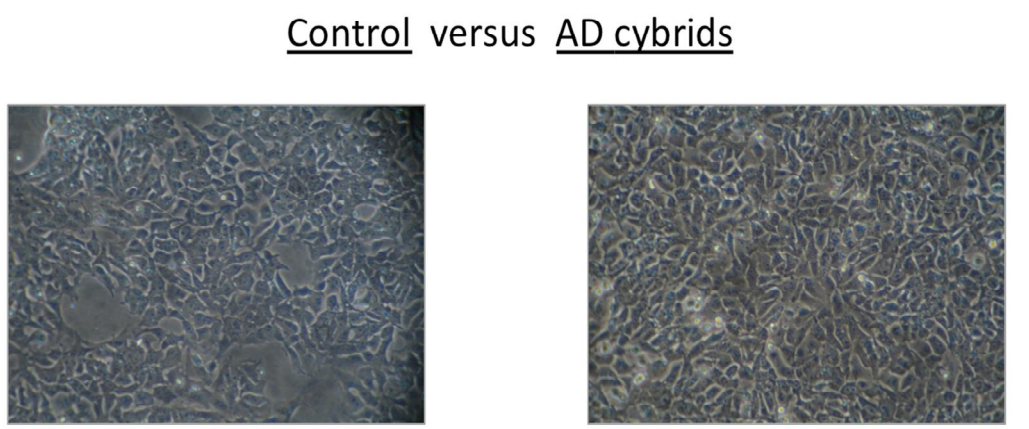
supporting the involvement of mitochondria in apoptosis triggered upon ER stress. However, the increase in these mitochondrial-related events was not detected in ρ0 cells lacking functional mitochondria.

Because caspase-4 was similarly activated in both lines, we could not implicate this ER-resident caspase in apoptotic cell death upon ER stress since it was not possible to observe caspase-9 or caspase-activation in ρ0 cells lacking functional mitochondria (Fig. 4.6). Our results are in agreement with those that reported a decreased susceptibility of mtDNA-depleted cells to apoptosis (Dey & Moraes 2000; Ferraresi *et al.* 2008). The absence of cell death by apoptosis in Aβ-, thapsigargin-, or brefeldin A treated ρ0 cells can be explained by the sequestration of pro-apoptotic proteins Bax, Bid, and BAD in the mitochondrial inner membrane (Biswas *et al.* 2005) and/or by the increment of the anti-apoptotic protein Bcl-2, a mitochondrial membrane protein that was shown to block apoptosis (Jacobson *et al.* 1993; Heath-Engel *et al.* 2008). Indeed, we have shown that resistance of mtDNA-depleted NT2 ρ0 cells against prion peptide-induced toxicity is associated with increased levels of Bcl-2 (Ferreiro *et al.* 2008b). Furthermore, it was observed an increment in MnSOD expression in ρ0 cells derived from SK-Hep1 hepatoma cell line (Park *et al.* 2004). In addition, the pro-apoptotic death associated protein-3 (DAP3), was found to be absent in ρ0 cells of different origin (osteosarcoma 143B, hepatocarcinoma HepG2, uterine carcinoma HeLaS3) when cells were treated with apoptotic inducers (Jacques *et al.* 2006). Our results further demonstrated that ER stress-mediated apoptosis was due to ER Ca^{2+} release induced by Aβ since dantrolene, an ER Ca^{2+} channel blocker, was able to prevent caspase-9 and caspase-3 activation in NT2 ρ+ with functional mitochondria (Figures 4.5-4.6), giving the idea that Ca^{2+} has a major role in the

communication between these two organelles. Our results are in accordance with Chami and colleagues (Chami *et al.* 2008) study, in which they show the importance of the SERCA- Ca^{2+} ATPase in the proapoptotic Ca^{2+} transfer from ER to mitochondria during ER stress. Further, in human myeloid cells caspase-3 and -9 were proven to be activated through ER Ca^{2+} release (Gonzalez *et al.* 2010). In conclusion, our study provided a newinsight about the interaction between ER and the mitochondria during apoptotic cell death. In particular, it was shown that the AD-associated Aβ peptide leads to ER stress and activates an ER stress-mediated apoptotic pathway by a mechanism that requires the presence of functional mitochondria. During Aβ-induced apoptotic cell death, ER stress, and ER Ca^{2+} release are upstream events that occurs before activation of the mitochondrial-mediated cell death pathway and could thus be potential targets for therapeutic intervention in AD.

CHAPTER 5

Amyloid β-induced endoplasmic reticulum stress is enhanced under mitochondrial dysfunction conditions



Based on: Rui O. Costa, Elisabete Ferreiro, Isaura Martins, Isabel Santana, Sandra M. Cardoso, Catarina R. Oliveira and Cláudia M.F. Pereira. (2011) Amyloid β-induced ER stress is enhanced under mitochondrial dysfunction conditions.
Published in Neurobiology of Aging (in press).

5.1. Summary.

Previously we reported that ER-mitochondria cross-talk is involved in Aβ-induced apoptosis. Now we show that mitochondrial dysfunction affects the ER stress response triggered by Aβ using cybrids that recreate the defect in mitochondrial COX activity detected in platelets from AD patients. AD and control cybrids were treated with Aβ or classical ER stressors and the ER stress-mediated apoptotic cell death pathway was accessed. Upon treatment, we found increased GRP78 levels and caspase-4 activation (ER stress markers), which were more pronounced in AD cybrids. Treated AD cybrids also exhibited decreased cell survival as well as increased caspase-3-like activity, PARP levels and TUNEL-positive apoptotic cells. Finally, we showed that Aβ-induced caspase-3 activation in both cybrid cell lines was prevented by dantrolene, thus implicating ER Ca^{2+} release in ER stress-mediated apoptosis.

Our results demonstrate that mitochondrial dysfunction occurring in AD patients due to COX inhibition potentiates cell susceptibility to Aβ-induced ER stress. This study further supports the close communication between ER and mitochondria during apoptosis in AD.

5.2. Introduction.

Aβ is the main component of extracellular senile plaques, one of the AD neuropathologic hallmarks (Selkoe 2001). Mitochondrial dysfunction has been demonstrated to be one of the mechanisms underlying Aβ-induced neurodegeneration (Moreira *et al.* 2006; Chen & Yan 2010; Ferreira *et al.* 2010). The presence of Aβ was confirmed in mitochondria from brains of transgenic mice with targeted neuronal overexpression of mutant human APP and AD patients and is associated with diminished enzymatic activity of respiratory chain complexes and a reduction in the rate of oxygen consumption (Caspersen *et al.* 2005). Recently, it was shown that RAGE-mediated signaling contributes to intraneuronal transport of Aβ and promotes neuronal degeneration through perturbation of mitochondrial function (Takuma *et al.* 2009). Other data also demonstrated that Aβ is transported into mitochondria via the TOM machinery (Hansson Petersen *et al.* 2008). In mitochondria isolated from brains of AD patients or transgenic mice, Aβ interacts with the ABAD (Lustbader *et al.* 2004), and this interaction promotes leakage of ROS, inhibits mitochondria activity and induces cell death (Takuma *et al.* 2005). Du and colleagues (Du *et al.* 2009) demonstrated that mitochondrial Aβ also interacts with CypD, an integral part of the mitochondrial PTP whose opening leads to cell death. In the same study, the authors have shown that CypD deficiency attenuates mitochondrial dysfunction induced by Aβ, improves learning and memory and synaptic function in an AD mouse model and alleviates Aβ-mediated reduction of LTP. Mitochondrial Aβ levels have been associated with the extent of mitochondrial dysfunction in different brain regions and the degree of cognitive impairment in AD transgenic mice (Dragicevic *et al.* 2010). Concomitantly, several in vitro evidences implicate

ER stress as an important cellular response triggered by Aβ, leading to neuronal cell death (Ferreiro *et al.* 2004; Ferreiro *et al.* 2006; Resende *et al.* 2008a; Resende *et al.* 2008b; Nishitsuji *et al.* 2009). Moreover, in transgenic mice modeling AD, ER stress-related genes were shown to be differentially regulated during the initial and intermediate stages of Aβ deposition (Selwood *et al.* 2009). Upon activation of processes such as the UPR (Imaizumi *et al.* 2001; Yoshida 2009), ER overload response (Pahl & Baeuerle 1997), ER-associated degradation (Travers *et al.* 2000) or even authophagy (Hoyer-Hansen & Jaattela 2007; Heath-Engel *et al.* 2008), ER is able to manage with different kinds of stress deciding cell fate (Yoshida 2009). Under conditions of severe or prolonged ER stress, these adaptive signalling pathways fail and apoptotic cell death is activated (reviewed in Kim *et al.* 2008a).

Through last years, several studies have pointed to an interaction between ER and mitochondria during cell death. It has been shown that physical interactions are established between these two organelles (Giorgi *et al.* 2009) and that Ca^{2+} released from ER and taken up by mitochondria is required to induce the mitochondrial apoptotic pathway under ER stress conditions (Pizzo & Pozzan 2007; Deniaud *et al.* 2008; Ferreiro *et al.* 2008a; Ferreiro *et al.* 2008b). Moreover, Bax/Bak translocation and oligomerization in the outer mitochondrial membrane, cytochrome c release, loss of mitochondrial membrane potential ($\Delta\Psi m$) and caspase-9 activation are common hallmarks of ER stress-induced apoptosis (Hacki *et al.* 2000; Zhang & Armstrong 2007). We have demonstrated that Aβ-induced ER stress activates an apoptotic pathway involving the translocation of Bax to mitochondria, loss of $\Delta\Psi m$, cytochrome c release and activation of caspases -9 and -3 (Ferreiro *et al.* 2008a; Ferreiro *et al.* 2008b). More recently, we provided evidence that the ER

stress-mediated apoptotic pathway induced by Aβ requires the presence of functional mitochondria (Costa *et al.* 2010), which is in accordance with data previously published by Cardoso and colleagues (Cardoso *et al.* 2001; Cardoso *et al.* 2002). These studiesdemonstrated that Aβ toxicity is abolished in mtDNA depleted ρ0 cells, supporting that mitochondrial dysfunction is a prominent feature of Aβ-induced cell death during the progression of AD.

Recent studies postulate that impaired energy metabolism and mitochondrial respiration abnormalities are early events in AD pathology (Small *et al.* 1995; Ferrer 2009). Defects in mitochondrial electron transport chain, namely in COX activity, have been reported to occur in this neurodegenerative disease (Kish *et al.* 1992; Bosetti *et al.* 2002; Cardoso *et al.* 2004a; Pickrell *et al.* 2009) and the accumulation of mtDNA mutations could be at the origin of this ETC malfunction (Lin *et al.* 2002; Coskun *et al.* 2003; Lin & Beal 2006; Kruger *et al.* 2010).

Since they were first introduced by Swerdlow and collegues (Swerdlow *et al.* 1997), AD cybrids have been used as a cellular model in order to better understand the importance of mitochondrial impairment in AD. This can be achieved given that these cytoplasmic hybrid cells replicate pathological characteristics occurring in AD patients, such as reduced COX activity, ATP depletion and increased oxidative stress (Ojaimi *et al.* 1999; Cardoso *et al.* 2004a; Cardoso *et al.* 2004b; Petrozzi *et al.* 2007). AD and control cybrids are obtained by the fusion of platelets from AD patients or age-matched control subjects with mtDNA-depleted ρ0 cells. The resultant cell line holds mitochondria, and consequently mtDNA, from AD or control donors. Therefore, the mitochondrial abnormalities observed in platelets obtained from AD patients are also present in AD cybrids.

This cell model was used in the present study to further investigate the relevance of AD-associated mitochondrial dysfunction to Aβ-induced ER stress and subsequent activation of apoptotic cell death. As positive control for ER stress, a parallel study was conducted in control and AD cybrids using brefeldin A and thapsigargin, two classical ER stressors. We provide evidence that mitochondrial dysfunction, arising from defects in mtDNA, increases the susceptibility of cells to ER stress-mediated apoptosis induced by Aβ, confirming the interaction between ER and mitochondria during the progression of AD.

5.3. Results.

Previous studies from our group established that Aβ peptide induces ER stress in primary cultured neurons, increasing the levels of ER stress markers and promoting ER Ca^{2+} release (Ferreiro *et al.* 2006; Resende *et al.* 2008a; Resende *et al.* 2008b). Concomitantly, Aβ activates a mitochondrial apoptotic pathway which involves the translocation of Bax to mitochondria, loss of $\Delta\Psi m$, cytochrome c release and activation of caspase-9 and -3 (Ferreiro *et al.* 2008b). Moreover, we showed that both Aβ-induced toxicity and activation of the ER stress-mediated apoptotic pathway require the presence of functional mitochondria since they are abolished in mtDNA-depleted ρ0 cells (Cardoso *et al.* 2001; Cardoso *et al.* 2002; Costa *et al.* 2010). Taken together, these studies highlight the important role of mitochondria in Aβ-induced cell death upon ER stress.

Here, to further characterize ER/mitochondria cross-talk in cell death triggered by Aβ, we analyzed the ER stress-mediated apoptotic cell death induced by this AD-associated peptide in AD cybrids containing mitochondria and mtDNA from AD patients, and thus the mitochondrial defect (COX inhibition) associated to the disease. The differential effect of Aβ in AD and control cybrids (the later with mtDNA from age-matched subjects) was compared with that of brefeldin A and thapsigargin, two classical ER stressors (Klausner *et al.* 1992; Rogers *et al.* 1995).

5.3.1. Mitochondrial dysfunction potentiates $A\beta_{1-40}$-induced endoplasmic reticulum stress.

ER stress was evaluated in AD and control cybrids treated with $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM) during 6 hours. The levels of the ER chaperone GRP78 were analysed by WB and caspase-4 activation was evaluated using the colorimetric substrate ac-LEVD-pNA, as described in Methods section. In control cybrids, $A\beta_{1-40}$, brefeldin A and thapsigargin slightly increased GRP78 levels after 6 hours incubation and this effect was significantly enhanced in AD cybrids (Fig. 5.1 A). The increase in GRP78 levels upon exposure of control cybrids to $A\beta_{1-40}$ became statistically significant after 24 hours and it was also shown to be potentiated in AD cybrids (Fig. 5.1 B). Caspase-4, a member of caspase-1 superfamily and human homolog of caspase-12 in rodent, has been implicated in ER stress-induced apoptosis (Hitomi *et al.* 2004). A raise in caspase-4-like activity occurred in both cell lines, treated as above, although this increment was much more pronounced in AD than in control cybrids (Fig. 5.2). These results indicate that the presence of a mitochondrial defect renders cells more susceptible to $A\beta_{1-40}$-induced ER stress.

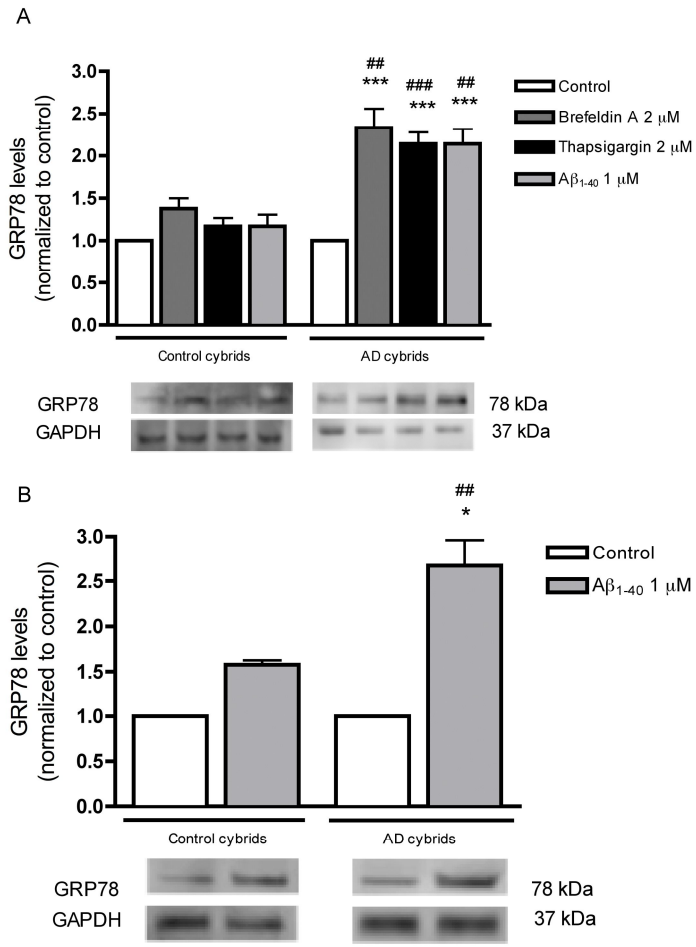


Figure 5.1 *The increase in the levels of the ER chaperone GRP78 upon $A\beta_{1-40}$, brefeldin A or thapsigargin exposure is more pronounced in AD cybrids than in control cybrids.*
Cybrids were mantained for 6 hours (A) at 37 ºC in the presence of $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM) or 24 hours (B) in the presence of $A\beta_{1-40}$ (1 μM). Total protein lysates were prepared and analysed by immunoblotting using an anti-GRP78 antibody; an antibody specific for GAPDH was used as a protein loading control. Results are the means ± SEM of at least 3 independent experiments and are normalized to control values. *p<0.05; ***p <0.001 compared with vehicle-treated cells. $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$, significantly different when compared to the same conditions in control cybrids.

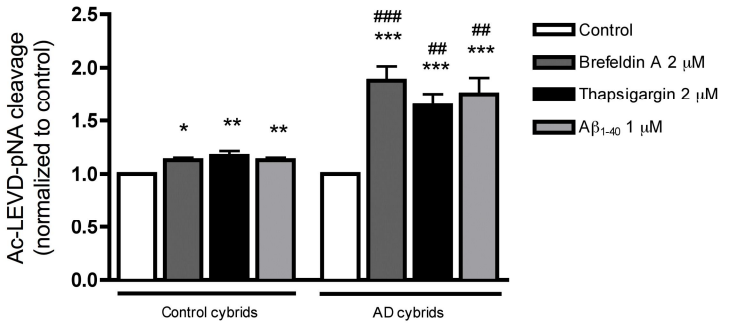


Figure 5.2 *The activation of the ER resident caspase-4 in response to $A\beta_{1-40}$, brefeldin A or thapsigargin is enhanced in AD cybrids relatively to control cybrids.*
Cybrid cells were incubated for 6 hours at 37 ºC with $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM). Caspase-4-like activity was determined in cell lysates by measuring the cleavage of the chromogenic substrate Ac-LEVD-pNA at 405 nm. Results are the means ± SEM of values corresponding at least to 3 independent experiments, performed in duplicate, and are normalized to controls. *p<0.05; **p <0.01; ***p <0.001, significantly different compared with respect to control values. $^{\#\#}p$<0.01, $^{\#\#\#}p$ <0.001, significantly different when compared to similar conditions in control cybrids.

5.3.2. $A\beta_{1-40}$-induced apoptotic cell death due to endoplasmatic reticulum stress is potentiated by impaired mitochondria.

To better elucidate the role of defective mitochondria in ER stress-mediated cell death induced by Aβ, cellular viability and apoptotic features were evaluated in control and AD cybrids. Once again, results were compared with those obtained in cells treated with thapsigargin or brefeldin A, two classical ER stressors. We first evaluated the metabolic capacity of cybrid cell lines by the MTT colorimetric assay, which can be correlated with cell survival. Upon $A\beta_{1-40}$, brefeldin A or thapsigargin treatment for 24 hours, a significant decrease in cell survival was determined (Fig. 5.3). It was also observed that decreased cell survival in treated AD cybrids is slightly potentiated in control

cybrids. Statistical comparisons between control and AD cybrids were not possible since the proliferative capacity of both cell lines is different.

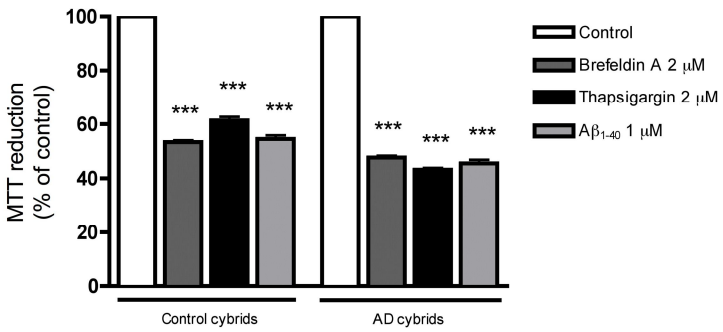


Figure 5.3 *Compromised cell survival upon $A\beta_{1-40}$, brefeldin A or thapsigargin treatment is more pronounced in AD cybrids than in control cybrids.*
Cybrid cells were incubated for 24 hours at 37 ºC with $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM). Cell viability was evaluated by the reduction of the tetrazolium salt MTT, as described in methods section. Results are the means ± SEM of values corresponding at least to 3 independent experiments, performed in duplicate, and are represented as the percentage (%) of absorbance measured in controls. ***p <0.001; significantly different when compared with respect to control values.

In both AD and control cybrids, $A\beta_{1-40}$ and ER stressors activated the apoptosis effector caspase-3 after 24 hours of treatment (Fig. 5.4 A). Moreover, caspase-3 activation was more pronounced in treated AD cybrids than in control cybrids. To confirm that ER stress is involved in Aβ-induced cell death, dantrolene, a blocker of ER Ca^{2+} channel associated with the RyR, was added before $A\beta_{1-40}$ treatment. Under these conditions, a significant decrease in caspase-3-like activity was observed in cybrid cell lines (Fig. 5.4 B), demonstrating that the release of Ca^{2+} from ER is involved in caspase-3 activation triggered by Aβ.

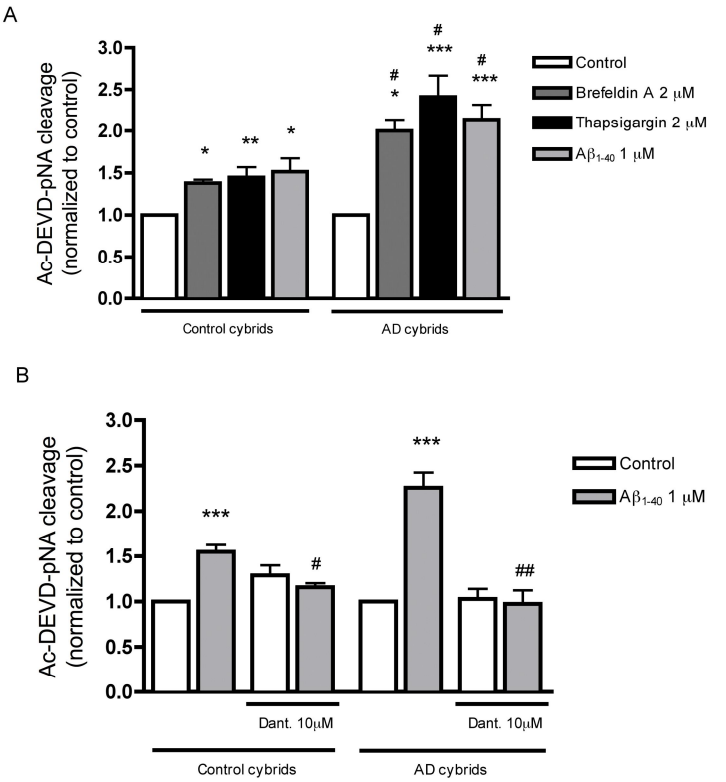


Figure 5.4 *Caspase-3-like activity upon $A\beta_{1\text{-}40}$, brefeldin A or thapsigargin treatment is increased in AD cybrids relatively to treated control cybrids. Inhibition of ER Ca^{2+} release prevents caspase-3 activation in both cybrid cell lines.*

Cybrid cells were incubated during 24 hours at 37 ºC in the presence of $A\beta_{1\text{-}40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM) (A). When tested (B), dantrolene (10 μM) was added 1 hour before $A\beta_{1\text{-}40}$ incubation. Caspase-3-like activity was determined by measuring the cleavage of the chromogenic substrate Ac-DEVD-pNA at 405 nm. Results are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays, and are normalized to control. $^{**}p<0.01$; $^{***}p<0.001$, significantly different with respect to control values.

In the apoptotic cascade, caspase-3 cleaves PARP leading to the increase of an 89 kDa fragments (Nicholson *et al.* 1995). After 24 hours treatment, $A\beta_{1-40}$, thapsigargin and brefeldin A increased caspase-3-mediated PARP cleavage analyzed by WB, which was enhanced in AD-treated cybrids when compared to control cybrids (Fig. 5.5). Finally, apoptosis was assessed by a fluorescent TUNEL assay (Fig. 5.6). Upon treatment with Aβ or ER stressors, it was observed a significant increase in the number of apoptotic cells in both control and AD cybrids (Fig. 5.6 A). Under these conditions, the total number of cells was not affected in control cybrids but, in contrast, a significant reduction in the total number of cells was measured in AD cybrids (Fig. 5.6 B). This fact is probably due to cell death and subsequent detachment from the coverslips revealing an enhanced susceptibility of AD cybrid cells to toxic insults.

Overall, the results described above demonstrate that mitochondrial dysfunction occurring in AD cybrids as a result of COX inhibition potentiates apoptosis induced under ER stress conditions.

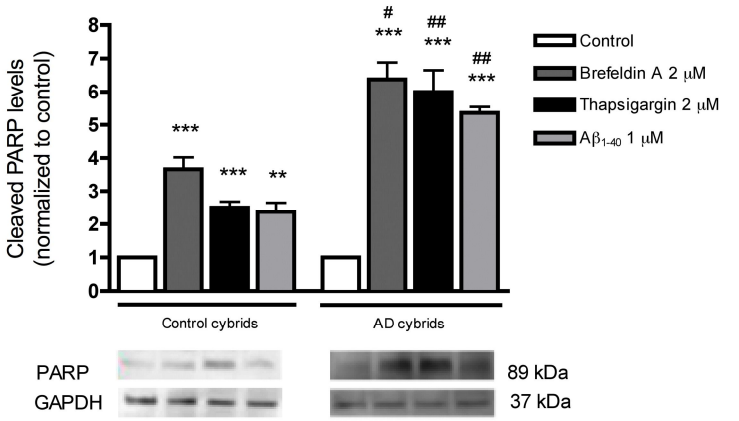


Figure 5.5 *PARP cleavage is enhanced in $A\beta_{1-40}$-, brefeldin A- or thapsigargin-treated AD cybrids in comparison with treated control cybrids.*
Cybrids were mantained for 24 hours at 37 ºC in the presence of $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM). Total protein lysates were prepared and analysed by immunoblotting using an anti-PARP antibody; as a protein loading control, an anti-GAPDH antibody was used. Values are the means ± SEM of at least 3 independent experiments and are normalized to control levels. $^{**}p<0.05$; $^{***}p<0.001$, significantly different compared with respect to control values. $^{\#}p<0.05$; $^{\#\#}p<0.01$, significantly different when compared to the same conditions in control cybrids.

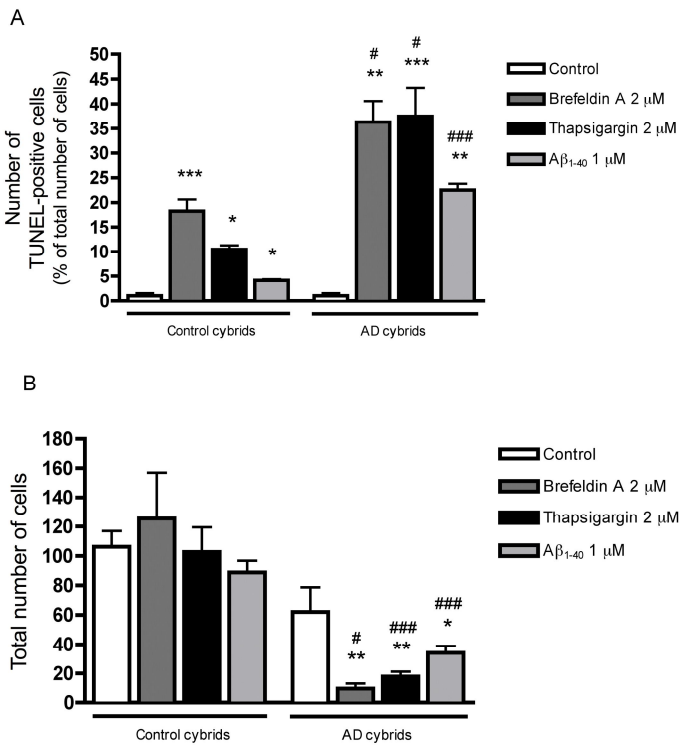


Figure 5.6 *$A\beta_{1\text{-}40}$-, brefeldin A- and thapsigargin-induced apoptosis is enhanced in AD cybrids relatively to treated control cybrids*.

TUNEL assay was performed, as described in methods section, in cybrid cells incubated during 24 hours at 37 ºC in the presence or in the absence of $A\beta_{1\text{-}40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM). Total number of TUNEL-positive cells in both cell lines was quantified and is represented in graph A and B. The results are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays. $*p<0.05$; $**p<0.01$; $***p<0.001$, significantly different with respect to control values. $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$, significantly different when compared to the same conditions in control cybrids.

5.4. Discussion.

The results here presented using AD cybrids, a cell line possessing the mitochondrial complex IV defect from AD patients (Ojaimi *et al.* 1999; Petrozzi *et al.* 2007), highlight the effect of mitochondrial dysfunction during ER stress-mediated apoptotic cell death induced by the Aβ peptide ($A\beta_{1–40}$ isoform) and emphasize the hypothesis of ER/mitochondria communication during Aβ-induced apoptosis.

Since it was first describe by King and Attardi (King & Attardi 1989), cybrid technique has been used as an in vitro model to study mitochondrial dysfunction in several neurodegenerative disorders, including AD (Swerdlow *et al.* 1997). It consists in the inclusion of mitochondria (and mtDNA) into host cells that have been depleted of their own mtDNA. Therefore, the differences between the cybrids lines (control and AD cybrids) are due to differences that have origin at mtDNA. Through last decade, several studies showed the capacity of AD cybrids to recreate many pathological features of AD. Among them, Sheehan and collegues (Sheehan *et al.* 1997) demonstrated decreased COX activity, increased basal cytosolic Ca^{2+} concentration and enhanced sensivity to IP3-mediated release of Ca^{2+} from ER. It was also reported reduced $\Delta\Psi m$, depletion of ATP levels, increased release of cytochrome c (Cassarino *et al.* 1998; Khan *et al.* 2000) as well as ROS production, lipid and protein oxidation, features that make these cells more susceptible to apoptosis (Cardoso *et al.* 2004a). Although impaired mitochondria are already present in these cells (Sheehan *et al.* 1997; Cassarino *et al.* 1998; Khan *et al.* 2000), deposition of $A\beta_{1\text{-}40/1\text{-}42}$ in AD cybrids can further potentiate mitochondrial dysfunction and cell death (Khan *et al.* 2000). In fact, AD cybrids are more vulnerable to Aβ toxicity, which induces a significant decrease in $\Delta\Psi m$,

mitochondrial release of cytochrome c and caspases activation (Cardoso *et al.* 2004a).

Using this AD cellular model, and also control cybrids obtained from platelets of age-matched control subjects, we evaluated the potential stressful effect of $A\beta_{1-40}$ peptide on ER and tested how mitochondrial dysfunction arising from COX inhibition affects $A\beta_{1-40}$-induced ER stress. As positive controls for ER stress, we used in our study two known ER stress inducers: thapsigargin, which disrupts ER Ca^{2+} homeostasis (Kuo *et al.* 1998), and brefeldin A, which blocks retrograde Golgi-derived vesicles leading to the accumulation of proteins in the ER lumen (Chardin & McCormick 1999).

In AD cybrids, characterized by the presence of a mitochondrial defect on the ETC COX, it was observed a significant increment in GRP78 expression levels upon treatment with $A\beta_{1-40}$ (1 μM), brefeldin A (2 μM) or thapsigargin (2 μM), in comparison with results obtained in treated control cybrids (Fig. 5.1 A-B). Under ER stress conditions, cells tend to increase the transcription of genes encoding ER-resident chaperones such as GRP78 as part of a defense mechanism termed unfolded protein response (Imaizumi *et al.* 2001). GRP78 promotes the correct folding of proteins that have been accumulated within the ER lumen and is considered to be an important molecule in the quality control of proteins, preventing unfolded proteins from being transported to the Golgi apparatus and further downstream (Kohno *et al.* 1993) and is thus considered a good marker for ER stress. Moreover, it has been shown to be increased in AD brains (Hoozemans *et al.* 2005) and to be up-regulated in cultured neurons upon Aβ treatment (Ferreiro *et al.* 2006).

Nakagawa and colleagues (Nakagawa *et al.* 2000) reported that caspase-12 knockout mice not only show resistance to ER stress but also to cell death

caused by Aβ. This study demonstrated that caspase-12 mediates an ER stress-specific apoptosis pathway (Cheung *et al.* 2006) and contributes to Aβ neurotoxicity, which was recently shown to be modulated via the UPS (Song *et al.* 2008). Despite this caspase is not present in humans, several studies suggested that caspase-4 is an ER stress responsive caspase in humans (Hitomi *et al.* 2004). Under ER stress conditions, caspase-4 is activated and can promote activation of caspase-3 (Kim *et al.* 2008a). However, other groups reported that ER stress only activates caspase-9 leading to apoptotic cell death by a mechanism that does not require caspase-4 (Obeng & Boise 2005). In the present study we measured caspase-4-like activity in both control and AD cybrid cells upon treatment with the ER stress inducers thapsigargin and brefeldin A, or $A\beta_{1-40}$ peptide. Under these conditions, we showed that this ER-resident caspase is activated in both cell lines (Fig. 5.2), but this increase is enhanced in AD cybrids, as observed for GRP78 levels (Fig. 5.1 A-B).

According to the literature, several groups point to the interaction between ER and mitochondria as an important process in normal cellular functioning, as well as during programmed cell death triggered by ER stress (Kim *et al.* 2006; Zhang & Armstrong 2007; Lei *et al.* 2008; Arduino *et al.* 2009; Klee *et al.* 2009; Sharaf El Dein *et al.* 2009; Vitiello *et al.* 2009). Our previous work supported the involvement of ER/mitochondria cross-talk during Aβ-induced neuronal death. We confirmed the induction of ER stress in cortical neurons by Aβ and the activation of an ER stress-mediated apoptotic pathway with mitochondrial involvement (Ferreiro *et al.* 2004; 2006; 2008b). Using NT2 cells treated with brefeldin A or tunicamycin (another ER stressor), we also observed that ER stress induces changes in the mitochondrial function, impairing mitochondrial membrane potential and activating caspase-9.

Moreover, stress stimuli at ER level evoked Ca^{2+} fluxes between ER and mitochondria (Arduíno *et al.* 2009). Further, the relevance of functional mitochondria to cellular death mediated by ER stress upon Aβ treatment was demonstrated in ρ cells, depleted of their mtDNA (Costa *et al.* 2010). Herein, we established that in both cybrid cell lines, $A\beta_{1-40}$, brefeldin A and thapsigargin induce apoptosis, since we observed caspase-3 activation (Fig. 5.4 A), PARP cleavage (Fig. 5.5) and a significant increase in TUNEL-positive apoptotic cells (Fig. 5.6). In AD cybrids, apoptosis triggered by Aβ, and by two ER stress inducers, was enhanced in comparison with control cybrids, indicating that compromised COX activity increases the susceptibility of these cells to ER stress-mediated cell death.

As previously described, activation of caspases, a family of cysteine proteases that cleave substrates at specific aspartate residues, is a central mechanism in the apoptotic cell death process (Thornberry & Lazebnik 1998). Upon cytochrome c release from mitochondria, apoptosome is formed and caspase-9 is activated initiating the caspase cascade leading to caspase-3 activation and consequent cell death (Li *et al.* 1997). Enhanced caspase-3 activation on treated cells supports the involvement of mitochondria in apoptosis triggered upon ER stress (Fig. 5.4 A). Our results further demonstrated that ER stress-mediated apoptosis was due to ER Ca^{2+} release induced by Aβ since dantrolene, an ER Ca^{2+} channel blocker, was able to prevent caspase-3 activation in both AD and control cybrids (Fig. 5.4 B). These results implicate Ca^{2+} in the communication between ER and mitochondria and are in accordance with our previous data (Costa *et al.* 2010) showing that in mtDNA-depleted ρ0 cells ER Ca^{2+} release and apoptotic cell death upon ER stress is abolished. Our results also agree with several reports that point ER

Ca^{2+} release and transfer to mitochondria, during ER stress, as pro-apoptotic (Chami *et al.* 2008) and responsible for the activation of caspase-3 (Gonzalez *et al.* 2010). Furthermore, Aβ-induced apoptosis is due, at least in part, to the perturbation of intracellular Ca^{2+} homeostasis (Scorziello *et al.* 1996; Huang *et al.* 2000; Ferreiro *et al.* 2008b; Resende *et al.* 2008b). We have previously demonstrated that in control cybrids Aβ induces a significant decrease in $\Delta\Psi m$, mitochondrial release of cytochrome c and caspases activation that occur to a greater extent in AD cybrids (Cardoso *et al.* 2004a; 2004b). Therefore, it cannot be excluded the possibility that in addition to their effects on ER, Aβ (and classical ER stressors) can also directly act on mitochondria, either in control and AD cybrids. However, in the present study, Aβ-induced caspase-3 activation occurring in both cybrids cell lines is prevented by dantrolene, demonstrating that Aβ effects on ER occur upstream of mitochondria, leading to cell death.

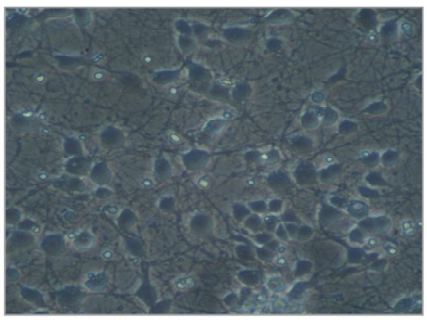
Taken together, the present study confirmed the interaction between ER and the mitochondria during apoptotic cell death that occurs in AD. Particularly, it was shown that the presence of a mitochondrial defect in complex IV of the ETC enhances cell death due to ER stress induced by the AD-associated Aβ peptide. These observations are in accordance with previous studies reporting that AD brains exhibit an increase in mtDNA control region mutations that can account for the sporadic appearance and mitochondrial defects seen in late-onset AD (Coskun *et al.* 2003). Further, variations in human mtDNA may contribute to individual differences in function and also to the increased susceptibility to various diseases such as AD (Shoffner *et al.* 1993; Hutchin & Cortopassi 1995; van der Walt *et al.* 2004). A reasonable explanation for this come from our group by Cardoso and colleagues (Cardoso *et al.* 2004a) who

demonstrated that complex IV inhibition in AD cybrids leads to increased ROS production. This could represent a potential mechanism underlying the increased cellular susceptibility to toxic insults, like ER stress, and might account for the neurodegenerative process that occurs in sporadic AD. By last, our results further suggest that the ER stress-mediated cell death pathway triggered by Aβ implicated in the activation of could be a potential target for therapeutic intervention in AD.

CHAPTER 6

Inhibition of mitochondrial cytochrome c oxidase potentiates endoplasmic reticulum Ca^{2+} deregulation and cell death induced by amyloid β-peptide.

Primary cultures of rat cortical neurons



Based on: Rui O. Costa, Catarina R. Oliveira and Cláudia M.F. Pereira. Inhibition of mitochondrial cytochrome c oxidase potentiates endoplasmic reticulum Ca^{2+} deregulation and cell death induced by amyloid β-peptide.
(Submitted for publication.)

6.1. Summary.

Previously we reported that Aβ leads to ER stress in cultured cortical neurons and that ER-mitochondria cross-talk is involved in Aβ-induced apoptotic neuronal cell death. Furthermore, we have shown that mitochondrial dysfunction affects the ER stress response triggered by Aβ using cybrid cells, which recreate the defect in COX activity present in platelets obtained from AD patients.

In order to further investigate the impact of COX inhibition on Aβ-induced ER dysfunction, a neuronal model was used. Primary cultures of rat brain cortical neurons were challenged with toxic concentrations of Aβ upon chemical inhibition of COX with KCN. In parallel, cultured cortical neurons were treated with the widely used ER stressor thapsigargin, which perturbs ER Ca^{2+} homeostasis. In Aβ-treated cortical neurons, a significant depletion of ER Ca^{2+} content was found and it was shown to be potentiated upon KCN-induced COX inhibition. KCN pre-incubation also enhanced the rise in cytosolic Ca^{2+} levels induced by Aβ or thapsigargin that was reverted in the presence of dantrolene, an inhibitor of ER Ca^{2+}-release through ryanodine receptors. Moreover, a significant decrease in cell survival and activation of apoptosis-related caspase-9 and -3 was observed in Aβ-or thapsigargin-treated neurons that were more pronounced after COX inhibition by KCN. These results demonstrate that the impairment of COX activity potentiates the Aβ-induced ER and cytosolic Ca^{2+} dyshomeostasis and activation of the mitochondria-mediated apoptotic cell death pathway.

Our data supports that mitochondria dysfunction arising from the disruption of COX activity in AD enhances the cellular susceptibility to toxic insults, namely to Aβ-induced ER stress. Therefore, this study strongly suggests that

the close communication between ER and mitochondria can be a valuable therapeutic target in AD.

6.2. Introduction.

AD is the most widespread form of dementia in the elderly that is due to synaptic and neuronal loss in the hippocampus and cerebral cortex. AD patients present extracellular senile plaques in susceptible brain regions, mainly composed of fibrillar Aβ that constitute one of the neuropathological hallmarks of this neurodegenerative disorder (Selkoe 2001).

Mitochondria plays a crucial role in providing energy to cells through ATP synthesis but also in activation of cell death pathways. Mitochondrial dysfunction has been implicated in several neurodegenerative diseases, including AD where it was described as a trigger for the early pathological events (Ienco *et al.* 2011). In fact, multiple defects in energy metabolism are associated with AD neuropathology and cognitive deficits (reviewed in Ferreira *et al.* 2010). In addition, alterations in the activity of several mitochondrial metabolic enzymes were reported in individuals with MCI (Valla *et al.* 2006). During this pre-clinical stage of the disease, synaptic alterations (Scheff *et al.* 2007) and brain glucose hypometabolism have been described (Mosconi *et al.* 2008). These metabolic alterations seem to involve the impairment of the ETC activity, in particular of COX (Small *et al.* 1995; Mosconi 2005; Ferrer 2009). Compromised energy metabolism increases the production of the AD-associated Aβ peptide (Velliquette *et al.* 2005). Along with these evidences, mitochondrial associated secretases were recently proved to be involved in APP processing and Aβ generation, showing that this peptide

can be produced locally in the mitochondria (Pavlov *et al.* 2010). The presence of Aβ in mitochondria has been demonstrated (Caspersen *et al.* 2005; Hansson Petersen *et al.* 2008) and it correlates with the extent of mitochondrial dysfunction due to inhibition of ETC complexes and the degree of cognitive impairment in AD transgenic mice (reviewed in Chen & Yan 2010; Dragicevic *et al.* 2010).

In addition to mitochondria, dysfunction of other cellular organelles such as the ER has been implicated in AD pathogenesis (Hoozemans *et al.* 2005). Previously, we provided evidences that Aβ can be a trigger for ER stress and demonstrated that ER/mitochondria cross-talk plays an essential role during Aβ-induced neuronal apoptosis (Ferreiro *et al.* 2006; Costa *et al.* 2010; 2011).

Using primary cultured rat brain cortical neurons as a neuronal model, here we investigated the role of the AD-associated mitochondrial impairment, in particular the inhibition of COX activity, in Aβ-induced ER Ca^{2+} dyshomeostasis and subsequent activation of apoptotic cell death. For this purpose, COX inhibition was induced by KCN treatment previously to neuronal exposure to toxic Aβ concentrations. Then, ER and cytosolic Ca^{2+} levels were monitored. Furthermore, cell viability was evaluated and activation of the apoptotic cell death was followed through the analysis of caspase-9- and caspase-3-like activities. As a positive control for ER Ca^{2+} dysregulation, a parallel study was conducted using thapsigargin, a classical disruptor of ER Ca^{2+} homeostasis (Rogers *et al.* 1995).

Overall, we demonstrated that mitochondrial impairment due to COX inhibition increases the cellular susceptibility to Aβ-induced apoptosis mediated by impaired ER Ca^{2+} homeostasis, supporting that ER/mitochondria cross-talk plays a crucial role during Aβ-induced cell death in AD.

6.3. Results.

In the present study, we used primary cortical neuronal cultures treated with the $A\beta_{1-40}$ upon mitochondrial COX inhibition to further disclose the role of ER/mitochondria cross-talk in cell death triggered by Aβ. In order to mimic the mitochondrial defect (COX inhibition) associated to AD, we used the chemical COX inhibitor KCN. The effect of Aβ in neuronal cells was compared with that of thapsigargin, a widely used ER stressor that disrupts ER Ca^{2+} homeostasis (Rogers *et al.* 1995; Kuo *et al.* 1998).

6.3.1. Mitochondrial COX inhibition enhances $A\beta_{1-40}$-induced ER Ca^{2+} dyshomeostasis

ER stress is associated with the disruption of ER Ca^{2+} homeostasis (Xu *et al.* 2005). Therefore, we monitored the levels of ER Ca^{2+} in cortical neurons treated with $A\beta_{1-40}$ (1 μM) during 3 hours, in the absence or in the presence of the COX inhibitor KCN (1 mM). We found that ER Ca^{2+} levels decrease by about 50% in $A\beta_{1-40}$-treated neurons in comparison with controls and this effect was enhanced in cells pre-incubated with KCN (Fig. 6.1 A-B). ER Ca^{2+} depletion was demonstrated to be followed by an increase in cytosolic Ca^{2+} concentration measured with the fluorescent probe Indo-1/AM after 6 hours incubation with $A\beta_{1-40}$. Similar results were obtained in neurons treated with thapsigargin (2 μM) for 6 hours (Fig. 6.2). Pre-incubation with KCN before $A\beta_{1-40}$ or thapsigargin treatment potentiated the increase in cytosolic Ca^{2+} levels that results from ER Ca^{2+} release since dantrolene (10 μM), an inhibitor of Ca^{2+} channels coupled to the ER RyR, significantly prevented the effect of

KCN (Fig. 6.2). These results indicate that a mitochondrial defect, in particular the inhibition of COX activity sensitizes cells to ER Ca^{2+} dyshomeostasis induced by neurotoxic concentrations of $A\beta_{1-40}$.

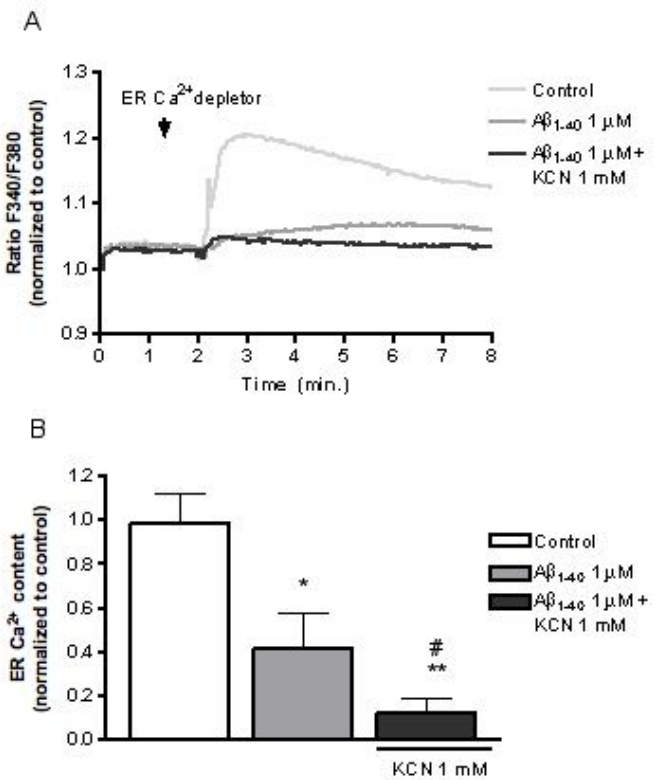


Figure 6.1 *COX inhibition upon KCN treatment potentiates the depletion of ER Ca^{2+} content induced by $A\beta_{1-40}$ in cortical neurons.*
Primary cultures of rat brain cortical neurons were incubated for 3 hours with $A\beta_{1-40}$ (1 μM), in the absence or in the presence of KCN (1mM) which was added 1 hour before $A\beta_{1-40}$. Then, levels of Ca^{2+} in ER stores were evaluated by monitoring the fluorescence of Fura-2 in the absence of external Ca^{2+}. The difference between Fura-2 fluorescence ratio 340/380 nm before and after the full depletion of ER Ca^{2+} was used to evaluate ER Ca^{2+} content. Results are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays. *$p<0.05$; **$p<0.01$; significantly different when compared to control values. $^{\#}p<0.05$; significantly different when compared to $A\beta_{1-40}$-treated cells.

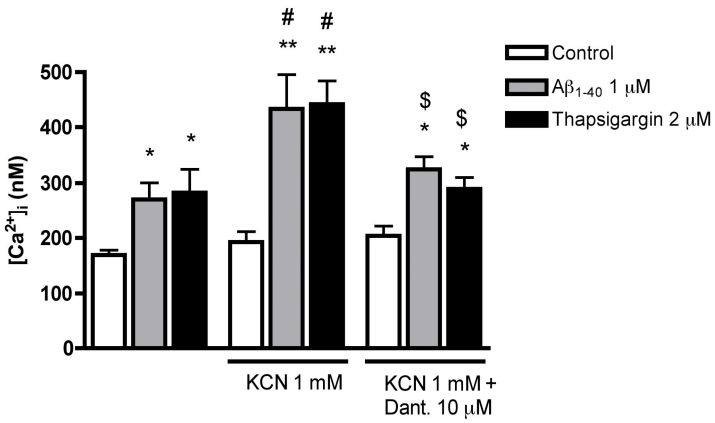


Figure 6.2 *COX inhibition by KCN potentiates the increase in basal cytosolic Ca^{2+} levels induced by $A\beta_{1-40}$ or thapsigargin.*

Primary cultures of rat brain cortical neurons were incubated for 6 hours with $A\beta_{1-40}$ or thapsigargin (2 μM), in the absence or in the presence of KCN (1 mM), which was added 1 hour before $A\beta_{1-40}$ and thapsigargin. When used, dantrolene (10 μM) was added 1 hour before $A\beta_{1-40}$ or thapsigargin incubation. Then, cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ was determined using the fluorescent Ca^{2+} indicator Indo-1/AM. Indo-1 fluorescence was measured fluorimetrically, at 350 nm excitation and 410 nm emission, and $[Ca^{2+}]_i$ i was calculated. Results are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays. *$p<0.05$; **$p<0.01$; significantly different when compared to controls. $^{\#}p<0.05$; significantly different when compared to $A\beta_{1-40}$- or thapsigargin-treated cells. $^{\$}p<0.05$; significantly different when compared to KCN pre-treated cells.

6.3.2. $A\beta_{1-40}$- and thapsigargin-induced apoptotic cell death is enhanced by COX inhibition

In order to better understand the role of mitochondrial COX inhibition in cell death induced by Aβ, we evaluated cellular viability and activation of apoptosis-associated caspases, namely caspase-9 and caspase-3, in primary cultured cortical neurons treated with $A\beta_{1-40}$. To implicate the impairment of ER Ca^{2+} homeostasis in Aβ-induced neuronal death under mitochondrial dysfunction conditions, a parallel study was performed with thapsigargin, which depletes ER Ca^{2+} stores.

First, we analyzed the metabolic ability of cortical neuronal cultures by the MTT colorimetric assay, which gives information about cell survival. Upon 24 hours of incubation with $A\beta_{1-40}$ or thapsigargin, a significant decrease in neuronal cell survival was observed, which was potentiated by KCN pre-treatment (Fig. 6.3).

Then, we explored the involvement of the pro-apoptotic caspase-9 and caspase-3 in $A\beta_{1-40}$- or thapsigargin-induced neurotoxicity. After 24 hours, both $A\beta_{1-40}$ and thapsigargin increased caspase-9-like (Fig. 6.4 A) and caspase-3-like (Fig. 6.4 B) activities, which were more pronounced in KCN pre-treated neurons (Fig. 6.4 A-B).

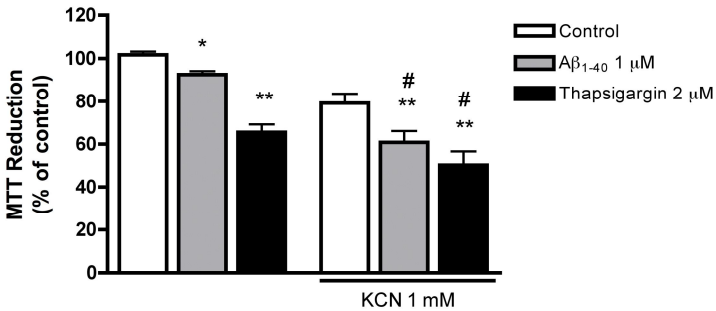


Figure 6.3 *Compromised cell survival upon $A\beta_{1-40}$ or thapsigargin treatment is enhanced by COX inhibition.*

Primary cultures of rat brain cortical neurons were incubated for 24 hours with $A\beta_{1-40}$ (1 μM) or thapsigargin (2 μM), in the absence or in the presence of KCN (1 mM) which was added 1 hour before the toxic insults. Cell viability was evaluated by measuring the reduction of the tetrazolium salt MTT. Results are the means ± SEM of values corresponding at least to 3 independent experiments, performed in duplicate, and are represented as the percentage (%) of absorbance measured in controls. *p <0.05; **p <0.01; significantly different when compared to control values. $^{\#}p < 0.05$; significantly different when compared to the same condition in the absence of KCN pre-treatment.

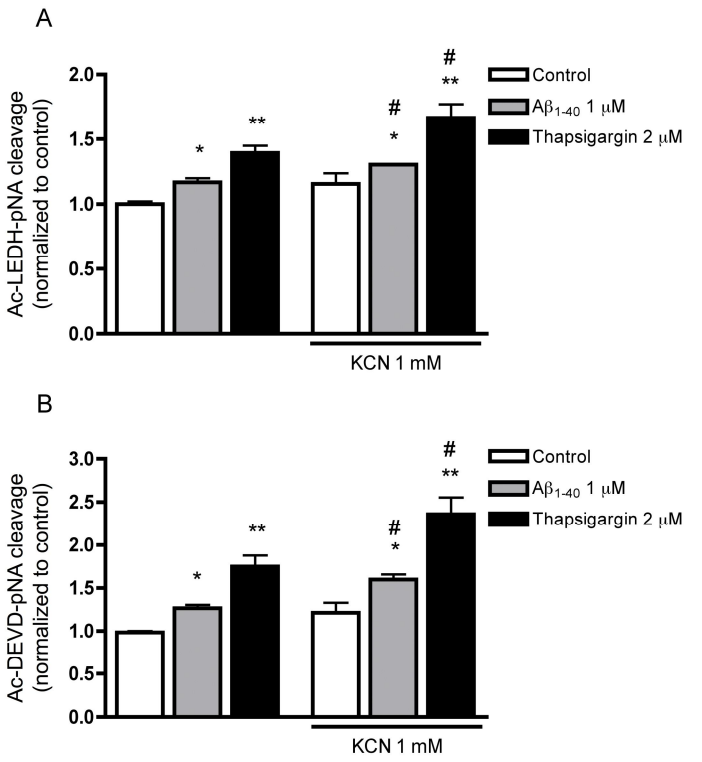


Figure 6.4 *Caspase-9 and caspase-3 activation upon $A\beta_{1-40}$ or thapsigargin treatment is potentiated under COX inhibition conditions.*
Primary cultures of rat brain cortical neurons were incubated for 24 hours with $A\beta_{1-40}$ (1 μM) or thapsigargin (2 μM), in the absence or in the presence of KCN (1 mM). When used, KCN was added 1 hour before $A\beta_{1-40}$ or thapsigargin. Caspase-9-like (A) and caspase-3-like (B) activities were determined by measuring at 405 nm the cleavage of the chromogenic substrates Ac-LEDH-pNA or Ac-DEVD-pNA, respectively. Results are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays. *$p<0.05$; **$p<0.01$; significantly different when compared with respect to control values. $^{\#}p<0.05$; significantly different when compared to $A\beta_{1-40}$–or thapsigargin-treated cells.

Overall, the results described above demonstrate that mitochondrial dysfunction occurring through KCN-induced COX inhibition potentiates apoptosis triggered by ER Ca^{2+} depletion in Aβ-treated cortical neurons. These findings are schematically represented in Fig. 6.5.

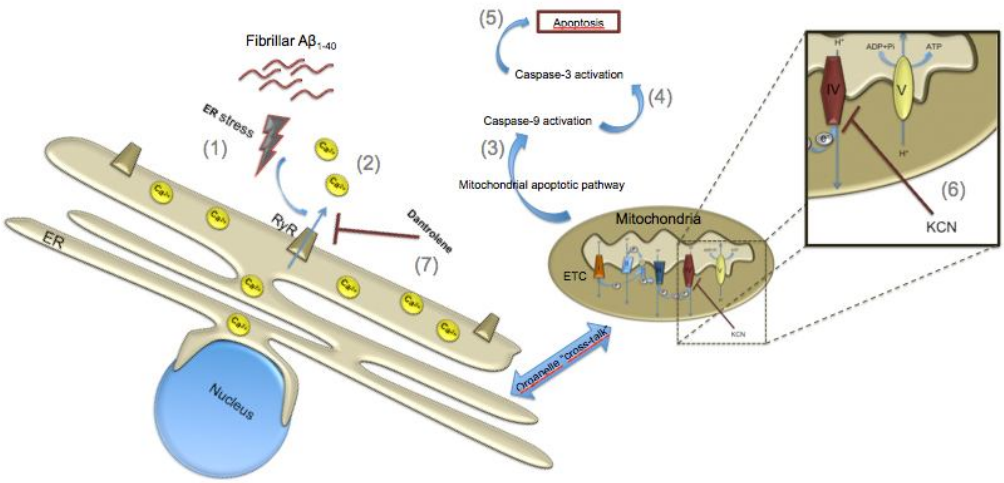


Figure 6.5 *Summary of the molecular mechanisms leading to enhanced Aβ-induced ER Ca^{2+} deregulation under mitochondrial dysfunction conditions in cortical neurons.*
$Aβ_{1-40}$ perturbs ER Ca^{2+} homeostasis, leading to ER stress, by increasing the release of Ca^{2+} through the ER RyR-associated Ca^{2+} channels (1). As a consequence, cytosolic Ca^{2+} levels increase (2). This culminates in decreased cellular survival and activation of a caspase-mediated apoptotic cell death pathway that involves caspase-9 (3) followed by caspase-3 (4), finally leading to cell death by apoptosis (5). These damaging effects triggered by $Aβ_{1-40}$ are potentiated when COX activity of the mitochondrial ETC is inhibited by KCN, thus mimicking a mitochondrial defect implicated in AD pathogenesis. One of the consequences of the communication between mitochondria and ER upon COX inhibition (6) is the increase in ER Ca^{2+} release through RyR-associated Ca^{2+} channels that can be antagonized by dantrolene (7).

6.4. Discussion.

Previously we reported that Aβ induces ER stress in primary cultured cortical neurons, promoting ER Ca^{2+} release and increasing the levels of several ER stress markers (Ferreiro *et al.* 2006; Resende *et al.* 2008a; 2008b). Other studies from our group demonstrated that ER-mitochondria cross-talk is involved in Aβ-induced apoptosis. First, we showed that Aβ-induced ER stress-mediated apoptotic pathway requires the presence of functional mitochondria using mtDNA-depleted ρ0 cells (Cardoso *et al.* 2001; 2002; 2004b; Costa *et al.* 2010). Second, we demonstrated that the activation of the mitochondrial apoptotic pathway by Aβ in cortical neurons triggers the translocation of Bax to mitochondria and consequent loss of $\Delta\Psi m$, cytochrome c release and activation of caspase-9 and -3 (Ferreiro *et al.* 2008a) and it is prevented by the ER Ca^{2+} release inhibitors dantrolene and xestospongin C. Furthermore, we provided evidences that mitochondrial dysfunction affects the ER stress response triggered by Aβ using cybrids that recreate the defect in COX activity present in platelets from AD patients (Costa *et al.* 2011). Taken together, these findings demonstrate that mitochondria are intimately involved in Aβ-induced cell death occurring through ER Ca^{2+} dyshomeostasis and stress.

This study was aimed to demonstrate that mitochondrial dysfunction arising from COX inhibition affects the neuronal response to ER Ca^{2+} dyshomeostasis and to cell death induced by the AD-associated Aβ peptide. In primary cortical neuronal cultures, we demonstrated that KCN-induced COX inhibition potentiates ER Ca^{2+} depletion, cytosolic Ca^{2+} rise due to ER Ca^{2+} release, caspase-9 and caspase-3 activation and compromised cell survival triggered by $A\beta_{1-40}$. Additionally, inhibition of COX activity with KCN enhanced the increase in Ca^{2+} levels and the activation of apoptotic cell death observed in

thapsigargin-treated neurons. These findings demonstrate that impaired COX activity potentiates the toxic effects of Aβ occurring through the perturbation of ER Ca^{2+} homeostasis in cortical neurons. Mitochondria, almost independent organelles containing their own DNA, are the main energy suppliers to neurons. This is accomplished through the ETC, a pentameric complex partially coded by mtDNA, which leads to ATP synthesis. Electron transport between complexes I to IV is coupled to proton extrusion generating a $\Delta\Psi m$, and the energy created by the protons gradient is used by complex V to synthesize ATP. Mitochondrial diseases are often caused by mutations of the mtDNA that affect mitochondrial function. Individual differences in human mtDNA were shown to be associated with increased susceptibility to various diseases including AD (Shoffner *et al.* 1993; Hutchin & Cortopassi 1995; van der Walt *et al.* 2004; Santoro *et al.* 2010). In this neurodegenerative disorder, defects in mitochondrial ETC, namely in COX activity, have been reported (Kish *et al.* 1992; Bosetti *et al.* 2002; Petrozzi *et al.* 2007; Pickrell *et al.* 2009). Several evidences obtained during the last decade, including observations in AD brains, led to the hypothesis that ETC malfunction could be due to the accumulation of mtDNA mutations (Lin *et al.* 2002; Coskun *et al.* 2003; Lin & Beal 2006; Kruger *et al.* 2010). ETC was also demonstrated to be an apoptosis modulator (Kwong *et al.* 2007). In fact, the interaction of toxic insults with ETC components, instead of energy failure due to ETC disruption, was shown to be determinant to the cellular vulnerability to apoptosis. In addition, the partial blockage of the ETC seems to be more deleterious to cell survival than its complete inhibition (Kwong *et al.* 2007). The partial ETC inhibition could contribute to severe ROS (reactive oxygen species) production, which along

with Ca^{2+} could play an important role in the interaction between ER and mitochondria during apoptosis (reviewed in Csordas & Hajnoczky 2009).

Using primary cultures of rat brain cortical neurons challenged with toxic concentrations of $A\beta_{1-40}$, we first evaluated the loss of ER Ca^{2+} homeostasis, a trigger for ER stress that has been implicated in AD pathogenesis (Scheper *et al.* 2011). Upon $A\beta_{1-40}$ treatment, we obtained evidences of Ca^{2+} release from ER to the cytosol, since a significant depletion ER Ca^{2+} stores was observed (Fig. 6.1), that was followed by an increase in cytosolic Ca^{2+} levels (Fig. 6.2). These results are in accordance with previous studies that demonstrated Ca^{2+} leakage from ER stores during Aβ-induced apoptosis (Scorziello *et al.* 1996; Huang *et al.* 2000; Ferreiro *et al.* 2004; Ferreiro *et al.* 2008b; Resende *et al.* 2008b; Costa *et al.* 2011). Significantly, we showed that the release of Ca^{2+} from the ER to the cytosol in $A\beta_{1-40}$-treated cortical neurons is potentiated in cells previously incubated with KCN, a COX inhibitor (Figures 6.1-6.2). Furthermore, dantrolene was able to revert the effect of KCN on cytosolic Ca^{2+} rise triggered by $A\beta_{1-40}$ or thapsigargin, a known ER stress inducer that impairs ER Ca^{2+} homeostasis (Fig. 6.2), demonstrating that ER Ca^{2+} release through ER receptor-associated Ca^{2+} channels is enhanced under COX inhibition conditions.

Our results are in agreement with a study from Sheehan and colleagues (1997) in which the increased basal cytosolic Ca^{2+} levels and the enhanced sensitivity to IP3R-mediated ER Ca^{2+} release correlate with decreased COX activity. We have recently demonstrated that compromised COX activity increases cellular susceptibility to $A\beta_{1-40}$-induced ER stress in AD cybrids which recreate the mitochondrial defect present in platelets from AD patients (Costa *et al.* 2011). In this cybrid model, we have also demonstrated that COX

inhibition potentiates $A\beta_{1-40}$-induced ROS production, decreased $\Delta\Psi m$ and cytochrome c release (Cardoso *et al.* 2004b). In turn, cytochrome c released from mitochondria, can bind to Ca^{2+} channels associated with ER receptors, namely with the IP3R, leading to Ca^{2+} release (Boehning *et al.* 2003; 2004). Under these conditions, the Ca^{2+} signal is transmitted from IP3R to mitochondria and promotes the opening of the mitochondrial PTP with consequent cytochrome c release and amplification of cell death (Boehning *et al.* 2005). On the other hand, a possible explanation for our results is that COX inhibition decrease ATP levels both as a consequence of ETC malfunction and also due to the production of ROS which can directly impair ATPases (reviewed in Mattson & Chan 2003). Lower ATP levels will affect ATP depend-Ca^{2+} pumps responsible for Ca^{2+} storage in ER compartment compromising Ca^{2+} homeostasis and decreasing ER Ca^{2+} content and subsequently increasing cytosolic Ca^{2+} levels, as we reported here.

Under ER stress conditions, apoptotic cell death pathways can be activated (Kudo 2011; Tabas & Ron 2011). ER stress-mediated apoptosis can occur through activation of the ER associated caspase-12/4 and subsequent caspase-3 activation (Kim *et al.* 2009) upon cytochrome c release from mitochondria, apoptosome formation and caspase-9 activation. However, some authors consider that the ER stress-mediated apoptotic cell death pathway is independent from caspase-12/4 and that Ca^{2+} released from ER can then be transferred to mitochondria, acting as a pro-apoptotic signal (Chami *et al.* 2008) responsible for the activation of caspase-3 (González *et al.* 2010).

Here, we showed that caspase-9 and the downstream apoptosis-effector caspase-3 are activated by toxic $A\beta_{1-40}$ concentrations (Fig. 6.4) that promote the release of Ca^{2+} from the ER (Figures 6.1-6.2). Caspases activation was

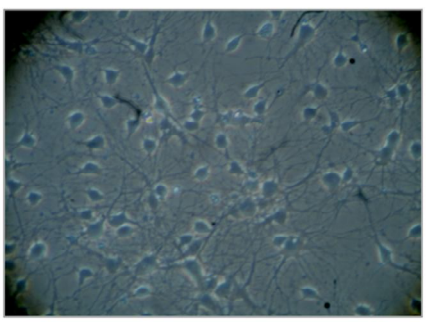
associated with a decrease in cell survival and both parameters were potentiated when cortical neurons were treated with $A\beta_{1-40}$ under COX inhibition conditions (Figures 6.3-6.4). However, we cannot exclude the possibility that in addition to the effect of $A\beta_{1-40}$ on ER Ca^{2+} homeostasis, it can directly acts on mitochondria leading to the release of pro-apoptotic factors, as suggest by several authors (Chen & Yan 2010; Pagani & Eckert 2011).

In conclusion, the results here described show that mitochondrial dysfunction, ocurring through COX inhibition, potentiates apoptotic neuronal death induced under ER stress conditions triggered by Aβ and support our previous evidences of a close communication between ER and mitochondria during cell death in AD. These findings further support that mitochondrial dysfunction represents a crucial mechanism underlying the neurodegenerative process that occurs in sporadic AD by increasing cellular susceptibility to toxic insults, in particular to ER stress.

CHAPTER 7

Endoplasmic reticulum stress occurs downstream of NR2B subunit of N-methyl-D-aspartate receptor in mature hippocampal cultures treated with amyloid-β oligomers

Primary cultures of rat hippocampal neurons



Based on: Rui O. Costa, Pascale N. Lacor, Ildete L. Ferreira, Yves P. Auberson, William L. Klein, Ana C. Rego, Catarina R. Oliveira and Cláudia M.F. Pereira. Endoplasmic reticulum stress occurs downstream of NR2B subunit of N-methyl-D-aspartate receptor in mature hippocampal cultures treated with amyloid-β oligomers.
(Submited for publication.)

7.1. Summary.

AD is a progressive neurodegenerative disorder affecting both the hippocampus and the cerebral cortex. Reduced synaptic density that occurs early in the disease process seems to be partially due to the overactivation of NMDARs leading to excitotoxicity. Recently, we demonstrated that AβO, the species implicated in synaptic loss during the initial disease stages, ER stress in cultured neurons. Here, we investigated whether AβO trigger ER stress by an NMDAR-dependent mechanism leading to neuronal dysfunction and analyzed the contribution of NR2A and NR2B subunits of this glutamate receptor. Our data revealed that AβO induces ER stress in mature hippocampal cultures, activating ER stress-associated sensors and increasing the levels of the ER chaperone GRP78. We also showed that AβO induces NOX-mediated superoxide production downstream of NR2B and impairs ER and cytosolic Ca^{2+} homeostasis. These events precede changes in cell viability and activation of the ER stress-mediated apoptotic pathway, which was associated with translocation of the transcription factor GADD153/CHOP to the nucleus and occurred by a caspase-12-independent mechanism. Significantly, ER stress took place after AβO interaction with NR2B subunits. In addition, AβO-induced ER stress and hippocampal dysfunction were prevented by ifenprodil, an antagonist of NR2B subunits while the NR2A antagonist NVP-AAM077 only slightly attenuated AβO-induced neurotoxicity. Taken together, our results highlight the role of NR2B subunit of NMDARs on ER stress-mediated hippocampal dysfunction caused by AβO suggesting that it might be a potential therapeutic target during the early stages of AD.

7.2. Introduction.

AD is the most prevalent neurodegenerative disorder that currently affects almost 35 million people worldwide. It is a chronic and progressive neurodegenerative illness characterized by memory deficits and cognitive decline due to synaptic and neuronal loss in the hippocampus and cerebral cortex. The abnormal deposition of Aβ in these brain regionshas suggested that this peptide plays an essential role in AD pathogenesis (Selkoe 2001). Initially, only Aβ deposited in extracellular plaques was assumed to be neurotoxic, but recent findings suggest that soluble AβO might be the culprits in AD pathology (Sakono & Zako 2010; Tomiyama *et al.* 2010). Indeed, AβO have been implicated in the impairment of synaptic plasticity and associated memory dysfunction during early AD stages and severe neuronal degeneration and dementia that occur during end disease stages (Lambert *et al.* 1998; Golde *et al.* 2006; Heinitz *et al.* 2006; Lesne *et al.* 2006).

In vitro and *in vivo* studies support that synaptic dysfunction and neuronal death occurring in AD are related with ER stress (Sato *et al.* 2001; LaFerla 2002; Katayama *et al.* 2004). Besides, reports revealed altered levels of ER stress markers in the brain of AD patients (Hoozemans *et al.* 2005). Concurrently, in a transgenic mice model of AD, ER stress-related genes were proven to be differentially regulated during the initial and intermediate stages of Aβ deposition (Selwood *et al.* 2009). Furthermore, fibrillar Aβ was shown to be involved in neuronal ER stress, triggering depletion of ER Ca^{2+} stores and activation of an ER-mediated apoptotic cell death pathway (Ferreiro *et al.* 2006; Costa *et al.* 2010). Recently, it was also demonstrated that AβO can act as a trigger for ER stress (Resende *et al.* 2008b; Nishitsuji *et al.* 2009).

There is growing consensus that the loss of glutamatergic synapses correlates with the onset and severity of memory impairment that precedes late neurodegeneration in AD (Parameshwaran *et al.* 2008; Shankar & Walsh 2009). Reduced synaptic density and selective neuronal dysfunction that occurs in the disease seems to be partially due to the overactivation of NMDARs and the consequent increase of intracellular Ca^{2+} leading to excitotoxicity (Kelly & Ferreira 2006). Aβ, including AβO, was shown to activate NMDARs (Dinamarca *et al.* 2008; Alberdi *et al.* 2010). However, little is known about the relative contribution of individual NMDAR subunits, namely NR2A and NR2B, to AβO-induced neuronal dysfunction and apoptotic cell death. Furthermore, it was suggested that AβO increase NOX-mediated superoxide production through activation of NMDARs (Shelat *et al.* 2008) and recent evidences demonstrate that NOX is the main source of superoxide radicals generated upon NMDAR activation (Brennan *et al.* 2009). Indirect interaction of Aβ with NMDARs was also demonstrated, namely with the extracellular domains of NR1 subunit, since this subunit was found in the AβO-pulled protein complex (De Felice *et al.* 2007; Lacor *et al.* 2007). AβO interaction with NMDARs has been suggested to lead to synapse damage and loss of hippocampal neurons (Klein 2006; Nathalie Lacor 2007). In both cortical and hippocampal neuronal cultures, Aβ was also demonstrated to modify cell surface expression of the NR1 and NR2B subunits of NMDARs (Snyder *et al.* 2005; Johansson *et al.* 2006; Lacor *et al.* 2007). Excitatory synapses containing the NR2B subunit of the NMDAR appear to be principal sites of AβO accumulation, which can be counteracted by NMDAR antagonists (Deshpande *et al.* 2009). Recently, it was also shown that selective antagonists of NMDARs containing the NR2B subunit prevent Aβ-mediated inhibition of

plasticity in the hippocampus *in vivo* (Hu *et al.* 2009). Additionally, the uncompetitive NMDARs antagonist memantine, used to improve cognitive function in moderate-severe AD patients (Lipton 2006), inhibits excitotoxicity due to overactivation of NMDARs (Parsons *et al.* 2007). Moreover, memantine prevents AβO-induced synapse loss, oxidative stress and Ca^{2+} influx in hippocampal neuronal cultures (De Felice *et al.* 2007; Lacor *et al.* 2007) and antagonizes the *in vivo* NMDAR-mediated activity in the hippocampus (Szegedi *et al.* 2010). Memantine also reduces neurodegeneration in rat hippocampus induced by Aβ (Miguel-Hidalgo *et al.* 2002), revealing the therapeutic importance of blocking NMDARs.

Here we investigated whether AβO trigger ER stress by a NMDAR-dependent mechanism in mature hippocampal cultures focusing on the contribution of NR2A and NR2B receptor subunits. Our results show that AβO-induced ER stress and hippocampal neuronal dysfunction are prevented by antagonists of NMDAR subunits, particularly by the NR2B antagonist ifenprodil. ER stress-mediated hippocampal dysfunction occurred after the close interaction between AβO and NR2B and subsequent NOX-mediated superoxide radical production.

The present study uncovers a novel pathway underlying hippocampal cell death triggered by AβO, thus contributing to elucidate the molecular mechanisms involved in the neurotoxic effect of soluble oligomeric Aβ, one of the crucial features in AD pathology.

7.3. Results.

In the present study, we investigated the hypothesis that AβO induce ER stress in hippocampal cells by a mechanism dependent on the activation of NMDARs. Furthermore, we discriminated the role of the NMDAR subunits NR2A and NR2B in this process. As a cellular model, we used primary mature hippocampal cultures with 17-21 days *in vitro* expressing both NR2A and NR2B at high levels (data not shown) as previously described (Luo *et al.* 2002).

7.3.1. Blockage of extracellular domains of NR2B subunits reduces AβO binding at synaptic terminals.

In order to analyze whether AβO can interact with NMDAR subunits, primary hippocampal cultures were treated with AβO together with antibodies directed against the extracellular termini of NR1, NR2A or NR2B. It was observed that AβO binding to mature hippocampal cells is prevented when the extracellular termini of NR1 and NR2B, but not of NR2A, is blocked. Indeed, a statistically significant reduction of AβO puncta density occurred in cells treated with AβO in the presence of anti-NR1 or anti-NR2B antibodies whereas antibodies directed against NR2A were ineffective (Fig. 7.1). AβO binding was not affected by anti-NR2A or NR2B antibodies previously pre-adsorbed with their respective control antigen peptide or by an antibody anti-C terminus of NR1 (Fig. 7.1).

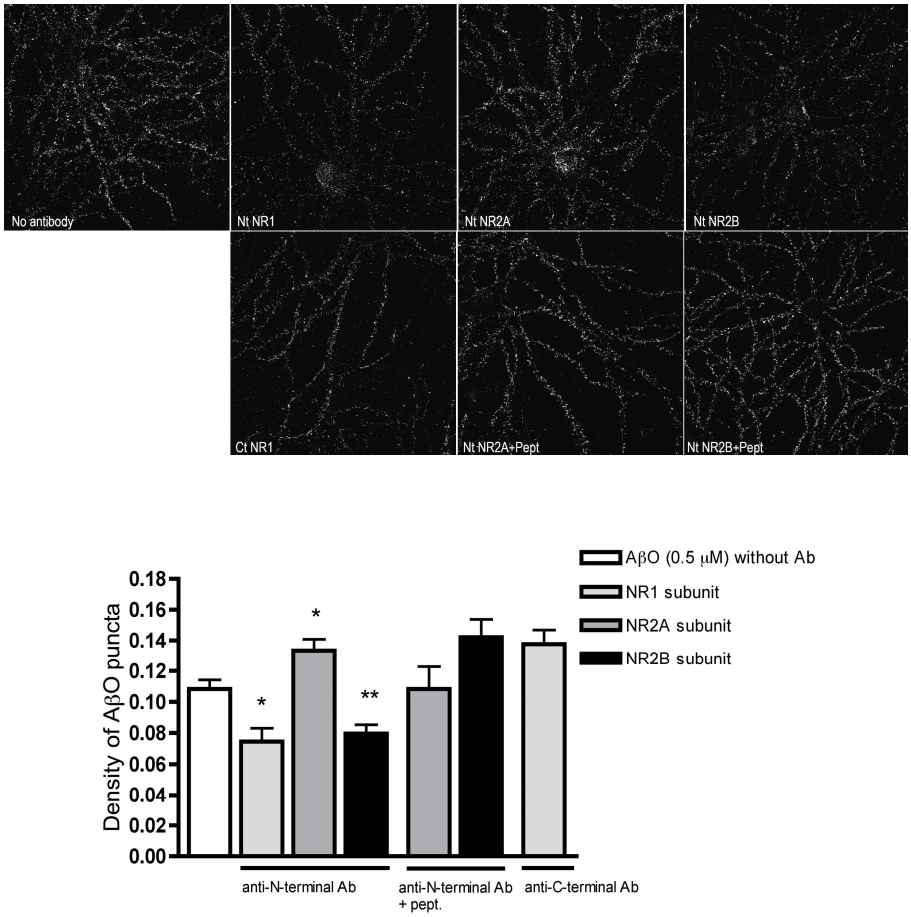


Figure 7.1 *AβO binding at synaptic terminals in mature hippocampal cultures*.
Cells were treated for 15 min with 0.5 μM AβO and binding was then evaluated by confocal microscopy with the NU-1 antibody. Blocking of AβO hot-spot binding was performed by incubating cells with 5 μg/mL of antibodies directed to the extracellular epitopes (nt) of NR1, NR2A or NR2B prior to AβO treatment. Controls included antibodies directed against the intracellular epitopes (ct) of these subunits or antibodies pre-adsorbed with the control peptide antigen. Values are the means ± SEM of at least 3 independent experiments. *$p<0.05$; **$p<0.01$ compared with AβO-treated cells in the absence of antibodies against NMDAR subunits.

7.3.2. Aβ oligomers-induced endoplasmic reticulum stress in hippocampal cultures is prevented by an NR2B antagonist.

Previous studies from our laboratory established that Aβ, including AβO, induces ER stress in primary cultured cortical neurons (Ferreiro *et al.* 2006; Resende *et al.* 2008a; Resende *et al.* 2008b). Here, we evaluated the ER stress response in hippocampal cultures challenged with AβO. After 6 hours treatment with 0.5 μM AβO, a significant increase in the levels of the ER chaperone GRP78 occurred as revealed by WB analysis (Fig. 7.2 A). Similar results were obtained for XBP-1 (Fig. 7.2 B), which indirectly evaluates the activation of IRE1α, one of the three main ER stress sensors, further providing evidences for altered ER function in the presence of AβO. Additionally, when hippocampal cells were incubated for 6 hours with 2 μM thapsigargin, a widely used ER stress inducer, GRP78 and XBP-1 levels significantly increased (Fig. 7.2 A-B) reaching levels similar to those obtained after AβO stimulation.

To investigate the role of NMDAR subunits in ER stress triggered by AβO in hippocampal cultures, we used the specific antagonists NVP-AAM077 and ifenprodil, which block NR2A and NR2B subunits, respectively (Reynolds & Miller 1989; Liu *et al.* 2004). Results demonstrated that ifenprodil, and to a lesser extent NVP-AAM077, can prevent ER stress that occurs after exposure of hippocampal cultures to AβO, restoring the levels of GRP78 (Fig. 7.2 A) and XBP-1 (Fig. 7.2 B). However, only ifenprodil was able to significantly protect cells from AβO-induced ER stress.

The disturbance of ER Ca^{2+} homeostasis is one of the early events implicated in the ER stress response (Kim *et al.* 2008b; Szegezdi *et al.* 2009). Therefore, the ER Ca^{2+} content was measured in hippocampal cells 3 hours after incubation with AβO (0.5 μM) or thapsigargin (2 μM) through single cell

Ca^{2+} imaging. Results confirmed that AβO impairs both ER and cytosolic Ca^{2+} homeostasis, leading to a significant depletion of ER Ca^{2+} stores (Fig. 7.2 C-C') accompanied by an increase in cytosolic Ca^{2+} levels (Fig. 7.2 D-D'). Similar results were obtained in thapsigargin-treated cells. In agreement with results obtained when ER stress markers were analysed, ifenprodil significantly inhibited the loss of ER Ca^{2+} content observed upon AβO treatment. Although NVP-AAM077 exhibited a tendency to prevent ER Ca^{2+} depletion after AβO incubation, results were not statistically significant (Fig. 7.2 C-C'). Concerning cytosolic Ca^{2+} levels, both NVP-AAM077 and ifenprodil were shown to protect against the deleterious effects of AβO. However, the most prominent effect was afforded by ifenprodil, since pre-treated cells with this NR2B antagonist presented cytosolic Ca^{2+} levels similar to control values (Fig. 7.2 D-D').

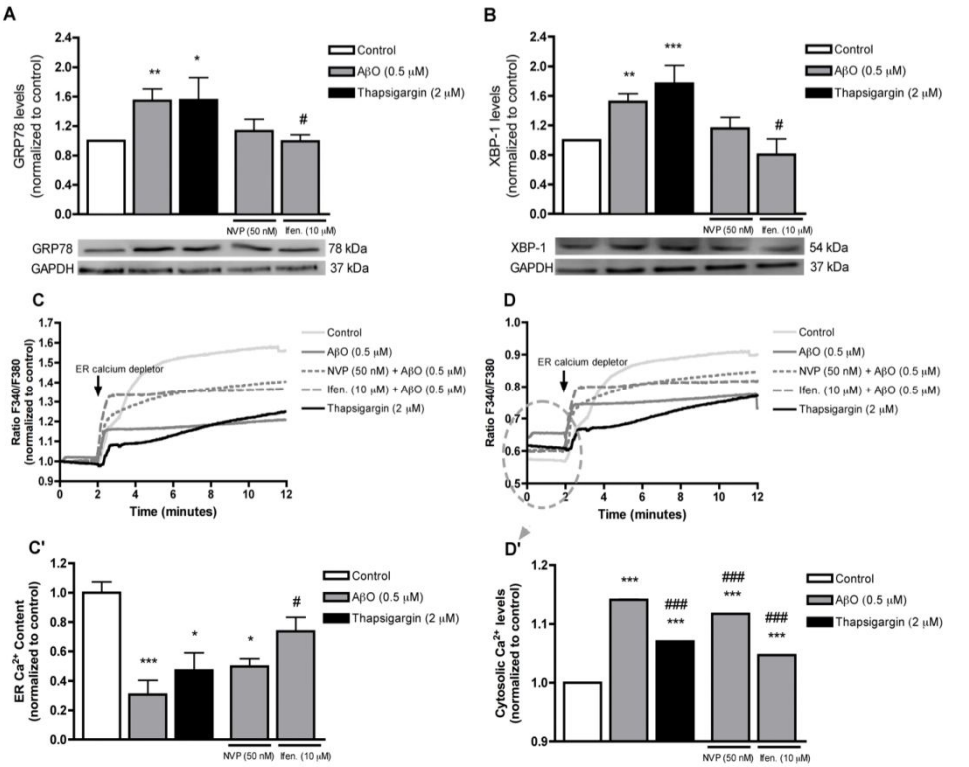


Figure 7.2 *Effect of the NR2A and NR2B subunits of the NMDARs on AβO-induced ER stress and ER versus cytosolic Ca^{2+} levels in hippocampal cells*.
Hippocampal cultures were treated during 3 or 6 hours with AβO (0.5 μM) for WB or Ca^{2+} measurements, respectively, in the absence or in the presence of NVP-AAM077 (50 nM) or ifenprodil (10 μM). Similarly, cells were treated with the widely used ER stressor thapsigargin (2 μM). Total protein lysates were prepared and analysed by immunoblotting using anti-GRP78 (A) or anti-XBP-1 (B) antibodies. Levels of Ca^{2+} in ER stores (C, C') and in the cytosol (D, D') were evaluated by monitoring the fluorescence of Fura-2. Initial Fura-2 fluorescence values were used to calculate cytosolic Ca^{2+} levels and the difference between Fura-2 fluorescence before and after the full depletion of ER Ca^{2+}content was used to evaluate ER Ca^{2+} levels. Values are the means ± SEM of at least 3 independent experiments. GAPDH was used for WB loading control purposes, and results were normalized to control values. *p<0.05; **p <0.01, ***p <0.001, compared with control condition. $^{\#}$p<0.05, $^{\#\#\#}$p <0.001; significantly different when compared to AβO-treated condition.

In order to better understand the mechanisms underlying AβO-NMDARs interaction and ER stress, we determined the activity of the membrane-bound enzymatic complex NOX, one of the main sources of superoxide radical (Bedard & Krause 2007). Our results showed NOX activation in AβO-treated mature hippocampal cultures that was significantly prevented by apocynin, a specific inhibitor for this enzyme. Ifenprodil, but not NVP-AAM077, abolished NOX activation in AβO-treated hippocampal cultures (Fig. 7.3 A). It was also observed that the increase in the levels of superoxide induced by AβO was significantly prevented by ifenprodil and by apocynin (Fig. 7.3 B). On the other hand, NVP-AAM077 was not able to inhibit AβO-induced superoxide production. These results suggest that AβO-induced NOX-mediated superoxide generation occurs through an NR2B-dependent and NR2A-independent pathway. Furthermore, it was observed that ER Ca^{2+} depletion is significantly prevented in the presence of apocynin (Fig. 7.3 C) demonstrating the involvement of NOX-mediated superoxide production on ER stress caused by AβO.

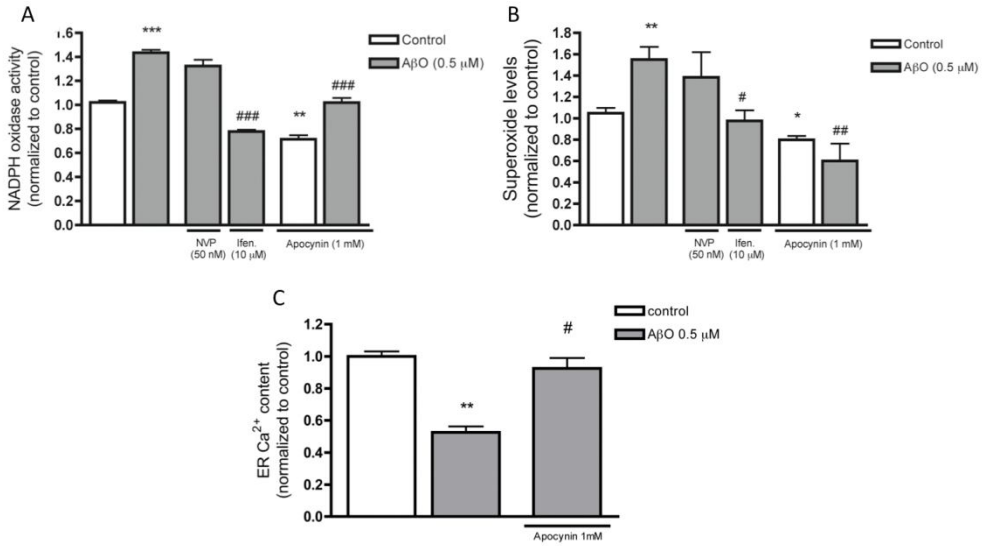


Figure 7.3 *Effect of the NR2A and NR2B subunits of NMDARs on AβO-induced NADPH oxidase activation, superoxide production and ER Ca^{2+} content in hippocampal cells.* Hippocampal cells were incubated for 6 hours with AβO (0.5 μM), in the absence or in the presence of NVP-AAM077 (50 nM) or ifenprodil (10 μM). The NOX inhibitor apocynin (1 mM) was added at the time of NOX activity assay or 15 min prior to the AβO stimulus. (A) NOX activity was evaluated by monitoring the chemiluminescence of the probe lucigenin. (B) Superoxide levels were measured using the fluorescent dihydroethidium (DHE) dye. (C) Levels of Ca^{2+} in ER stores were accessed by monitoring the fluorescence of Fura-2 in the absence of external Ca^{2+}. The difference between Fura-2 fluorescence ratio 340/380 nm before and after the addition of thapsigargin (2.5 μM) was used to evaluate ER Ca^{2+} levels. Values are the means ± SEM of at least 3 independent experiments. ** $p<0.01$; *** $p<0.001$, significantly different compared to control values. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$; significantly different when compared to AβO-treated cells.

7.3.3. An NR2B antagonist suppresses the compromise in cell survival and activation of the endoplasmic reticulum stress-mediated apoptotic cell death pathway induced by Aβ oligomers.

After demonstrating that ER stress was activated in hippocampal cells by AβO through an NR2B-dependent mechanism, we next evaluated its role in cell survival, which was analysed by the MTT assay. Upon AβO (0.5 μM) or thapsigargin (2 μM) treatment for 6 hours, a significant decrease in cell survival was observed (Fig. 7.4), that was even more evident when AβO treatment was extended for 24 hours (data not shown). Ifenprodil was demonstrated to rescue hippocampal cells from the decrease in cell viability induced by AβO incubation during 6 hours (Fig. 7.4).

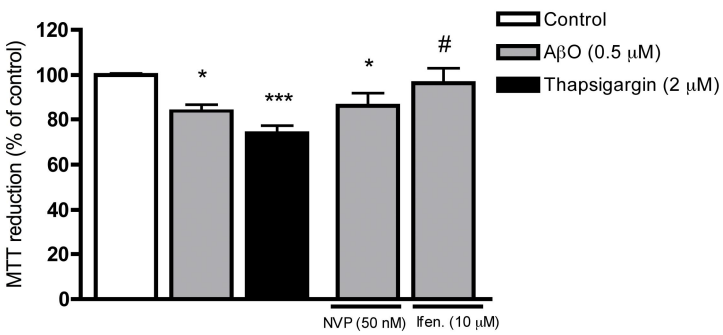


Figure 7.4 *Role of NR2A and NR2B subunits of NMDARs on AβO-induced loss of hippocampal cell survival.*
Hippocampal cells were incubated for 6 hours with AβO (0.5 μM), in the absence or in the presence of NVP-AAM077 (50 nM) or ifenprodil (10 μM). Thapsigargin (2 μM) treatment during 6 hours was used as a positive control for ER stress. Cell viability in treated cells was evaluated by the MTT assay. Results are the means ± SEM of values corresponding at least to 3 independent experiments, performed in duplicate. *p<0.05; **p <0.01; ***p <0.001; significantly different with respect to control values; $^{\#}$p <0.05, significantly different when compared to AβO-treated cells.

Then, we investigated by WB and immunocytochemistry the levels and the sub-cellular localization of GADD153/CHOP, a pro-apoptotic transcription factor that is an important mediator of ER stress-induced cell death (Wang *et al.* 1996; Oyadomari & Mori 2004). Indeed, after an incubation period of 6 hours with AβO (0.5 μM), the total levels of GADD153/CHOP were significantly increased when compared to control condition but were not changed in the presence of antagonists of NR2A or NR2B subunits (Fig. 7.5 B). However, in AβO-treated hippocampal cells, GADD153/CHOP upregulation was associated with enhanced translocation of this transcription factor to the nucleus (Fig. 7.5 C) that was abolished in ifenprodil pre-treated cells. Although caspase-12 has been implicated in AβO-induced apoptosis (Nakagawa *et al.* 2000), hippocampal cell death triggered by AβO seems to occur independently of this ER-resident caspase. While thapsigargin (2 μM) significantly decreased the levels of pro-caspase-12 (Fig. 7.5 A), these levels were not affected upon treatment with AβO (0.5 μM) for 6 hours. Furthermore, pro-caspase-12 levels were not different between AβO-treated and untreated cells, in the absence or in the presence of NMDAR antagonists (Fig. 7.5 A).

Overall, the results described above report the neuroprotective effect accomplished by the blockage of NMDARs, in particular of the NR2B subunit, on ER stress-mediated hippocampal dysfunction caused by AβO as depict in fig 7.6.

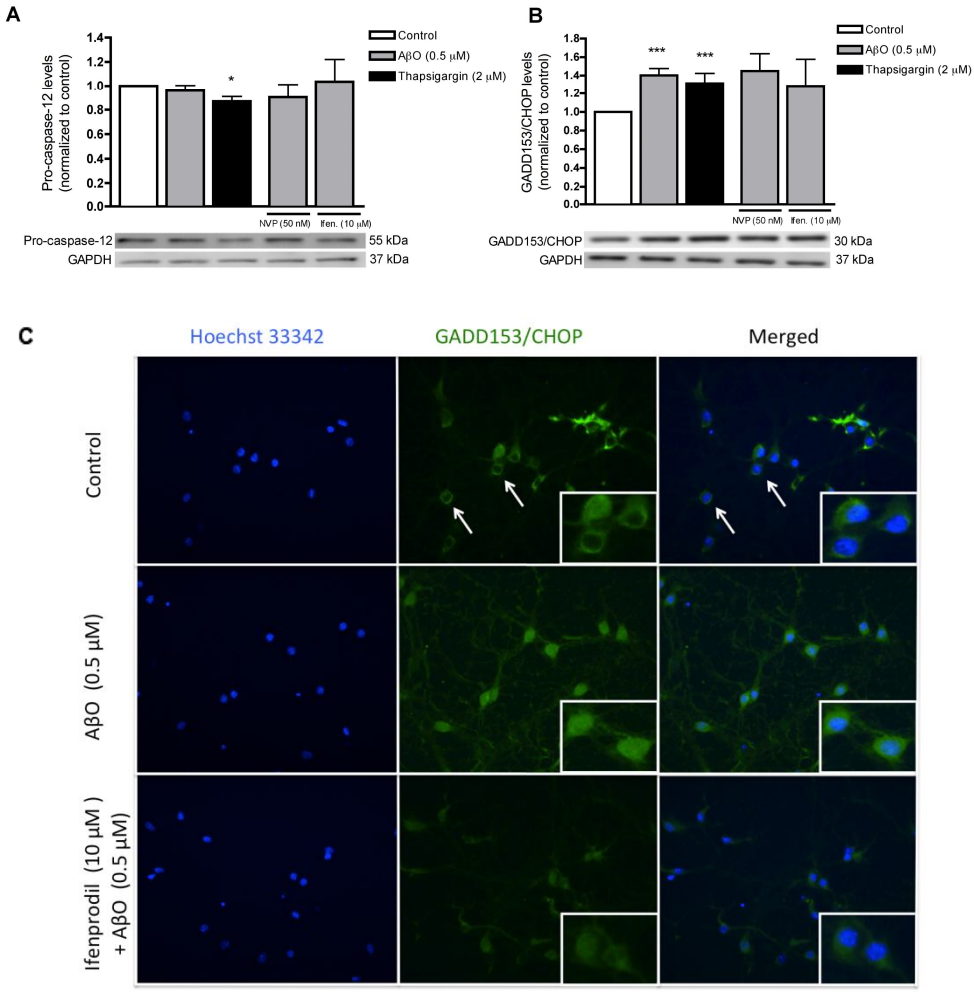


Figure 7.5 *Role of NR2A and NR2B subunits of NMDARs on ER stress-mediated apoptotic cell death pathway triggered by AβO in hippocampal cells*.
Hippocampal cells were treated for 6 hours with AβO (0.5μM), in the absence or in the presence of NVP-AAM077 (50 nM) or ifenprodil (10 μM). In parallel, cells were treated for 6 hours with the widely used ER stress inducer thapsigargin (2 μM). Levels of pro-caspase-12 (A) or GADD153/CHOP (B) were analysed by immunoblotting in total protein lysates. (C) GADD153/CHOP sub-cellular localization was evaluated by fluorescence microscopy in cells co-labelled with an anti-GADD153/CHOP antibody (green) and Hoechst 33342 (blue), using a 400x magnification. Arrows indicate absence of nuclear localization of GADD153/CHOP. Values are the means ± SEM of at least 3 independent experiments, normalized to control. GAPDH was used as loading control. *p<0.05; ***p <0.001; significantly different when compared to control conditions.

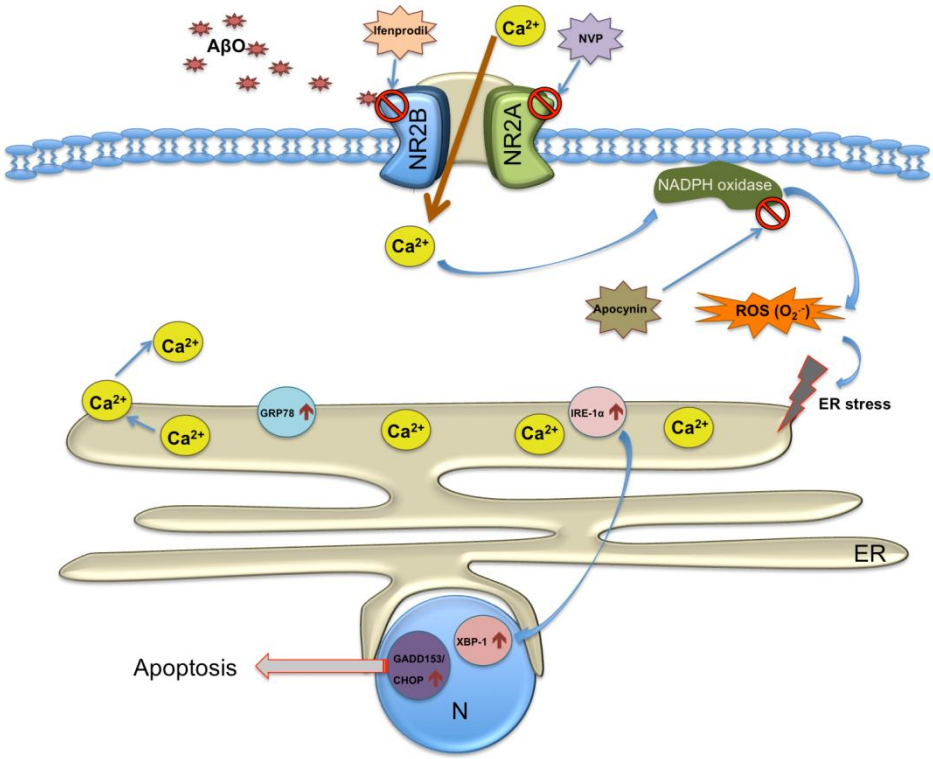


Figure 7.6 *Summary of the NMDAR-dependent mechanism proposed for AβO-induced ER stress in mature hippocampal cells.*
AβO can interact with or close to the NR2B subunit of NMDAR, subsequently leading to the early rise of cytosolic Ca^{2+} levels. This increment leads to the activation of NOX and consequent production of superoxide radicals leading to ER Ca^{2+} depletion. Then, cellular response to ER stress is activated in order to re-establish its normal functioning through activation of ER stress sensors, including IRE-1α and downstream XBP-and expression of chaperones, such as GRP78. However, when the AβO toxic insult is prolonged, the ER stress-mediated apoptotic pathway is induced increasing the levels of the pro-apoptotic transcription factor GADD153/CHOP that is translocated to the nucleus (N). AβO-induced ER stress occurs mainly by an NR2B-dependent mechanism since it can be prevented by ifenprodil, an antagonist of NR2B subunits but not by NVP-AAM077, an antagonist of NR2A subunits of NMDARs.

7.4. Discussion.

Several studies highlight the importance of ER stress during the progression of amyloid pathology in AD (Katayama *et al.* 2004; Ferreiro *et al.* 2007; Nishitsuji *et al.* 2009; Selwood *et al.* 2009). We have previously shown that fibrillar Aβ induces ER stress and activates an ER stress-mediated apoptotic pathway with mitochondrial involvement (Ferreiro *et al.* 2006; 2008a; Costa *et al.* 2010). However, it is now well accepted that other Aβ species, specifically AβO, are the main neurotoxic species involved in AD (Klein 2006; Haass & Selkoe 2007; Sakono & Zako 2010). Taking these evidences into account, we have recently demonstrated that the ER stress response is also activated by AβO treatment in primary cultured cortical neurons (Resende *et al.* 2008a; 2008b).

A strong correlation between AβO levels and the extent of synaptic damage and cognitive impairment has been demonstrated in AD (Klein *et al.* 2001; Nathalie Lacor 2007). Furthermore, several evidences support that synaptic targeting by AβO and resulting synaptic alterations are involved in memory loss in early stages of the disease (Lacor *et al.* 2004; Klein 2006; Lacor *et al.* 2007) and correlates with binding and activation of NMDARs in mature hippocampal neurons (De Felice *et al.* 2007; Lacor *et al.* 2007). In this work we addressed whether AβO can cause ER stress in hippocampal cultures by a mechanism involving the NMDAR. First, we evaluated the induction of ER stress in AβO-treated primary cultures of hippocampal cells. Then, we investigated the role of NR2A and NR2B subunits of NMDAR on AβO-induced ER stress and neurotoxicity using NVP-AAM077 and ifenprodil to

antagonize NR2A and NR2B, respectively. As positive control, we used the known ER stress inducer thapsigargin (Kuo *et al.* 1998).

Under ER stress conditions, cells tend to recover by activating the UPR (Imaizumi *et al.* 2001; Naidoo 2009; Yoshida 2009). However, under severe or prolonged ER stress, these adaptive signaling pathways fail leading to activation of apoptotic cell death (reviewed in Kim *et al.* 2008a). During UPR, the expression of ER-resident chaperones is up-regulated and can be used to monitor ER stress. Among these chaperones, GRP78 is considered a valuable ER stress marker (Liu *et al.* 2010). Moreover, GRP78 has been shown to be increased in AD brains (Hoozemans *et al.* 2005) and to be up-regulated in cultured neurons upon fibrillar or oligomeric Aβ treatment (Ferreiro *et al.* 2006; Resende *et al.* 2008a). Under ER stress conditions, the ER stress sensor IRE-1α is activated increasing the specific splicing of XBP-1 mRNA. Subsequently, XBP-1 induces the expression of chaperones and other proteins involved in protein folding and degradation (Zhang & Kaufman 2006; Yoshida 2007; Naidoo 2009). Results presented here show increased levels of GRP78 (Fig. 7.2 A) and XBP-1 (Fig. 7.2 B) in hippocampal cells treated with AβO, strongly suggesting that AβO induce ER stress in these cells.

Disturbance of ER Ca^{2+} homeostasis is commonly associated with ER stress (Szegezdi *et al.* 2009). Previous reports demonstrated that Aβ, including AβO, depletes ER Ca^{2+} content in cortical neurons, increasing cytosolic Ca^{2+} levels and finally leading to apoptosis (Scorziello *et al.* 1996; Huang *et al.* 2000; Ferreiro *et al.* 2008b; Resende *et al.* 2008b). Furthermore, ER Ca^{2+} dyshomeostasis is also implicated in the communication between ER and mitochondria, acting as a pro-apoptotic signal (Chami *et al.* 2008; Costa *et al.* 2010; Gonzalez *et al.* 2010). Here, we depict the early impairment of ER Ca^{2+}

homeostasis in mature hippocampal cells in the presence of AβO, comparatively to what occurs in thapsigargin-treated cells. In fact, a significant depletion of ER Ca^{2+} content occurred 3 hours after cell treatment (Fig. 7.2 C-C'), accompanied by an increase in cytosolic Ca^{2+} levels (Fig. 7.2 D-D').

When ER stress is too severe, apoptotic cell death is induced through the activation of the transcription growth arrest factor and DNA damage gene GADD153/CHOP (Schapansky *et al.* 2007; Pino *et al.* 2009). Our results confirmed the activation of this pathway by AβO since enhanced protein levels as well as nuclear localization of this pro-apoptotic transcription factor was observed upon treatment with AβO during 6 hours, as revealed by WB analysis and fluorescence microscopy (Fig. 7.5 B-C). The translocation of GADD153/CHOP to the nucleus was associated with a significant decrease in cell survival (Fig. 7.4).

Interestingly, despite the ER-resident caspase-12 has been implicated in cell death upon Aβ exposure (Nakagawa *et al.* 2000; Song *et al.* 2008), we did not find evidences for the conversion of the ER membrane-localized procaspase-12 into its cytosolic active form in AβO-treated hippocampal cultures (Morishima *et al.* 2002) (Fig. 7.5 A). These results suggest that AβO-induced ER stress in hippocampal cells occurs by a caspase-12-independent process. Accordingly, Obeng and Boise (Obeng & Boise 2005) reported that ER stress activates caspase-9 leading to apoptotic cell death by a mechanism that does not require caspase-12, which is in agreement with the results presented here.

Besides demonstrating that AβO induces ER stress in hippocampal cells, we also focused on the possible role of NMDARs. A current therapeutic approach to slow disease progression in moderate-severe AD patients involves the use of memantine, an uncompetitive antagonist of NMDARs (Lipton 2006).

Memantine, which has been shown to improve cognitive function in AD patients (Reisberg *et al.* 2003), is able to re-establish the glutamatergic system homeostasis (Robinson & Keating 2006; Parsons *et al.* 2007) and to prevent oxidative stress and Ca^{2+} influx induced by AβO in hippocampal neuronal cultures (De Felice *et al.* 2007), as well as Aβ-induced synaptotoxicity (Lacor *et al.* 2007) and neurodegeneration (Miguel-Hidalgo *et al.* 2002). *In vivo*, memantine was also shown to reduce the neurodegenerative process in rat hippocampus (Szegedi *et al.* 2010), revealing the therapeutic importance of blocking this receptor. NMDARs activation by AβO is associated with synaptic dysfunction preceding neurodegeneration (Kelly & Ferreira 2006), suggesting that NMDARs activation also occurs in early phases of AD, when AβO assume a great importance. Furthermore, AβO were demonstrated to modify cell surface expression of NMDARs, in particular of the NR1 and NR2B subunits (Snyder *et al.* 2005; Lacor *et al.* 2007).

Pre-incubation with NR2A and NR2B antagonists previous to the AβO stimulus, especially with the NR2B antagonist ifenprodil, significantly prevented ER stress, cytosolic and ER Ca^{2+} dyshomeostasis, superoxide production as well as cell death. Indeed, in the presence of ifenprodil, GRP78 and XBP-1 (Fig. 7.2 A-B) levels remained similar to controls and were significantly different from those determined in AβO-treated cells. NVP-AAM077 also presented a protective effect but it did not reach statistical significance. When Ca^{2+} homeostasis was evaluated, ifenprodil significantly prevented the depletion of ER Ca^{2+} content (Fig. 7.2 C-C') and the increase in cytosolic Ca^{2+} levels (Fig. 7.2 D-D') induced by AβO. On the other hand, NVP-AAM077 only slightly decreased cytosolic Ca^{2+} rise (Fig. 7.2 D-D'). These results suggest that both NR2A and NR2B subunits are involved in Ca^{2+}

entry and subsequent increase in cytosolic levels upon NMDAR activation triggered by AβO but that only NR2B subunits are implicated in the loss of ER Ca^{2+} homeostasis. An increase in NOX activity was also observed in AβO-treated hippocampal cultures, which was prevented by NRB blockage (Fig. 7.3 A). NOX activation was demonstrated to be implicated in AβO-induced superoxide production since it was abolished in the presence of apocynin, a NOX inhibitor (Fig. 7.3 B). Moreover, ifenprodil significantly prevented superoxide production in AβO-treated hippocampal cultures (Fig. 7.3 B) demonstrating the involvement of NR2B subunit. These results are in agreement with previous studies demonstrating NOX as the main source of superoxide upon NMDARs activation (Brennan *et al.* 2009) and in cultured neurons exposed to either oligomeric Aβ or NMDA (Shelat *et al.* 2008). Interestingly, NOX activation was shown to be involved in the depletion of ER Ca^{2+} stores in mature hippocampal cultures treated with AβO (Fig. 7.3 C). Nevertheless, the depletion of ER Ca^{2+} stores in AβO-treated hippocampal cultures can also be explained by the mechanism of Ca^{2+}-induced Ca^{2+} release in which cytosolic Ca^{2+} can sensitize the ER Ca^{2+} release through IP3R or RyR channels (Berridge *et al.* 2000). The fact that NVP-AAM077 prevented the increase in cytosolic Ca^{2+} levels without significant effects on ER Ca^{2+} depletion, suggests that the Ca^{2+} threshold level required for CICR activation is still reached in the presence of NVP-AAM077, allowing AβO-induced ER Ca^{2+} release and depletion of its content. This is an early event that could trigger several mechanisms culminating in acute cell damage. In fact, our results highlight the loss of cellular viability by AβO that was prevented by the NR2B subunit antagonist ifenprodil (Fig. 7.4). The protein levels of the pro-apoptotic transcription factor GADD153/CHOP increased in hippocampal

cultures upon AβO exposure, but it was not significantly prevented when NMDARs were blocked with ifenprodil (Fig. 7.5 B). However, the nuclear translocation of GADD153/CHOP induced by AβO was partially attenuated in the presence of this NR2B antagonist (Fig. 7.5 C).

Amelioration of AβO-induced ER stress by ifenprodil in mature hippocampal cultures is well correlated with our previous findings showing a decrease in cell surface levels of NR2B subunit upon AβO treatment, in the absence of changes in total protein levels, which we believe to be associated with its activation followed by internalization (Lacor *et al.* 2007). Accordingly, other studies demonstrated that AβO can alter the sub-cellular localization of NMDAR subunits, namely of NR1 (Snyder *et al.* 2005; Lacor *et al.* 2007). Furthermore, we observed a selective increase in total NR2A levels, attributed to *de novo* protein synthesis without changes in its plasma membrane localization (data not shown) that might explain the fact that NVP-AAM077 only exerted a slightly protective effect in AβO-treated hippocampal cultures. The relevance of NR2B subunit for AβO-induced toxicity in hippocampal cultures was also proved using an antibody competition assay. Pre-incubation of cells with antibodies against the extracellular epitopes of NR1 and NR2B subunits decreased AβO binding, whereas the NR2A antibody was not effective (Fig. 7.1). Similar results have been reported for NR1 (De Felice *et al.* 2007) as well as for other synaptic receptors, such as mGluR5 (Renner *et al.* 2010) and PrP^C (Laurén *et al.* 2009), suggesting that AβO-binding sites are part of a multi-protein complex (Renner *et al.* 2010). Further, the localization of the NR1/NR2B and NR1/NR2A complexes have been shown to be different, with predominant localization of NR1/NR2B at perisynaptic sites, outside of the postsynaptic density (Zhang & Diamond 2006), which may be the AβO-

binding site. Similar reduction of AβO binding is also afforded by blocking mGluR5, another glutamate receptor located on the outside ring of the postsynaptic density (Lujan *et al.* 1997). However, so far it has been difficult to determine which synaptic receptor could serve as a binding site for AβO as neither antibody competition or selective receptors knockout (e.g. PrP or mGluR5) have fully eliminated AβO binding (Laurén *et al.* 2009; Renner *et al.* 2010).

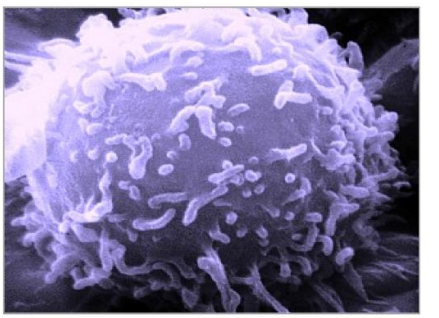
In conclusion, the present study provided a novel perspective about the interaction and neurotoxicity of AβO on hippocampal cells, as summarized in Fig. 7.6. First, AβO was demonstrated to trigger ER stress by an NMDAR-dependent mechanism. Second, it was possible to discriminate the role of the NR2A and NR2BNMDAR subunits, proving that NR2B has an important role in AβO-induced hippocampal ER stress and neurotoxicity that occurs after NMDAR/NR2B-AβO interaction. Finally, NOX-mediated superoxide production downstream of NR2B-AβO interaction was shown to be involved in the loss of ER Ca^{2+} homeostasis. These results suggest that NR2B subunits can thus be a potential target for therapeutic intervention in early stages of AD progression.

CHAPTER 8

3xTg-AD mice and human lymphocytes: different strategies for studying Alzheimer's disease pathogenesis

3xTg-AD mouse model

Human lymphocytes



8.1. Summary

Previously, we demonstrated that ER is involved in cellular stress induced by the AD-associated Aβ peptide. Furthermore, the ER stress response triggered by Aβ was shown to activate an apoptotic cell death pathway by a mechanism involving the cross-talk between ER and mitochondria. These results were obtained using several in vitro models which were exposed to toxic concentrations of Aβ or well-known ER stress inducers, namely primary cultured rat brain cortical and hippocampal neurons, mtDNA-depleted ρ0 cells without functional mitochondria and also cybrid cell lines obtained from platelets of AD patients and characterized by mitochondrial COX inhibition.

In an attempt to further clarify the role of ER stress in AD pathogenesis, we recurred to different approaches. First, we studied the age-dependent induction of ER stress and ER stress-mediated apoptotic cell death pathway in 3xTg-AD mouse model for AD that closely mimics AD pathology. Second, in collaboration with the "Hospitais da Universidade de Coimbra" we used human PBMCs, namely lymphocytes, isolated from mild, moderate and severe AD patients and individuals with MCI *versus* non-demented age-matched subjects in order to study the relationship between the different stages of cognitive impairment and markers of peripheral cell injury. For this purpose we analyzed ER Ca^{2+} levels and data were correlated with changes in the expression of the ER stress markers Grp78 and XBP-1, and also of the apoptosis-related ER proteins caspase-12 and GADD153/CHOP.

Together, our results obtained in 3xTg-AD mice and PBMCs highlight the role of ER stress in the early stages of AD pathology, suggesting that it might be aneffective therapeutic target for AD.

8.2. Introduction.

AD is the most prevalent neurodegenerative disorder that currently affects almost 35 million people worldwide. It is a chronic and progressive neurodegenerative illness characterized by memory deficits and cognitive decline that arise from synaptic and cholinergic/glutamatergic neuronal loss in the hippocampus and cerebral cortex (Selkoe 2001; Mattson 2004). As main neuropathologic hallmarks, AD brains present an abnormal deposition of extracellular senile plaques, composed essentially by Aβ peptide, and intracellular NFTs of hyperphosphorylated tau.

The perturbation of intracellular Ca^{2+} homeostasis has been reported both in brain and in peripheral cells obtained from AD patients (Mattson 2002). In part, ER dysfunction could account for cellular Ca^{2+} deregulation leading to activation of cell death pathways. In fact, several studies established a correlation between the abnormal ER function and the progression of the disease (Katayama *et al.* 2004; Selwood *et al.* 2009). These alterations seem to result from Aβ-induced ER Ca^{2+} deregulation (Ferreiro *et al.* 2006; Costa *et al.* 2010) and activation of ER stress signalling pathways (Naidoo 2009) to re-establish normal cell functioning (Schroder & Kaufman 2005). However, when ER stress is too prolonged or severe, the ER stress-mediated apoptotic cell death pathway is induced (Oyadomari & Mori 2004; Hoozemans *et al.* 2005; Scheper *et al.* 2011).

Since ER stress is assumed to be involved in AD pathogenesis, here we investigated the induction of the ER stress response and disturbance of ER Ca^{2+} homeostasis during the progression of the pathology and cognitive alterations. Extensively validated markers for ER stress, such as GRP78, XBP-1, caspase-12 and GADD153/CHOP (Yoshida 2007; 2009) and ER Ca^{2+} levels (Ferreiro *et*

al. 2006) were analysed in the 3xTg-AD transgenic mice modelling AD and in human PBMCs isolated from non-demented or MCI subjects or AD patients. We provided evidences that ER stress and Ca^{2+}dyshomeostasis is an early event during AD development and therefore, we believe that ER dysfunction could assume an important role for future detection and therapeutical intervention in AD.

8.3. Results.

In the present study, we evaluated the hypothesis that ER dysfunction can be correlated with the progression of AD pathology and cognitive impairment. For this purpose, we used an AD mouse model, 3xTg-AD (Oddo *et al.* 2003a; 2003b) *versus* non-Tg mice and also PBMCs isolated from non-demented controls, individuals with MCI, which corresponds to a pre-clinical stage of the disease, and also AD patients presenting different degrees of cognitive impairment (mild and moderate-severestages).

8.3.1. Studies in male and female 3xTg-AD *versus* non-Tg micewith 3-, 6- and 12-months of age

ER stress markers (Grp78, XBP-1, pro-caspase-12 and GADD153/CHOP) were analyzed in brain homogenates prepared from cerebral cortex and hippocampus of 3-, -6- and 12-month old male and female 3xTg-AD mice and were compared with that determined in aged-matched male and female non-Tg mice. In the cerebral cortex, Grp78 and XBP-1 levels only significantly increased in 12-month old 3xTg-AD males comparatively with non-Tg mice and pro-caspase-12 and GADD153/CHOP levels remained unchanged in all ages analyzed (Fig. 8.1).

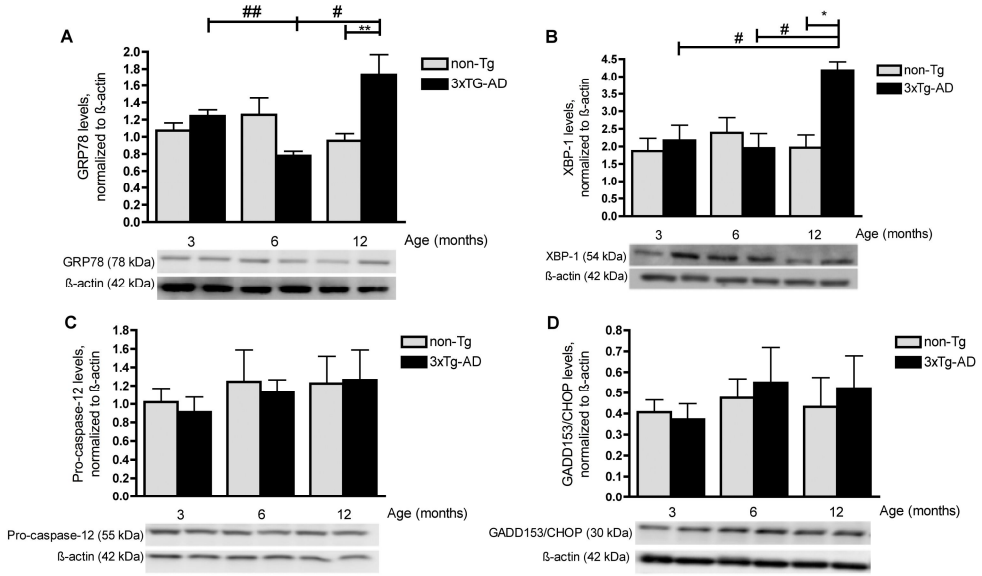


Figure 8.1 *Levels of ER stress markers in the cerebral cortex of male 3xTg-AD mice.*
Brain homogenates were prepared from cerebral cortex of 3xTg-AD and age-matched non-Tg male mice with 3-, 6- and 12-months of age. Proteins from cell lysates (30µg) were analysed by immunoblotting with the following antibodies: (A) anti-GRP78, against the ER chaperone GRP78, (B) anti-XBP-1, against the ER chaperone genes regulator, XBP-1, (C) anti-GADD153/CHOP against the ER stress-induced pro-apoptotic transcription factor GADD153/CHOP, (D) anti-pro-caspase-12, against the precursor of the ER-resident caspase-12. Values expressed in arbitrary units, are the means ± SEM of 2-6 experiments (1 animal per experiment), normalized to β-actin levels. *$p<0.05$, **$p<0.01$, statistically significant when compared with the correspondent age-matched non-Tg mice; $^{\#}p<0.05$, $^{\#\#}p<0.01$, statistically significant when compared with animals of the same genotype but with different ages.

In 3- and 6-month old female 3xTg-AD mice increased levels of Grp78 and XBP-1 were detected comparatively with non-Tg mice; however, these levels were similar at 12-months of age. Pro-caspase-12 levels significantly decreased in 3-, -6 and 12-month old 3xTg-AD female mice in comparison with non-Tg-AD mice while GADD153/CHOP levels were not affected (Fig. 8.2).

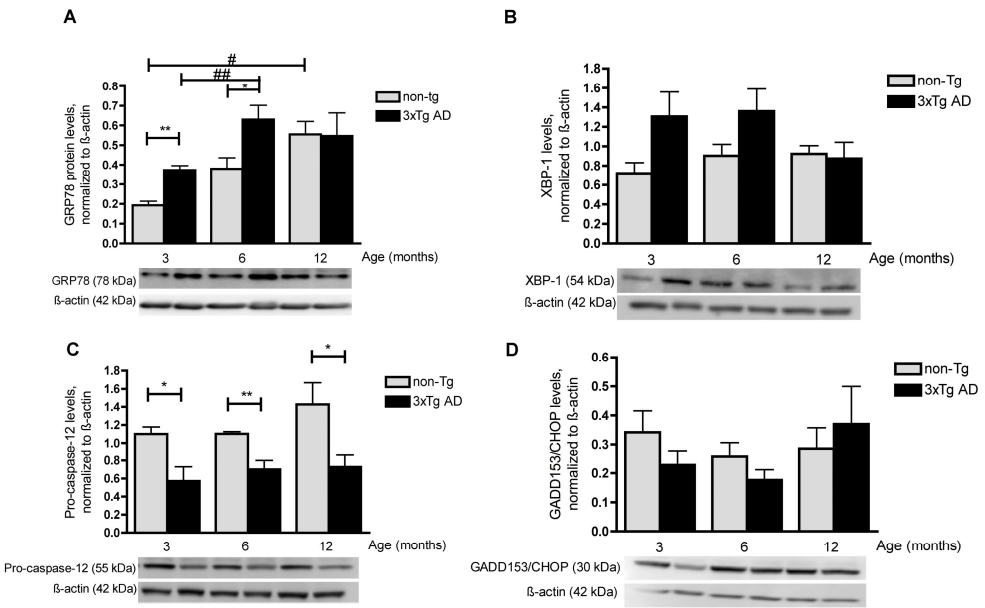


Figure 8.2 *Levels of ER stress markers in the cerebral cortex of female 3xTg-AD mice.*
Brain homogenates were prepared from the cerebral cortex of 3xTg-AD and age-matched non-Tg female mice with 3-, 6- and 12-months of age. Cell lysates (30 µg of protein) were prepared and analysed by immunoblotting with the following antibodies: (A) anti-GRP78, against the ER chaperone GRP78, (B) anti-XBP-1, against the ER chaperone genes regulator, XBP-1, (C) anti-GADD153/CHOP against the ER stress-induced pro-apoptotic transcription factor GADD153/CHOP, (D) anti-pro-caspase-12, against the precursor of the ER-resident caspase-12. Values expressed in arbitrary units, are the means ± SEM of 2-5 experiments (1 animal per experiment), normalized to β-actin levels. *$p<0.05$, **$p<0.01$, statistically significant when compared with the correspondent age-matched non-Tg mice; $^{\#}p<0.05$, $^{\#\#}p<0.01$, statistically significant when compared with animals of the same genotype but with different ages.

Comparatively with non-Tg mice, changes in ER stress markers were more pronounced in the hippocampus than in the cerebral cortex, both in female and male 3xTg-AD mice. In the hippocampus of 3xTg-AD males, an increase in Grp78, XBP-1 and GADD153/CHOP levels was observed in 3-month old animals that were accompanied by a decrease in pro-caspase-12 levels (Fig. 8.3).

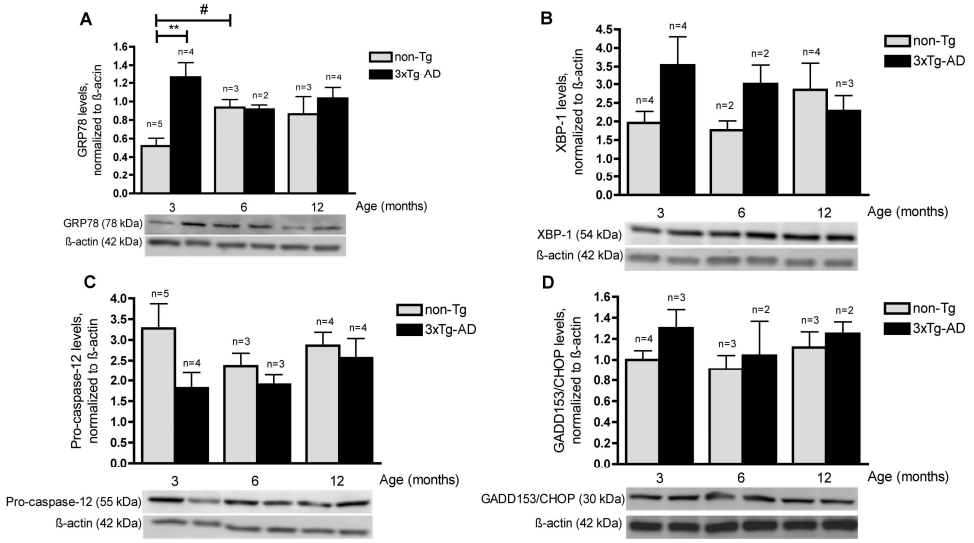


Figure 8.3 *Levels of ER stress markers in the hippocampus of 3xTg-AD male mice*.
Brain homogenates were prepared from cerebral hippocampi of 3xTg-AD and age-matched non-Tg male mice with 3-, 6- and 12-months of age. Proteins from cell lysates (30µg) were analysed by immunoblotting with the following antibodies: (A) anti-GRP78, against the ER chaperone GRP78, (B) anti-XBP-1, against the ER chaperone genes regulator, XBP-1, (C) anti-GADD153/CHOP against the ER stress-induced pro-apoptotic transcription factor GADD153/CHOP, (D) anti-pro-caspase-12, against the precursor of the ER-resident caspase-12. Values expressed in arbitrary units, are the means ± SEM of 2-5 experiments (1 animal per experiment), normalized to β-actin levels. **$p<0.01$, statistically significant when compared with the correspondent age-matched non-Tg mice; $^{\#}p<0.05$, statistically significant when compared with animals of the same genotype but with different ages.

Increased XBP-1 and GADD153/CHOP levels were detected in the hippocampus of 3-, 6- and 12-month old 3xTg-AD females comparatively with non-Tg mice. Pro-caspase-12 levels decreased in 3- and 6-month old 3xTg-AD females in comparison with non-Tg mice. Surprisingly, Grp78 levels in 3- and 6-month old females remained similar to those determined in age-matched non-Tg animals and a significant increase was only observed in 12-month old 3xTg-AD mice (Fig. 8.4). All the evidences obtained through WB analysis

showing different patterns of ER stress relative to age, gender or studied brain area were summarized in table 8.1, in which a comparison between 3xTg-AD and non-Tg is made.

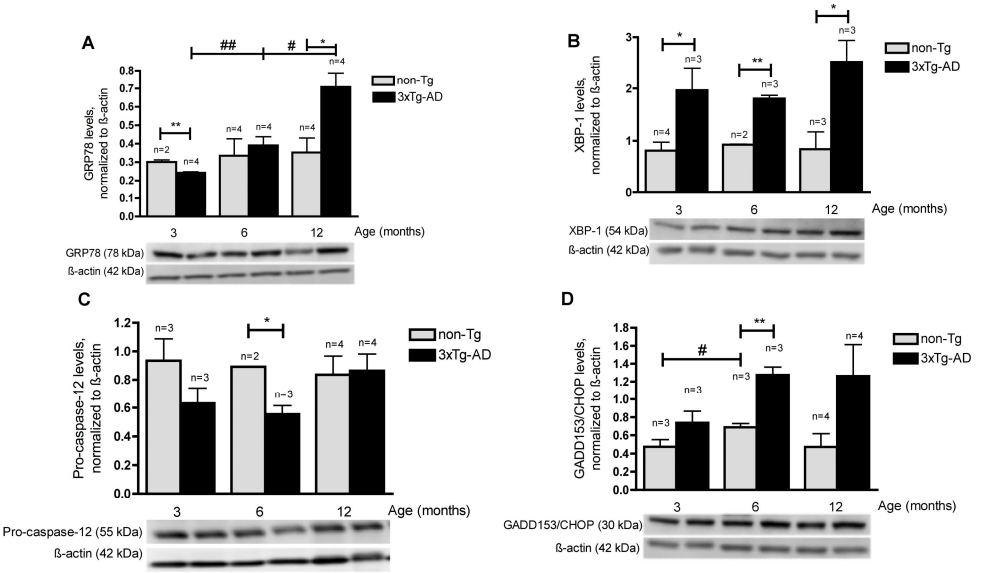


Figure 8.4 *Levels of ER stress markers in the hippocampus of 3xTg-AD female mice.*
Brain homogenates were prepared from cerebral hippocampi of 3xTg-AD and age-matched non-Tg female mice with 3-, 6- and 12-months of age. Cell lysates were prepared and proteins (30μg) were analysed by immunoblotting with the following antibodies: (A) anti-GRP78, against the ER chaperone GRP78, (B) anti-XBP-1, against the ER chaperone genes regulator, XBP-1, (C) anti-GADD153/CHOP against the ER stress-induced pro-apoptotic transcription factor GADD153/CHOP, (D) anti-pro-caspase-12, against the precursor of the ER-resident caspase-12. Values expressed in arbitrary units, are the means ± SEM of 2-5 experiments (1 animal per experiment), normalized to β-actin levels. *p<0.05, **p <0.01, statistically significant when compared with the correspondent age-matched non-Tg mice; $^{\#}$p <0.05, $^{\#\#}$p <0.01, statistically significant when compared with animals of the same genotype but with different ages.

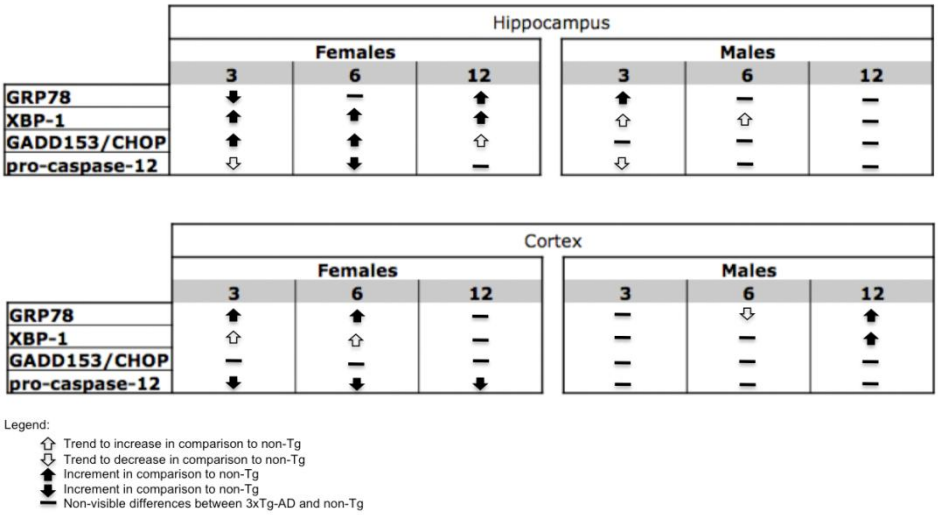


Table 8.1 *Gender, brain region and age affect the levels of ER stress markers in 3xTg-AD mice.*

Hippocampal and cortical extracts were analysed in both male and female mice at different ages (3-, 6- and 12-months). This table summarizes the data collected from WB analysis using different antibodies reactive against ER stress-related proteins. Black arrows represent statistical differences between 3xTg-AD and non-Tg. Trends for the increase or decrease in the levels of the studied proteins are marked with white arrows. Lines represent the absence of differences between 3xTg-AD and non-Tg.

8.3.2. Studies in peripheral blood lymphocytes from non-demented controls, individuals with mild cognitive impairment and Alzheimer's disease patients.

ER and cytosolic Ca^{2+} levels were analyzed to evaluate possible abnomalities that could be correlated with the stage of cognitive impairment. Furthermore, this data was correlated with changes in expression of ER stress markers and apoptotic-related proteins.

A decrease in ER Ca^{2+} content was observed in PBMCs obtained from MCI individuals and mild AD patients, which was more pronounced in the former

cells (Fig. 8.5 A-B). This increment was correlated with changes in cytosolic Ca^{2+} levels determined in the absence of external Ca^{2+} (Fig. 8.5 C-D), that were also more pronounced in mild AD patients.

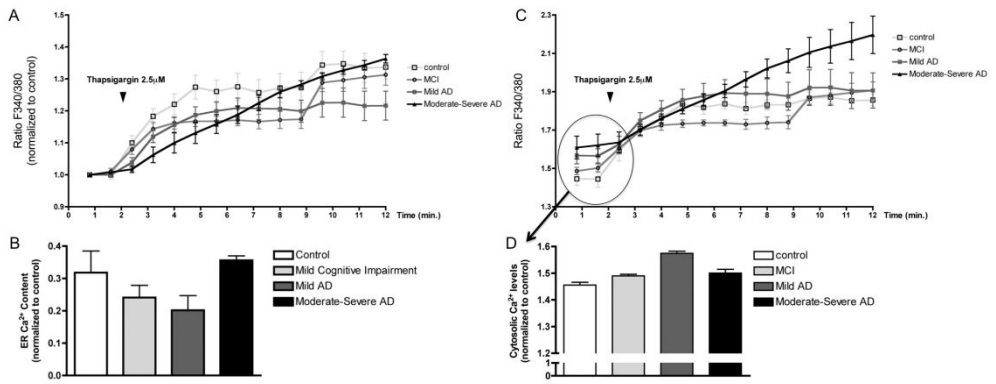


Figure. 8.5 *ER and cytosolic Ca^{2+} levels in human peripheral blood mononuclear cells (PBMCs).*
PBMCs obtained from peripheral blood of 66 to 90 years-old individuals diagnosed with Mild Cognitive Impairment (MCI) (n=5) or probable AD in different stages: mild AD (n=4) or moderate-severe AD (n=2). Age-matched control subjects were neurologically healthy and the mean age was 78 ± 9 (n=2).
Levels of Ca^{2+} in ER stores (A, B) and in the cytosol (C, D) were evaluated by monitoring the fluorescence of Fura-2 in the absence of external Ca^{2+}. The difference between Fura-2 fluorescence ratio 340/380 nm before and after the addition of the irreversible inhibitor of the ER Ca^{2+}-ATPase thapsigargin to fully deplete its content was used to evaluate ER Ca^{2+} levels (B). Cytosolic Ca^{2+} levels were obtained by analyzing the Fura-2 fluorescence ratio at 340 and 380 nm before thapsigargin (2.5 μM) addition (at t=2 min) (D). Data was expressed as the mean ± SEM.

Activation of ER stress sensors and downstream signalling pathways is a cellular response triggered in an attempt to restore homeostasis and avoid further damage. In PBMCs obtained from MCI individuals and mild AD patients, protein levels of the ER chaperone GRP78 (Fig. 8.6 A) and of XBP-1, activated downstream of the IRE-1alpha ER stress sensor (Fig. 8.6 B), were demonstrated to be increased. In PBMCs from moderate-severe AD patients,

the levels of GRP78 and XBP-1 were similar to that measured in control PBMCs, suggesting induction of an ER stress response during the early stages of the disease. Concomitantly, the ER stress-mediated apoptotic pathway was induced, as demonstrated by an increase in the levels of GADD153/CHOP, a pro-apoptotic transcription factor that is an important mediator of ER stress-induced cell death (Fig. 8.6 C).

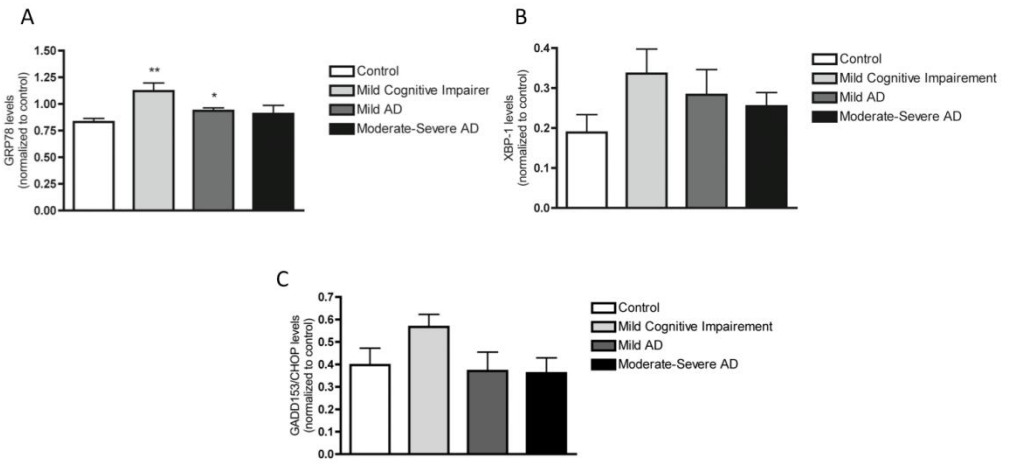


Figure 8.6 *Levels of ER stress markers in human PBMCs.*
Homogenates were prepared from PBMCs isolated from 66 to 90 years-old patients diagnosed with Mild Cognitive Impairment (MCI) or probable AD in different stages (mild AD or moderate-severe AD. Age-matched control subjects were neurologically healthy and the mean age was 78 ± 9. Proteins from cell lysates (30μg) were analysed by immunoblotting with the following antibodies: (A) anti-GRP78, against the ER chaperone GRP78, (B) anti-XBP-1, against the ER chaperone genes regulator, XBP-1, (C) anti-GADD153/CHOP against the ER stress-induced pro-apoptotic transcription factor GADD153/CHOP. Values are the means ± SEM expressed in arbitrary units. Values were normalized to GAPDH levels. *$p<0.05$, **$p<0.01$, statistically significant when compared with healthy age-matched subjects.

8.4. Discussion.

Previous studies from our laboratory established that ER stress is induced in primary cultures of rat brain cortical neurons by the AD-associated Aβ peptide (Ferreiro *et al.* 2006; Resende *et al.* 2008a; 2008b). This peptide was shown to be an ER stressor that deregulates ER Ca^{2+} homeostasis and activates the ER stress-mediated apoptotic cell death pathway (Ferreiro *et al.* 2006). Here, we used an AD *in vivo* model, the 3xTg-AD mice that develops amyloid and tau pathologies and also cognitive deficits in an age- and region-dependent manner mimicking disease progression in humans (Oddo *et al.* 2003a; 2003b; Billings *et al.* 2005), and also PBMCs, namely lymphocytes collected from human patients with probable AD in different stages of cognitive deterioration, in order to better understand the role of ER stress during the progression of AD.

ER stress was assessed through measuring the levels of GRP78 and XBP-1. Both can be considered valuable markers for monitoring ER stress in AD since they are up-regulated under these conditions (Zhang & Kaufman 2006; Yoshida 2009) and were already described to be increased in AD brains (Hoozemans *et al.* 2005). Activation of apoptotic cell death in AD seems to occur through activation of the transcription factor GADD153/CHOP, an important mediator of ER stress-induced cell death (Schapansky *et al.* 2007; Pino *et al.* 2009). Caspase-12, resultant from the conversion of the ER membrane-localized pro-caspase-12 into its active form (Morishima *et al.* 2002), has also been implicated in this apoptotic pathway (Song *et al.* 2008) and resistance to ER stress and Aβ-induced cell death was reported in knockout mice for this caspase (Nakagawa *et al.* 2000). However, the role of caspase-12 is still controversial since ER stress could activate apoptotic cell mechanisms dependent on caspase-9 but independent of caspase-12 (Obeng & Boise 2005).

Our first group of results were obtained inbrain extracts from the hippocampus and cerebral cortex of 3-, 6- or 12-month old male and female non-Tg versus 3xTg-AD mice. This model harbours mutations on PS-1 (PS1M146V) and APP (APPswe) to modulate Aβ overproduction and in tau protein (tauP301L) to induce the formation of NFTs. Although the familial AD form is not the most prevalent, it is indistinguishable from sporadic AD in terms of clinical and neuropathological characteristics. In addition, both forms of the disease are associated with increased Aβ levels, either due to increased amyloidogenic APP processing or to deficient peptide clearance. Aβ triggers several pathogenic mechanisms leading to synaptic and neuronal dysfunction that underlie cognitive deficits and dementia, as previously described in the "amyloid cascade hypothesis" (Blennow *et al.* 2006). Here we report that comparatively with non-Tg mice, changes in ER stress markers were more pronounced in the hippocampus than in the cerebral cortex, both in female and male 3xTg-AD mice, and females were apparently more susceptible of ER stress than males (Table 8.1). These results are in accordance with previous epidemiologic studies reporting higher incidence of AD in women (Andersen *et al.* 1999). Furthermore, we have shown that induction of ER stress occurs in an age-dependent manner suggesting that it represents an early event during the progression of the pathology.

Interestingly, here we report that in comparison to non-Tg mice the hippocampus of both male and female 3xTg-AD mice, present altered levels of ER stress markers at 3 months of age, despite extracellular Aβ deposits only appear in 6 months-old mice and tau alterations are detected at 12 months of age (Oddo *et al.* 2003a). Previously, it was reported that ER stress is activated during pre-NFTs deposition (Hoozemans *et al.* 2009). Here we showed that ER

stress occurs even before Aβ deposition in plaques supporting the role of soluble Aβ oligomers in these alterations (Lambert *et al.* 1998; Klein *et al.* 2001; Sakono & Zako 2010). Aβ oligomerization was detected in the brain of 3xTg-AD mice between 2 and 6 months (Oddo *et al.* 2006) and we have shown that Aβ oligomers induce ER stress in primary cultured cortical neurons (Resende *et al.* 2008b). However, intraneuronal APP has recently gained a great importance in the mechanisms responsible for neuronal dysfunction in these 3xTg-AD mice (Winton *et al.* 2011). Taking into consideration these evidences, aberrant intracellular APP accumulation could contribute to the early ER stress here observed.

Another mechanism that could account for the earlier ER dysfunction occurring in the hippocampus and cerebral cortex of 3xTg-AD mice is Ca^{2+} deregulation. Mutant presenilin, which is overexpressed in the 3xTg-AD brain, was implicated in Ca^{2+} dyshomeostasis in AD (Stutzmann *et al.* 2007). Also, the RyR-associated Ca^{2+} channel type 2 was demonstrated to be selectively increased in the hippocampus of 3xTg-AD mice, relatively to the non-Tg controls, affecting both synaptic transmission and plasticity (Chakroborty *et al.* 2009) through a mechanism involving the NMDAR (Goussakov *et al.* 2010).

The data obtained in vivo with 3xTg-AD mice was further supported by results obtained with a human peripheral model. In PBMCs, we demonstrated that ER stress markers and ER Ca^{2+} homeostasis are affected in pre-clinical and initial stages of the disease. We showed altered expression of ER stress markers (Fig. 8.6 A-B) and apoptotic-related ER proteins (Fig. 8.6 C), as well as decreased ER Ca^{2+} content and increased cytosolic levels (Fig. 8.5), especially in MCI subjects and mild AD patients. Although these cells were used as a blood peripheral model for a brain disorder, our study is corroborated

by others that described several cytogenetic alterations in AD lymphocytes (Petrozzi *et al.* 2002).

The disturbance of ER Ca^{2+} homeostasis is considered one of the earliest events implicated in the ER stress response (Kim *et al.* 2008b). Concerning AD, previous reports demonstrated that Aβ depletes ER Ca^{2+} content in cortical neurons, increasing cytosolic Ca^{2+} levels and finally leading to apoptosis (Huang *et al.* 2000; Ferreiro *et al.* 2008b; Resende *et al.* 2008b). Furthermore, Ca^{2+} signals were previously shown to be altered in AD lymphocytes, in a mechanism assumed to correspond to the loss of neuronal plasticity (Bondy *et al.* 1996). Our study further confirms the loss of Ca^{2+} homeostasis in AD peripheral blood cells and shows the involvement of ER Ca^{2+} depletion (Fig. 8.5 A-B) on the deregulation of cytosolic Ca^{2+} levels (Fig. 8.5 C-D) that were more evident in PBMCs obtained from MCI individuals and mild AD patients. Our results also suggest that ER Ca^{2+} release can be a trigger for the communication between ER and mitochondria, acting as a pro-apoptotic signal (Chami *et al.* 2008; Costa *et al.* 2010; González *et al.* 2010).

In opposition, moderate-severe AD patients presented GRP78 and XBP-1 levels similar to controls, indicating that ER stress response occurs essentially during the early stages of the disease. However, this response was shown to be followed by activation of ER stress-mediated cell death, as shown by increased GADD153/CHOP levels (Fig. 8.6 C). Accordingly with our results, previous reports have shown that patients with MCI present high levels of pro-apoptotic Bax (Gatta *et al.* 2009), revealing activation of apoptotic cell death pathways during preclinical stages in peripheral blood cells. In addition, synaptic alterations were also observed in MCI subjects and mild AD patients (Scheff *et al.* 2007). In lymphocytes from MCI subjects and AD patients, higher basal

ROS levels were detected and were associated with DNA damage by oxidative stress andenhanced apoptosis (Mecocci *et al.* 1998; Leuner *et al.* 2007). Therefore, a possible interaction between Ca^{2+} and ROS could be the trigger for cellular dysfunction (Boehning *et al.* 2003; 2004). Indeed, several studies demonstrate that ER stress promotes oxidative stress (Santos *et al.* 2009). In cultured neurons treated with Aβ, we have shown that ER Ca^{2+} release leads to the generation of ROS and subsequent activation of mitochondrial-mediated apoptosis (Ferreiro *et al.* 2008b).

In conclusion, we provided evidences of early ER dysfunction in AD. Using human and non-human models, namely the 3xTg-AD mice model and PBMCs from AD patients and also control and MCI subjects, we observed that ER stress and ER Ca^{2+} dyshomeostasis occurs in an age-, gender- and regional-dependent manner, supporting that alterations occurring at ER levels can be used as a biomarker for the diagnosis of this neurodegenerative disorder or even as a possible target for therapeutic intervention.

CHAPTER 9 - General Discussion and Conclusions

In order to better understand the molecular mechanisms underlying the toxic effects of Aβ, we investigated its role in the induction of ER stress response and analyzedthe ER/mitochondria cross-talk during Aβ-induced cell death and also the role of the NMDARs for glutamate, in particular of its NR2A and NR2B subunits, as an upstream mechanism leading to Aβ-induced ER stress. Fot this purpose, several human and non-human cellular and animal models with different complexity were employed.

The first model used was the mtDNA-depleted NT2 human teratocarcinoma ρ0 cellular line, characterized by the absence of functional mitochondria, that has been reported as an excellent model to study the role of mitochondria in ageing and age-related neurodegenerative diseases such as AD. The results presented in chapter 4 show that Aβ leads to ER stress and activates an ER stress-mediated apoptotic pathway by a mitochondria-dependent mechanism. It also reveals that Aβ-induced ER stress and ER Ca^{2+} release act as upstream events that activate the mitochondrial-mediated cell death pathway suggesting that the ER/mitochondria cross-talk might be a promising target for therapeutic intervention in AD.

To further characterize the ER/mitochondria cross-stalk in cell death triggered by Aβ, Alzheimer *versus* control cybridswere used. These cytoplasmic hybrids, or cybrids, result from the fusion of mtDNA-depleted human teratocarcinoma NT2 ρ0 cells with platelets isolated from peripheral blood samples obtained from sporadic AD patients or aged-matched control subjects. Data described in chapter 5 demonstrate that mitochondrial dysfunction occurring in AD cybrids due to COX inhibition enhances apoptosis induced under ER stress conditions triggered by Aβ.

The role of mitochondrial dysfunction arising from COX inhibition on the cellular susceptibility to ER stress induced by Aβ was also investigated in a neuronal cell model. In primary cultures of rat brain cortical neurons, it was demonstrated that ER Ca^{2+} dyshomeostasis and activation of caspase-dependent apoptotic cell death triggered by Aβ are potentiated by COX inhibition achieved through KCN pre-treatment (chapter 6).

Next, the role of AβO in ER stress was studiedin hippocampal neuronal cultures. Our results, described in detail in chapter 7, demonstrate the ability of AβO to trigger ER stress by an NMDAR-dependent mechanism. Moreover, we were able to discriminate the role of the NR2A and NR2Bsubunits of NMDAR, proving that NR2B has an important role in AβO-induced hippocampal ER stress and neurotoxicity that occurs after NR2B-AβO interaction. Furthermore, it was shown that NOX-mediated superoxide production downstream of NR2B-AβO interaction is involved in the loss of ER Ca^{2+} homeostasis. These results suggest that NR2B subunits can thus be another potential target for therapeutic intervention in early stages of AD progression.

The role of ER stress in AD was also studied *in vivo* using an AD animal model, the triple transgenic mice model (3xTg-AD) (chapter 8). It was shown that an early increase in ER stress markers occurs inthe brain of male and female 3xTg-AD mice in comparison with non-Tg mice and that these alterations were more pronounced in the hippocampus than in the cerebral cortex. In addition, female 3xTg-AD mice were shown to be more susceptible to ER stress. These results were in line with data obtained in a peripheral model of AD. In human PBMCs, namely lymphocytes, isolated from patients in different stages of AD (mild, moderate and severe) and individuals with

mild cognitive impairment *versus* non-demented age-matched subjects (chapter 8) it was observed that ER Ca^{2+} homeostasis is impaired and that the levels of ER stress increase during pre-clinical and early disease stages.

Overall, results presented in this thesis contribute to better understand the role of ER stress in AD pathogenesis. It was describedthat ER is susceptible to toxic oligomeric and fibrillar Aβ, and that upon ER stress, the close communication between ER and mitochondria leads to the activation of apoptotic cell death. Furthermore, it was shown that mitochondrial dysfunction occurring in AD as a result of COX inhibition increases the cellular susceptibility to ER stress induced by Aβ. Additionally, the interaction of Aβ with NR2B subunits of the NMDAR for glutamate and subsequent production of superoxide radicals through a NOX-mediated mechanism was shown to be involved in the activation of the ER stress response and induction of neuronal cell death. Finally, results obtained in an AD transgenic mice model and in a human peripheral model support that ER stress is an early event during the progression of AD.

REFERENCES

Agostinho P, Oliveira CR (2003). Involvement of calcineurin in the neurotoxic effects induced by amyloid-beta and prion peptides. **17**, 1189-1196.

Aihara Y, Inoue T, Tashiro T, Okamoto K, Komiya Y, Mikoshiba K (2001). Movement of endoplasmic reticulum in the living axon is distinct from other membranous vesicles in its rate, form, and sensitivity to microtubule inhibitors. *J Neurosci Res*. **65**, 236-246.

Albensi BC, Mattson MP (2000). Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. *Synapse*. **35**, 151-159.

Alberdi E, Sanchez-Gomez MV, Cavaliere F, Perez-Samartin A, Zugaza JL, Trullas R, Domercq M, Matute C (2010). Amyloid beta oligomers induce Ca^{2+} dysregulation and neuronal death through activation of ionotropic glutamate receptors. *Cell Calcium*. **47**, 264-272.

Ambrosio AF, Silva AP, Malva JO, Mesquita JF, Carvalho AP, Carvalho CM (2000). Role of desensitization of AMPA receptors on the neuronal viability and on the $[Ca^{2+}]i$ changes in cultured rat hippocampal neurons. *Eur J Neurosci*. **12**, 2021-2031.

Anandatheerthavarada HK, Biswas G, Robin MA, Avadhani NG (2003). Mitochondrial targeting and a novel transmembrane arrest of Alzheimer's amyloid precursor protein impairs mitochondrial function in neuronal cells. *J Cell Biol*. **161**, 41-54.

Andersen K, Launer LJ, Dewey ME, Letenneur L, Ott A, Copeland JR, Dartigues JF, Kragh-Sorensen P, Baldereschi M, Brayne C, Lobo A, Martinez-Lage JM, Stijnen T, Hofman A (1999). Gender differences in the incidence of AD and vascular dementia: The EURODEM Studies. EURODEM Incidence Research Group. *Neurology*. **53**, 1992-1997.

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG (1981). Sequence and organization of the human mitochondrial genome. *Nature*. **290**, 457-465.

Appleby RD, Porteous WK, Hughes G, James AM, Shannon D, Wei YH, Murphy MP (1999). Quantitation and origin of the mitochondrial membrane potential in human cells lacking mitochondrial DNA. *Eur J Biochem*. **262**, 108-116.

Arduino DM, Esteves AR, Cardoso SM, Oliveira CR (2009). Endoplasmic reticulum and mitochondria interplay mediates apoptotic cell death: relevance to Parkinson's disease. *Neurochem Int*. **55**, 341-348.

Area-Gomez E, de Groof AJ, Boldogh I, Bird TD, Gibson GE, Koehler CM, Yu WH, Duff KE, Yaffe MP, Pon LA, Schon EA (2009). Presenilins are enriched in endoplasmic reticulum membranes associated with mitochondria. *Am J Pathol*. **175**, 1810-1816.

Aridor M, Balch WE (1999). Integration of endoplasmic reticulum signaling in health and disease. *Nat Med*. **5**, 745-751.

Auberson YP, Allgeier H, Bischoff S, Lingenhoehl K, Moretti R, Schmutz M (2002). 5-Phosphonomethylquinoxalinediones as competitive NMDA receptor antagonists with a preference for the human 1A/2A, rather than 1A/2B receptor composition. *Bioorg Med Chem Lett*. **12**, 1099-1102.

Bai X, Wang X, Xu Q (2010). Endothelial damage and stem cell repair in atherosclerosis. *Vascul Pharmacol*. **52**, 224-229.

Bandeira-Duarte C, Carvalho CA, Cragoe Junior EJ, Carvalho AP (1990). Influence of isolation media on synaptosomal properties: intracellular pH, pCa, and Ca^{2+} uptake. *Neurochem Res*. **15**, 313-320.

Baumann O , Walz B (2001). Endoplasmic reticulum of animal cells and its organization into structural and functional domains. *Int Rev Cytol*. **205**, 149-214.

Baumgartner HK, Gerasimenko JV, Thorne C, Ferdek P, Pozzan T, Tepikin AV, Petersen OH, Sutton R, Watson AJ, Gerasimenko OV (2009). Calcium elevation in mitochondria is the main Ca^{2+} requirement for mitochondrial permeability transition pore (mPTP) opening. *J Biol Chem*. **284**, 20796-20803.

Bayir H, Kagan VE (2008). Bench-to-bedside review: Mitochondrial injury, oxidative stress and apoptosis--there is nothing more practical than a good theory. *Crit Care*. **12**, 206.

Bedard K, Krause KH (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. **87**, 245-313.

Berg L (1988). Clinical Dementia Rating (CDR). *Psychopharmacol Bull*. **24**, 637-639.

Berridge MJ (1995). Inositol trisphosphate and calcium signaling. *Ann N Y Acad Sci*. **766**, 31-43.

Berridge MJ, Lipp P, Bootman MD (2000). The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol*. **1**, 11-21.

Bhatia R, Lin H, Lal R (2000). Fresh and globular amyloid beta protein (1-42) induces rapid cellular degeneration: evidence for AbetaP channel-mediated cellular toxicity. *FASEB J*. **14**, 1233-1243.

Billings LM, Oddo S, Green KN, McGaugh JL, LaFerla FM (2005). Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron*. **45**, 675-688.

Biswas G, Anandatheerthavarada HK, Avadhani NG (2005). Mechanism of mitochondrial stress-induced resistance to apoptosis in mitochondrial DNA-depleted C2C12 myocytes. *Cell Death Differ*. **12**, 266-278.

Blass JP (2001). Brain metabolism and brain disease: is metabolic deficiency the proximate cause of Alzheimer dementia? *J Neurosci Res*. **66**, 851-856.

Blennow K, de Leon MJ, Zetterberg H (2006). Alzheimer's disease. *Lancet*. **368**, 387-403.

Boddapati S, Levites Y, Sierks MR (2011). Inhibiting beta-secretase activity in Alzheimer's disease cell models with single-chain antibodies specifically targeting APP. *J Mol Biol*. **405**, 436-447.

Boehning D, Patterson RL, Sedaghat L, Glebova NO, Kurosaki T, Snyder SH (2003). Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol*. **5**, 1051-1061.

Boehning D, Patterson RL , Snyder SH (2004). Apoptosis and calcium: new roles for cytochrome c and inositol 1,4,5-trisphosphate. *Cell Cycle*. **3**, 252-254.

Boehning D, van Rossum DB, Patterson RL, Snyder SH (2005). A peptide inhibitor of cytochrome c/inositol 1,4,5-trisphosphate receptor binding blocks intrinsic and extrinsic cell death pathways. *Proc Natl Acad Sci U S A*. **102**, 1466-1471.

Bondy B, Hofmann M, Muller-Spahn F, Witzko M, Hock C (1996). The PHA-induced calcium signal in lymphocytes is altered after blockade of K(+)-channels in Alzheimer's disease. *J Psychiatr Res*. **30**, 217-227.

Bonifacino JS, Weissman AM (1998). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu Rev Cell Dev Biol*. **14**, 19-57.

Bosetti F, Brizzi F, Barogi S, Mancuso M, Siciliano G, Tendi EA, Murri L, Rapoport SI, Solaini G (2002). Cytochrome c oxidase and mitochondrial F1F0-ATPase (ATP synthase) activities in platelets and brain from patients with Alzheimer's disease. *Neurobiol Aging*. **23**, 371-376.

Brennan AM, Suh SW, Won SJ, Narasimhan P, Kauppinen TM, Lee H, Edling Y, Chan PH, Swanson RA (2009). NADPH oxidase is the primary source of superoxide induced by NMDA receptor activation. *Nat Neurosci*. **12**, 857-863.

Brookmeyer R, Gray S, Kawas C (1998). Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. *Am J Public Health*. **88**, 1337-1342.

Buchet K, Godinot C (1998). Functional F1-ATPase essential in maintaining growth and membrane potential of human mitochondrial DNA-depleted rho degrees cells. *J Biol Chem*. **273**, 22983-22989.

Cardoso SM, Santana I, Swerdlow RH, Oliveira CR (2004b). Mitochondria dysfunction of Alzheimer's disease cybrids enhances Abeta toxicity. *J Neurochem*. **89**, 1417-1426.

Cardoso SM, Santana I., Swerdlow R.H., C.R. O (2004a). Cytochrome c oxidase is decreased in Alzheimer's disease cybrids enhances Abeta toxicity. *J Neurochem*. **25**, 105-110.

Cardoso SM, Santos S, Swerdlow RH, Oliveira CR (2001). Functional mitochondria are required for amyloid beta-mediated neurotoxicity. *FASEB J*. **15**, 1439-1441.

Cardoso SM, Swerdlow RH, Oliveira CR (2002). Induction of cytochrome c-mediated apoptosis by amyloid beta 25-35 requires functional mitochondria. *Brain Res*. **931**, 117-125.

Caspersen C, Wang N, Yao J, Sosunov A, Chen X, Lustbader JW, Xu HW, Stern D, McKhann G, Yan SD (2005). Mitochondrial Abeta: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease. *FASEB J*. **19**, 2040-2041.

Cassarino DS, Swerdlow RH, Parks JK, Parker WD, Jr., Bennett JP, Jr. (1998). Cyclosporin A increases resting mitochondrial membrane potential in SY5Y cells and reverses the depressed mitochondrial membrane potential of Alzheimer's disease cybrids. *Biochem Biophys Res Commun*. **248**, 168-173.

Chafekar SM, Zwart R, Veerhuis R, Vanderstichele H, Baas F, Scheper W (2008). Increased Abeta1-42 production sensitizes neuroblastoma cells for ER stress toxicity. *Curr Alzheimer Res*. **5**, 469-474.

Chakroborty S, Goussakov I, Miller MB, Stutzmann GE (2009). Deviant ryanodine receptor-mediated calcium release resets synaptic homeostasis in presymptomatic 3xTg-AD mice. *J Neurosci*. **29**, 9458-9470.

Chami M, Oules B, Szabadkai G, Tacine R, Rizzuto R, Paterlini-Brechot P (2008). Role of SERCA1 truncated isoform in the proapoptotic calcium transfer from ER to mitochondria during ER stress. *Mol Cell*. **32**, 641-651.

Chapman R, Sidrauski C, Walter P (1998). Intracellular signaling from the endoplasmic reticulum to the nucleus. *Annu Rev Cell Dev Biol*. **14**, 459-485.

Chardin P, McCormick F (1999). Brefeldin A: the advantage of being uncompetitive. *Cell*. **97**, 153-155.

Chen JX, Yan SS (2010). Role of mitochondrial amyloid-beta in Alzheimer's disease. *J Alzheimers Dis*. **20 Suppl 2**, S569-578.

Cheung HH, Lynn Kelly N, Liston P, Korneluk RG (2006). Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. *Exp Cell Res*. **312**, 2347-2357.

Chevet E, Cameron PH, Pelletier MF, Thomas DY, Bergeron JJ (2001). The endoplasmic reticulum: integration of protein folding, quality control, signaling and degradation. *Curr Opin Struct Biol*. **11**, 120-124.

Cirrito JR, Holtzman DM (2003). Amyloid beta and Alzheimer disease therapeutics: the devil may be in the details. *J Clin Invest*. **112**, 321-323.

Citron M (2004). Strategies for disease modification in Alzheimer's disease. *Nat Rev Neurosci*. **5**, 677-685.

Clapham DE (2007). Calcium signaling. *Cell*. **131**, 1047-1058.

Cnop M, Ladriere L, Igoillo-Esteve M, Moura RF, Cunha DA (2010). Causes and cures for endoplasmic reticulum stress in lipotoxic beta-cell dysfunction. *Diabetes Obes Metab*. **12 Suppl 2**, 76-82.

Copanaki E, Schurmann T, Eckert A, Leuner K, Muller WE, Prehn JH, Kogel D (2007). The amyloid precursor protein potentiates CHOP induction and cell death in response to ER Ca^{2+} depletion. *Biochim Biophys Acta*. **1773**, 157-165.

Coskun PE, Ruiz-Pesini E, Wallace DC (2003). Control region mtDNA variants: longevity, climatic adaptation, and a forensic conundrum. *Proc Natl Acad Sci U S A*. **100**, 2174-2176.

Costa RO, Ferreiro E, Cardoso SM, Oliveira CR, Pereira CM (2010). ER stress-mediated apoptotic pathway induced by Abeta peptide requires the presence of functional mitochondria. *J Alzheimers Dis*. **20**, 625-636.

Costa RO, Ferreiro E, Martins I, Santana I, Cardoso SM, Oliveira CR, Pereira CMF (2011). Amyloid β-induced ER stress is enhanced under mitochondrial dysfunction conditions *Neurobiol Aging*. **In press**.

Cowburn RF, Wiehager B, Trief E, Li-Li M, Sundstrom E (1997). Effects of beta-amyloid-(25-35) peptides on radioligand binding to excitatory amino acid receptors and voltage-dependent calcium channels: evidence for a selective affinity for the glutamate and glycine recognition sites of the NMDA receptor. *Neurochem Res*. **22**, 1437-1442.

Cregan SP, MacLaurin JG, Craig CG, Robertson GS, D.W. N, Park DS, Slack RS (1999). Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. *J Neurosci.* . **19**, 7860-7869.

Csordas G, Hajnoczky G (2009). SR/ER-mitochondrial local communication: calcium and ROS. *Biochim Biophys Acta*. **1787**, 1352-1362.

Cudna RE, Dickson AJ (2003). Endoplasmic reticulum signaling as a determinant of recombinant protein expression. *Biotechnol Bioeng*. **81**, 56-65.

Czirr E, Cottrell BA, Leuchtenberger S, Kukar T, Ladd TB, Esselmann H, Paul S, Schubenel R, Torpey JW, Pietrzik CU, Golde TE, Wiltfang J, Baumann K, Koo EH, Weggen S (2008). Independent generation of Abeta42 and Abeta38 peptide species by gamma-secretase. *J Biol Chem*. **283**, 17049-17054.

Dahlgren KN, Manelli AM, Stine WB, Jr., Baker LK, Krafft GA, LaDu MJ (2002). Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J Biol Chem*. **277**, 32046-32053.

De Felice FG, Velasco PT, Lambert MP, Viola K, Fernandez SJ, Ferreira ST, Klein WL (2007). Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. *J Biol Chem*. **282**, 11590-11601.

Deniaud A, Sharaf el dein O, Maillier E, Poncet D, Kroemer G, Lemaire C, Brenner C (2008). Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. *Oncogene*. **27**, 285-299.

Deshpande A, Kawai H, Metherate R, Glabe CG, Busciglio J (2009). A role for synaptic zinc in activity-dependent Abeta oligomer formation and accumulation at excitatory synapses. *J Neurosci*. **29**, 4004-4015.

Dey R, Moraes CT (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential decrease susceptibility to apoptosis and do not modulate the protective effect of Bcl-x(L) in osteosarcoma cells. *J Biol Chem*. **275**, 7087-7094.

DiMauro S (2006). Mitochondrial myopathies. *Curr Opin Rheumatol*. **18**, 636-641.

Dinamarca MC, Colombres M, Cerpa W, Bonansco C, Inestrosa NC (2008). Beta-amyloid oligomers affect the structure and function of the postsynaptic region: role of the Wnt signaling pathway. *Neurodegener Dis*. **5**, 149-152.

Dragicevic N, Mamcarz M, Zhu Y, Buzzeo R, Tan J, Arendash GW, Bradshaw PC (2010). Mitochondrial amyloid-beta levels are associated with the extent of mitochondrial dysfunction in different brain regions and the degree of cognitive impairment in Alzheimer's transgenic mice. *J Alzheimers Dis*. **20 Suppl 2**, S535-550.

Du H, Guo L, Zhang W, Rydzewska M, Yan S (2009). Cyclophilin D deficiency improves mitochondrial function and learning/memory in aging Alzheimer disease mouse model. *Neurobiol Aging*.

Dulin F, Leveille F, Ortega JB, Mornon JP, Buisson A, Callebaut I, Colloc'h N (2008). P3 peptide, a truncated form of A beta devoid of synaptotoxic effect, does not assemble into soluble oligomers. *FEBS Lett*. **582**, 1865-1870.

Endo M (1977). Calcium release from the sarcoplasmic reticulum *Physiol Rev.***57**, 71-108.

Esler WP, Wolfe MS (2001). A portrait of Alzheimer secretases--new features and familiar faces. *Science*. **293**, 1449-1454.

Fan MM, Raymond LA (2007). N-methyl-D-aspartate (NMDA) receptor function and excitotoxicity in Huntington's disease. *Prog Neurobiol*. **81**, 272-293.

Ferraresi R, Troiano L, Pinti M, Roat E, Lugli E, Quaglino D, Taverna D, Bellizzi D, Passarino G, Cossarizza A (2008). Resistance of mtDNA-depleted cells to apoptosis. *Cytometry A*. **73**, 528-537.

Ferreira IL, Resende R, Ferreiro E, Rego AC, Pereira CF (2010). Multiple defects in energy metabolism in Alzheimer's disease. *Curr Drug Targets*. **11**, 1193-1206.

Ferreiro E, Costa R, Marques S, Cardoso SM, Oliveira CR, Pereira CM (2008a). Involvement of mitochondria in endoplasmic reticulum stress-induced apoptotic cell death pathway triggered by the prion peptide PrP(106-126). *J Neurochem*. **104**, 766-776.

Ferreiro E, Eufrasio A, Pereira C, Oliveira CR, Rego AC (2007). Bcl-2 overexpression protects against amyloid-beta and prion toxicity in GT1-7 neural cells. *J Alzheimers Dis*. **12**, 223-228.

Ferreiro E, Oliveira CR, Pereira C (2004). Involvement of endoplasmic reticulum Ca^{2+} release through ryanodine and inositol 1,4,5-triphosphate receptors in the neurotoxic effects induced by the amyloid-beta peptide. *J Neurosci Res*. **76**, 872-880.

Ferreiro E, Oliveira CR, Pereira CM (2008b). The release of calcium from the endoplasmic reticulum induced by amyloid-beta and prion peptides activates the mitochondrial apoptotic pathway. *Neurobiol Dis*. **30**, 331-342.

Ferreiro E, Resende R, Costa R, Oliveira CR, Pereira CM (2006). An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity. *Neurobiol Dis*. **23**, 669-678.

Ferrer I (2009). Altered mitochondria, energy metabolism, voltage-dependent anion channel, and lipid rafts converge to exhaust neurons in Alzheimer's disease. *J Bioenerg Biomembr*. **41**, 425-431.

Finch EA, Turner TJ, Goldin SM (1991). Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science*. **252**, 443-446.

Folstein MF, Folstein SE, McHugh PR (1975). "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res*. **12**, 189-198.

Forman MS, Lee VM, Trojanowski JQ (2003). 'Unfolding' pathways in neurodegenerative disease. *Trends Neurosci*. **26**, 407-410.

Foyouzi-Youssefi R, Arnaudeau S, Borner C, Kelley WL, Tschopp J, Lew DP, Demaurex N, Krause KH (2000). Bcl-2 decreases the free Ca^{2+} concentration within the endoplasmic reticulum. *Proc Natl Acad Sci U S A*. **97**, 5723-5728.

Friel DD, Tsien RW (1992). A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurones modulates effects of Ca^{2+} entry on $[Ca^{2+}]i$. *J Physiol*. **450**, 217-246.

Gatta L, Cardinale A, Wannenes F, Consoli C, Armani A, Molinari F, Mammi C, Stocchi F, Torti M, Rosano GM, Fini M (2009). Peripheral blood mononuclear cells from mild cognitive impairment patients show deregulation of Bax and Sod1 mRNAs. *Neurosci Lett*. **453**, 36-40.

Gilmore TD (2006). Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene*. **25**, 6680-6684.

Giorgi C, De Stefani D, Bononi A, Rizzuto R, Pinton P (2009). Structural and functional link between the mitochondrial network and the endoplasmic reticulum. *Int J Biochem Cell Biol*. **41**, 1817-1827.

Golde TE (2003). Alzheimer disease therapy: can the amyloid cascade be halted? *J Clin Invest*. **111**, 11-18.

Golde TE, Dickson D, Hutton M (2006). Filling the gaps in the abeta cascade hypothesis of Alzheimer's disease. *Curr Alzheimer Res*. **3**, 421-430.

Gong Y, Chang L, Viola KL, Lacor PN, Lambert MP, Finch CE, Krafft GA, Klein WL (2003). Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss. *Proc Natl Acad Sci U S A*. **100**, 10417-10422.

Gonzalez D, Espino J, Bejarano I, Lopez JJ, Rodriguez AB, Pariente JA (2010). Caspase-3 and -9 are activated in human myeloid HL-60 cells by calcium signal. *Mol Cell Biochem*. **333**, 151-157.

González D, Espino J, Bejarano I, Lopez JJ, Rodriguez AB, Pariente JA (2010). Caspase-3 and -9 are activated in human myeloid HL-60 cells by calcium signal. *Mol Cell Biochem*. **333**, 151-157.

Goto Y, Niidome T, Akaike A, Kihara T, Sugimoto H (2006). Amyloid beta-peptide preconditioning reduces glutamate-induced neurotoxicity by promoting endocytosis of NMDA receptor. *Biochem Biophys Res Commun*. **351**, 259-265.

Goussakov I, Miller MB, Stutzmann GE (2010). NMDA-mediated Ca(2+) influx drives aberrant ryanodine receptor activation in dendrites of young Alzheimer's disease mice. *J Neurosci*. **30**, 12128-12137.

Guo Q, Sopher BL, Furukawa K, Pham DG, Robinson N, Martin GM, Mattson MP (1997). Alzheimer's presenilin mutation sensitizes neural cells to apoptosis induced by trophic factor withdrawal and amyloid

beta-peptide: involvement of calcium and oxyradicals. *J Neurosci*. **17**, 4212-4222.

Haass C, Selkoe DJ (2007). Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol*. **8**, 101-112.

Hacki J, Egger L, Monney L, Conus S, Rosse T, Fellay I, Borner C (2000). Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene*. **19**, 2286-2295.

Hansson Petersen CA, Alikhani N, Behbahani H, Wiehager B, Pavlov PF, Alafuzoff I, Leinonen V, Ito A, Winblad B, Glaser E, Ankarcrona M (2008). The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae. *Proc Natl Acad Sci U S A*. **105**, 13145-13150.

Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D (2000). Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell*. **5**, 897-904.

Harding HP, Zhang Y, Ron D (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature*. **397**, 271-274.

Hardy J, Selkoe DJ (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. **297**, 353-356.

Harkany T, Abraham I, Timmerman W, Laskay G, Toth B, Sasvari M, Konya C, Sebens JB, Korf J, Nyakas C, Zarandi M, Soos K, Penke B, Luiten PG (2000). beta-amyloid neurotoxicity is mediated by a glutamate-triggered excitotoxic cascade in rat nucleus basalis. *Eur J Neurosci*. **12**, 2735-2745.

He S, Yaung J, Kim YH, Barron E, Ryan SJ, Hinton DR (2008). Endoplasmic reticulum stress induced by oxidative stress in retinal pigment epithelial cells. *Graefes Arch Clin Exp Ophthalmol*. **246**, 677-683.

Heath-Engel HM, Chang NC, Shore GC (2008). The endoplasmic reticulum in apoptosis and autophagy: role of the BCL-2 protein family. *Oncogene*. **27**, 6419-6433.

Heinitz K, Beck M, Schliebs R, Perez-Polo JR (2006). Toxicity mediated by soluble oligomers of beta-amyloid(1-42) on cholinergic SN56.B5.G4 cells. *J Neurochem*. **98**, 1930-1945.

Herrmann N, Li A, Lanctot K (2011). Memantine in dementia: a review of the current evidence. *Expert Opin Pharmacother*. **12**, 787-800.

Hertz E, Yu CH, Hertz L, Juurlink BHJ, Schousboe A (1989). Preparation of primary cultures of mouse cortical neurons. *In: Shahar,. A., De Vellis, J., Vernadakis, A., Haber, B. (Eds.), A Dissection and tissue culture manual of the nervous system. Alan. Liss, New York*.

Hetz CA, Soto C (2006). Stressing out the ER: a role of the unfolded protein response in prion-related disorders. *Curr Mol Med*. **6**, 37-43.

Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagishi S, Bando Y, Imaizumi K, Tsujimoto Y, Tohyama M (2004). Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. *J Cell Biol*. **165**, 347-356.

Hoozemans JJ, van Haastert ES, Nijholt DA, Rozemuller AJ, Eikelenboom P, Scheper W (2009). The unfolded protein response is activated in pretangle neurons in Alzheimer's disease hippocampus. *Am J Pathol*. **174**, 1241-1251.

Hoozemans JJ, Veerhuis R, Van Haastert ES, Rozemuller JM, Baas F, Eikelenboom P, Scheper W (2005). The unfolded protein response is activated in Alzheimer's disease. *Acta Neuropathol*. **110**, 165-172.

Hotamisligil GS (2010). Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell*. **140**, 900-917.

Hoyer-Hansen M, Jaattela M (2007). Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ*. **14**, 1576-1582.

Hu NW, Klyubin I, Anwyl R, Rowan MJ (2009). GluN2B subunit-containing NMDA receptor antagonists prevent Abeta-mediated synaptic plasticity disruption in vivo. *Proc Natl Acad Sci U S A*. **106**, 20504-20509.

Hu Y, Moraes CT, Savaraj N, Priebe W, Lampidis TJ (2000). Rho(0) tumor cells: a model for studying whether mitochondria are targets for rhodamine 123, doxorubicin, and other drugs. *Biochem Pharmacol*. **60**, 1897-1905.

Huang HM, Ou HC, Hsieh SJ (2000). Antioxidants prevent amyloid peptide-induced apoptosis and alteration of calcium homeostasis in cultured cortical neurons. *Life Sci*. **66**, 1879-1892.

Huang HM, Zhang H, Ou HC, Chen HL, Gibson GE (2004). alpha-keto-beta-methyl-n-valeric acid diminishes reactive oxygen species and alters endoplasmic reticulum Ca(2+) stores. *Free Radic Biol Med*. **37**, 1779-1789.

Hummasti S, Hotamisligil GS (2010). Endoplasmic reticulum stress and inflammation in obesity and diabetes. *Circ Res*. **107**, 579-591.

Hutchin T, Cortopassi G (1995). A mitochondrial DNA clone is associated with increased risk for Alzheimer disease. *Proc Natl Acad Sci U S A*. **92**, 6892-6895.

Ienco EC, Simoncini C, Orsucci D, Petrucci L, Filosto M, Mancuso M, Siciliano G (2011). May "mitochondrial eve" and mitochondrial haplogroups play a role in neurodegeneration and Alzheimer's disease? *Int J Alzheimers Dis*. **2011**, 709061.

Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R (2001). An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell*. **105**, 891-902.

Imai Y, Soda M, Takahashi R (2000). Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J Biol Chem*. **275**, 35661-35664.

Imaizumi K, Miyoshi K, Katayama T, Yoneda T, Taniguchi M, Kudo T, Tohyama M (2001). The unfolded protein response and Alzheimer's disease. *Biochim Biophys Acta*. **1536**, 85-96.

Inagi R (2010). Endoplasmic reticulum stress as a progression factor for kidney injury. *Curr Opin Pharmacol*. **10**, 156-165.

Jacobson MD, Burne JF, King MP, Miyashita T, Reed JC, Raff MC (1993). Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature*. **361**, 365-369.

Jacques C, Chevrollier A, Loiseau D, Lagoutte L, Savagner F, Malthiery Y, Reynier P (2006). mtDNA controls expression of the Death Associated Protein 3. *Exp Cell Res*. **312**, 737-745.

Jiang S, Cai J, Wallace DC, Jones DP (1999). Cytochrome c-mediated apoptosis in cells lacking mitochondrial DNA. Signaling pathway involving release and caspase 3 activation is conserved. *J Biol Chem*. **274**, 29905-29911.

Johansson S, Radesater AC, Cowburn RF, Thyberg J, Luthman J (2006). Modelling of amyloid beta-peptide induced lesions using roller-drum incubation of hippocampal slice cultures from neonatal rats. *Exp Brain Res*. **168**, 11-24.

Katayama T, Imaizumi K, Manabe T, Hitomi J, Kudo T, Tohyama M (2004). Induction of neuronal death by ER stress in Alzheimer's disease. *J Chem Neuroanat*. **28**, 67-78.

Kaufman RJ (2002). Orchestrating the unfolded protein response in health and disease. *J Clin Invest*. **110**, 1389-1398.

Kelly BL, Ferreira A (2006). beta-Amyloid-induced dynamin 1 degradation is mediated by N-methyl-D-aspartate receptors in hippocampal neurons. *J Biol Chem*. **281**, 28079-28089.

Khan SM, Cassarino DS, Abramova NN, Keeney PM, Borland MK, Trimmer PA, Krebs CT, Bennett JC, Parks JK, Swerdlow RH, Parker WD, Jr., Bennett JP, Jr. (2000). Alzheimer's disease cybrids replicate beta-amyloid abnormalities through cell death pathways. *Ann Neurol*. **48**, 148-155.

Kim I, Xu W, Reed JC (2008a). Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov*. **7**, 1013-1030.

Kim J, Choi TG, Ding Y, Kim Y, Ha KS, Lee KH, Kang I, Ha J, Kaufman RJ, Lee J, Choe W, Kim SS (2008b). Overexpressed cyclophilin B suppresses apoptosis associated with ROS and Ca^{2+} homeostasis after ER stress. *J Cell Sci*. **121**, 3636-3648.

Kim SJ, Zhang Z, Hitomi E, Lee YC, Mukherjee AB (2006). Endoplasmic reticulum stress-induced caspase-4 activation mediates apoptosis and neurodegeneration in INCL. *Hum Mol Genet*. **15**, 1826-1834.

Kim SM, Park HS, Jun DY, Woo HJ, Woo MH, Yang CH, Kim YH (2009). Mollugin induces apoptosis in human Jurkat T cells through endoplasmic reticulum stress-mediated activation of JNK and caspase-12 and subsequent activation of mitochondria-dependent caspase cascade regulated by Bcl-xL. *Toxicol Appl Pharmacol*. **241**, 210-220.

Kincaid MM, Cooper AA (2007). ERADicate ER stress or die trying. *Antioxid Redox Signal*. **9**, 2373-2387.

King MP, Attardi G (1989). Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science*. **246**, 500-503.

Kish SJ, Bergeron C, Rajput A, Dozic S, Mastrogiacomo F, Chang LJ, Wilson JM, DiStefano LM, Nobrega JN (1992). Brain cytochrome oxidase in Alzheimer's disease. *J Neurochem*. **59**, 776-779.

Klafki HW, Wiltfang J, Staufenbiel M (1996). Electrophoretic separation of ßA4 peptides (1-40) and (1-42). *Analytical Biochemistry*. **237**, 24-29.

Klausner RD, Donaldson JG, Lippincott-Schwartz J (1992). Brefeldin A: insights into the control of membrane traffic and organelle structure. *J Cell Biol*. **116**, 1071-1080.

Klee M, Pallauf K, Alcala S, Fleischer A, Pimentel-Muinos FX (2009). Mitochondrial apoptosis induced by BH3-only molecules in the exclusive presence of endoplasmic reticular Bak. *EMBO J*. **28**, 1757-1768.

Klein WL (2006). Synaptic targeting by A beta oligomers (ADDLS) as a basis for memory loss in early Alzheimer's disease. *Alzheimers Dement*. **2**, 43-55.

Klein WL, Krafft GA, Finch CE (2001). Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? *Trends Neurosci*. **24**, 219-224.

Koch GL (1990). The endoplasmic reticulum and calcium storage. *Bioessays*. **12**, 527-531.

Kohno K, Normington K, Sambrook J, Gething MJ, Mori K (1993). The promoter region of the yeast KAR2 (BiP) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. *Mol Cell Biol*. **13**, 877-890.

Kokubo H, Kayed R, Glabe CG, Yamaguchi H (2005). Soluble Abeta oligomers ultrastructurally localize to cell processes and might be related to synaptic dysfunction in Alzheimer's disease brain. *Brain Res*. **1031**, 222-228.

Kozutsumi Y, Segal M, Normington K, Gething MJ, Sambrook J (1988). The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature*. **332**, 462-464.

Kruger J, Hinttala R, Majamaa K, Remes AM (2010). Mitochondrial DNA haplogroups in early-onset Alzheimer's disease and frontotemporal lobar degeneration. *Mol Neurodegener*. **5**, 8.

Kudo T (2011). Therapeutic strategies for Alzheimer disease based on endoplasmic reticulum stress. *Nihon Shinkei Seishin Yakurigaku Zasshi*. **30**, 163-168.

Kumar A, Zou L, Yuan X, Long Y, Yang K (2002). N-methyl-D-aspartate receptors: transient loss of NR1/NR2A/NR2B subunits after traumatic brain injury in a rodent model. *J Neurosci Res*. **67**, 781-786.

Kuo TH, Kim HR, Zhu L, Yu Y, Lin HM, Tsang W (1998). Modulation of endoplasmic reticulum calcium pump by Bcl-2. *Oncogene*. **17**, 1903-1910.

Kwong JQ, Henning MS, Starkov AA, Manfredi G (2007). The mitochondrial respiratory chain is a modulator of apoptosis. *J Cell Biol*. **179**, 1163-1177.

Lacor PN, Buniel MC, Chang L, Fernandez SJ, Gong Y, Viola KL, Lambert MP, Velasco PT, Bigio EH, Finch CE, Krafft GA, Klein WL (2004). Synaptic targeting by Alzheimer's-related amyloid beta oligomers. *J Neurosci*. **24**, 10191-10200.

Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, Viola KL, Klein WL (2007). Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *J Neurosci*. **27**, 796-807.

LaFerla FM (2002). Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. *Nat Rev Neurosci*. **3**, 862-872.

Lai CS, Preisler J, Baum L, Lee DH, Ng HK, Hugon J, So KF, Chang RC (2009). Low molecular weight Abeta induces collapse of endoplasmic reticulum. *Mol Cell Neurosci*. **41**, 32-43.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998). Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci U S A*. **95**, 6448-6453.

Lambert MP, Velasco PT, Chang L, Viola KL, Fernandez S, Lacor PN, Khuon D, Gong Y, Bigio EH, Shaw P, De Felice FG, Krafft GA, Klein WL (2007). Monoclonal antibodies that target pathological assemblies of Abeta. *J Neurochem*. **100**, 23-35.

Lanner JT, Georgiou DK, Joshi AD, Hamilton SL (2010). Ryanodine receptors: structure, expression, molecular details, and function in calcium release. *Cold Spring Harb Perspect Biol*. **2**, a003996.

Laurén J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM (2009). Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature*. **457**, 1128-1132.

Laurén J., David A. Gimbel, Haakon B. Nygaard, John W. Gilbert , Strittmatter SM (2010). Replying to: H. W. Kessels, L. N. Nguyen, S. Nabavi & R. Malinow Nature 466, 10.1038/nature09217; Nature 466, E4-E5 .

Lei X, Zhang S, Bohrer A, Ramanadham S (2008). Calcium-independent phospholipase A2 (iPLA2 beta)-mediated ceramide generation plays a key role in the cross-talk between the endoplasmic

reticulum (ER) and mitochondria during ER stress-induced insulin-secreting cell apoptosis. *J Biol Chem*. **283**, 34819-34832.

Lesne S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH (2006). A specific amyloid-beta protein assembly in the brain impairs memory. *Nature*. **440**, 352-357.

Leuner K, Pantel J, Frey C, Schindowski K, Schulz K, Wegat T, Maurer K, Eckert A, Muller WE (2007). Enhanced apoptosis, oxidative stress and mitochondrial dysfunction in lymphocytes as potential biomarkers for Alzheimer's disease. *J Neural Transm Suppl*, 207-215.

Li BS, Sun MK, Zhang L, Takahashi S, Ma W, Vinade L, Kulkarni AB, Brady RO, Pant HC (2001). Regulation of NMDA receptors by cyclin-dependent kinase-5. *Proc Natl Acad Sci U S A*. **98**, 12742-12747.

Li F, Tsien JZ (2009). Memory and the NMDA receptors. *N Engl J Med*. **361**, 302-303.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*. **91**, 479-489.

Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA (1998). Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem*. **273**, 2015-2023.

Lin JH, Walter P, Yen TS (2008). Endoplasmic reticulum stress in disease pathogenesis. *Annu Rev Pathol*. **3**, 399-425.

Lin MT, Beal MF (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*. **443**, 787-795.

Lin MT, Simon DK, Ahn CH, Kim LM, Beal MF (2002). High aggregate burden of somatic mtDNA point mutations in aging and Alzheimer's disease brain. *Hum Mol Genet*. **11**, 133-145.

Lindholm D, Wootz H, Korhonen L (2006). ER stress and neurodegenerative diseases. *Cell Death Differ*. **13**, 385-392.

Ling ZQ, Tian Q, Wang L, Fu ZQ, Wang XC, Wang Q, Wang JZ (2009). Constant illumination induces Alzheimer-like damages with endoplasmic reticulum involvement and the protection of melatonin. *J Alzheimers Dis*. **16**, 287-300.

Lipton SA (2006). Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond. *Nat Rev Drug Discov*. **5**, 160-170.

Liu H, Qian J, Wang F, Sun X, Xu X, Xu W, Zhang X (2010). Expression of two endoplasmic reticulum stress markers, GRP78 and GADD153, in rat retinal detachment model and its implication. *Eye (Lond)*. **24**, 137-144.

Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science*. **304**, 1021-1024.

Lujan R, Roberts JD, Shigemoto R, Ohishi H, Somogyi P (1997). Differential plasma membrane distribution of metabotropic glutamate receptors mGluR1 alpha, mGluR2 and mGluR5, relative to neurotransmitter release sites. *J Chem Neuroanat*. **13**, 219-241.

Luo JH, Fu ZY, Losi G, Kim BG, Prybylowski K, Vissel B, Vicini S (2002). Functional expression of distinct NMDA channel subunits tagged with green fluorescent protein in hippocampal neurons in culture. *Neuropharmacology*. **42**, 306-318.

Lustbader JW, Cirilli M, Lin C, Xu HW, Takuma K, Wang N, Caspersen C, Chen X, Pollak S, Chaney M, Trinchese F, Liu S, Gunn-Moore F, Lue LF, Walker DG, Kuppusamy P, Zewier ZL, Arancio O, Stern D, Yan SS, Wu H (2004). ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease. *Science*. **304**, 448-452.

Mancuso M, Coppede F, Murri L, Siciliano G (2007). Mitochondrial cascade hypothesis of Alzheimer's disease: myth or reality? *Antioxid Redox Signal*. **9**, 1631-1646.

Manczak M, Anekonda TS, Henson E, Park BS, Quinn J, Reddy PH (2006). Mitochondria are a direct site of A beta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. *Hum Mol Genet*. **15**, 1437-1449.

Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K, Harding HP , Ron D (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev*. **18**, 3066-3077.

Masliah E, Terry RD, Mallory M, Alford M, Hansen LA (1990). Diffuse plaques do not accentuate synapse loss in Alzheimer's disease. *Am J Pathol*. **137**, 1293-1297.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985). Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci U S A*. **82**, 4245-4249.

Mattson MP (2002). Oxidative stress, perturbed calcium homeostasis, and immune dysfunction in Alzheimer's disease. *J Neurovirol*. **8**, 539-550.

Mattson MP (2004). Pathways towards and away from Alzheimer's disease. *Nature*. **430**, 631-639.

Mattson MP, Chan SL (2003). Neuronal and glial calcium signaling in Alzheimer's disease. *Cell Calcium*. **34**, 385-397.

Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE (1992). beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J Neurosci*. **12**, 376-389.

Mattson MP, Gary DS, Chan SL, Duan W (2001). Perturbed endoplasmic reticulum function, synaptic apoptosis and the pathogenesis of Alzheimer's disease. *Biochem Soc Symp*, 151-162.

Mattson MP, LaFerla FM, Chan SL, Leissring MA, Shepel PN, Geiger JD (2000). Calcium signaling in the ER: its role in neuronal plasticity and neurodegenerative disorders. *Trends Neurosci*. **23**, 222-229.

Maxfield FR, Wustner D (2002). Intracellular cholesterol transport. *J Clin Invest*. **110**, 891-898.

McGuckin MA, Eri RD, Das I, Lourie R, Florin TH (2010). ER stress and the unfolded protein response in intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol*. **298**, G820-832.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984). Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology*. **34**, 939-944.

Mecocci P, Polidori MC, Ingegni T, Cherubini A, Chionne F, Cecchetti R, Senin U (1998). Oxidative damage to DNA in lymphocytes from AD patients. *Neurology*. **51**, 1014-1017.

Meldolesi J, Pozzan T (1998). The endoplasmic reticulum Ca^{2+} store: a view from the lumen. *Trends Biochem Sci*. **23**, 10-14.

Merksamer PI, Papa FR (2010). The UPR and cell fate at a glance. *J Cell Sci*. **123**, 1003-1006.

Michalak M, Robert Parker JM, Opas M (2002). Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium*. **32**, 269-278.

Miguel-Hidalgo JJ, Alvarez XA, Cacabelos R, Quack G (2002). Neuroprotection by memantine against neurodegeneration induced by beta-amyloid(1-40). *Brain Res*. **958**, 210-221.

Miller SW, Trimmer PA, Parker WD, Jr., Davis RE (1996). Creation and characterization of mitochondrial DNA-depleted cell lines with "neuronal-like" properties. *J Neurochem*. **67**, 1897-1907.

Minamino T , Kitakaze M (2010). ER stress in cardiovascular disease. *J Mol Cell Cardiol*. **48**, 1105-1110.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron*. **12**, 529-540.

Moreira PI, Cardoso SM, Santos MS, Oliveira CR (2006). The key role of mitochondria in Alzheimer's disease. *J Alzheimers Dis*. **9**, 101-110.

Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y (2002). An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem*. **277**, 34287-34294.

Mosconi L (2005). Brain glucose metabolism in the early and specific diagnosis of Alzheimer's disease. FDG-PET studies in MCI and AD. *Eur J Nucl Med Mol Imaging*. **32**, 486-510.

Mosconi L, Pupi A, De Leon MJ (2008). Brain glucose hypometabolism and oxidative stress in preclinical Alzheimer's disease. *Ann N Y Acad Sci*. **1147**, 180-195.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. **65**, 55-63.

Myhrer T (1998). Adverse psychological impact, glutamatergic dysfunction, and risk factors for Alzheimer's disease. *Neurosci Biobehav Rev*. **23**, 131-139.

Naidoo N (2009). ER and aging-Protein folding and the ER stress response. *Ageing Res Rev*. **8**, 150-159.

Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature*. **403**, 98-103.

Nathalie Lacor P (2007). Advances on the Understanding of the Origins of Synaptic Pathology in AD. *Curr Genomics*. **8**, 486-508.

Nathalie P, Jean-Noel O (2008). Processing of amyloid precursor protein and amyloid peptide neurotoxicity. *Curr Alzheimer Res*. **5**, 92-99.

Newman M, Musgrave IF, Lardelli M (2007). Alzheimer disease: amyloidogenesis, the presenilins and animal models. *Biochim Biophys Acta*. **1772**, 285-297.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*. **376**, 37-43.

Niederer KE, Morrow DK, Gettings JL, Irick M, Krawiecki A, Brewster JL (2005). Cypermethrin blocks a mitochondria-dependent apoptotic signal initiated by deficient N-linked glycosylation within the endoplasmic reticulum. *Cell Signal*. **17**, 177-186.

Nijtmans LG, Spelbrink JN, Van Galen MJ, Zwaan M, Klement P, Van den Bogert C (1995). Expression and fate of the nuclearly encoded subunits of cytochrome-c oxidase in cultured human cells depleted of mitochondrial gene products. *Biochim Biophys Acta*. **1265**, 117-126.

Nishitoh H, Kadowaki H, Nagai A, Maruyama T, Yokota T, Fukutomi H, Noguchi T, Matsuzawa A, Takeda K, Ichijo H (2008). ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. *Genes Dev*. **22**, 1451-1464.

Nishitsuji K, Tomiyama T, Ishibashi K, Ito K, Teraoka R, Lambert MP, Klein WL, Mori H (2009). The E693Delta mutation in amyloid precursor protein increases intracellular accumulation of amyloid beta oligomers and causes endoplasmic reticulum stress-induced apoptosis in cultured cells. *Am J Pathol*. **174**, 957-969.

Nuss JE, Choksi KB, DeFord JH, Papaconstantinou J (2008). Decreased enzyme activities of chaperones PDI and BiP in aged mouse livers. *Biochem Biophys Res Commun*. **365**, 355-361.

Nutt LK, Pataer A, Pahler J, Fang B, Roth J, McConkey DJ, Swisher SG (2002). Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca^{2+} stores. *J Biol Chem*. **277**, 9219-9225.

O'Nuallain B, Shivaprasad S, Kheterpal I, Wetzel R (2005). Thermodynamics of A beta(1-40) amyloid fibril elongation. *Biochemistry*. **44**, 12709-12718.

Obeng EA, Boise LH (2005). Caspase-12 and caspase-4 are not required for caspase-dependent endoplasmic reticulum stress-induced apoptosis. *J Biol Chem*. **280**, 29578-29587.

Oddo S, Caccamo A, Kitazawa M, Tseng BP, LaFerla FM (2003a). Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiol Aging*. **24**, 1063-1070.

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM (2003b). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron*. **39**, 409-421.

Oddo S, Caccamo A, Tran L, Lambert MP, Glabe CG, Klein WL, LaFerla FM (2006). Temporal profile of amyloid-beta (Abeta) oligomerization in an in vivo model of Alzheimer disease. A link between Abeta and tau pathology. *J Biol Chem*. **281**, 1599-1604.

Oida Y, Izuta H, Oyagi A, Shimazawa M, Kudo T, Imaizumi K, Hara H (2008). Induction of BiP, an ER-resident protein, prevents the neuronal death induced by transient forebrain ischemia in gerbil. *Brain Res*. **1208**, 217-224.

Ojaimi J, Masters CL, McLean C, Opeskin K, McKelvie P, Byrne E (1999). Irregular distribution of cytochrome c oxidase protein subunits in aging and Alzheimer's disease. *Ann Neurol*. **46**, 656-660.

Oyadomari S, Mori M (2004). Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ*. **11**, 381-389.

Pagani L, Eckert A (2011a). Amyloid-Beta interaction with mitochondria. *Int J Alzheimers Dis*. **2011**, 925050.

Pahl HL, Baeuerle PA (1995). A novel signal transduction pathway from the endoplasmic reticulum to the nucleus is mediated by transcription factor NF-kappa B. *EMBO J*. **14**, 2580-2588.

Pahl HL, Baeuerle PA (1996). Activation of NF-kappa B by ER stress requires both Ca^{2+} and reactive oxygen intermediates as messengers. *FEBS Lett*. **392**, 129-136.

Pahl HL, Baeuerle PA (1997). The ER-overload response: activation of NF-kappa B. *Trends Biochem Sci*. **22**, 63-67.

Pahl HL, Sester M, Burgert HG, Baeuerle PA (1996). Activation of transcription factor NF-kappaB by the adenovirus E3/19K protein requires its ER retention. *J Cell Biol*. **132**, 511-522.

Parameshwaran K, Dhanasekaran M, Suppiramaniam V (2008). Amyloid beta peptides and glutamatergic synaptic dysregulation. *Exp Neurol*. **210**, 7-13.

Parihar MS, Hemnani T (2004). Alzheimer's disease pathogenesis and therapeutic interventions. *J Clin Neurosci*. **11**, 456-467.

Park MK, Choi YM, Kang YK, Petersen OH (2008). The endoplasmic reticulum as an integrator of multiple dendritic events. *Neuroscientist*. **14**, 68-77.

Park SY, Chang I, Kim JY, Kang SW, Park SH, Singh K, Lee MS (2004). Resistance of mitochondrial DNA-depleted cells against cell death: role of mitochondrial superoxide dismutase. *J Biol Chem*. **279**, 7512-7520.

Parsons CG, Stoffler A, Danysz W (2007). Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system--too little activation is bad, too much is even worse. *Neuropharmacology*. **53**, 699-723.

Paschen W (2001). Dependence of vital cell function on endoplasmic reticulum calcium levels: implications for the mechanisms underlying neuronal cell injury in different pathological states. *Cell Calcium*. **29**, 1-11.

Pavlov PF, Wiehager B, Sakai J, Frykman S, Behbahani H, Winblad B, Ankarcrona M (2010). Mitochondrial gamma-secretase participates in the metabolism of mitochondria-associated amyloid precursor protein. *FASEB J*. **25**, 78-88.

Pereira C, Agostinho P, Moreira PI, Cardoso SM, Oliveira CR (2005). Alzheimer's disease-associated neurotoxic mechanisms and neuroprotective strategies. *Curr Drug Targets CNS Neurol Disord*. **4**, 383-403.

Perreault S, Bousquet O, Lauzon M, Paiement J, Leclerc N (2009). Increased association between rough endoplasmic reticulum membranes and mitochondria in transgenic mice that express P301L tau. *J Neuropathol Exp Neurol*. **68**, 503-514.

Petrozzi L, Lucetti C, Scarpato R, Gambaccini G, Trippi F, Bernardini S, Del Dotto P, Migliore L, Bonuccelli U (2002). Cytogenetic alterations in lymphocytes of Alzheimer's disease and Parkinson's disease patients. *Neurol Sci*. **23 Suppl 2**, S97-98.

Petrozzi L, Ricci G, Giglioli NJ, Siciliano G, Mancuso M (2007). Mitochondria and neurodegeneration. *Biosci Rep*. **27**, 87-104.

Pickrell AM, Fukui H, Moraes CT (2009). The role of cytochrome c oxidase deficiency in ROS and amyloid plaque formation. *J Bioenerg Biomembr*. **41**, 453-456.

Pino SC, O'Sullivan-Murphy B, Lidstone EA, Yang C, Lipson KL, Jurczyk A, diIorio P, Brehm MA, Mordes JP, Greiner DL, Rossini AA, Bortell R

(2009). CHOP mediates endoplasmic reticulum stress-induced apoptosis in Gimap5-deficient T cells. *PLoS One*. **4**, e5468.

Pizzo P, Pozzan T (2007). Mitochondria-endoplasmic reticulum choreography: structure and signaling dynamics. *Trends Cell Biol*. **17**, 511-517.

Porter KR, Claude A, Fullam EF (1945). A Study of Tissue Culture Cells by Electron Microscopy : Methods and Preliminary Observations. *J Exp Med*. **81**, 233-246.

Racay P, Kaplan P, Lehotsky J, Mezesova V (1995). Rabbit brain endoplasmic reticulum membranes as target for free radicals. Changes in Ca(2+)-transport and protection by stobadine. *Biochem Mol Biol Int*. **36**, 569-577.

Rainer M, Wuschitz A, Jagsch C, Erb C, Chirikdjian JJ, Mucke HA (2011). Memantine in moderate to severe Alzheimer's disease: an observational post-marketing study. *J Neural Transm*.

Rao RV, Hermel E, Castro-Obregon S, del Rio G, Ellerby LM, Ellerby HM, Bredesen DE (2001). Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. *J Biol Chem*. **276**, 33869-33874.

Recuero M, Serrano E, Bullido MJ, Valdivieso F (2004). Abeta production as consequence of cellular death of a human neuroblastoma overexpressing APP. *FEBS Lett*. **570**, 114-118.

Reddy PH (2006). Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. *J Neurochem*. **96**, 1-13.

Reijonen S, Putkonen N, Norremolle A, Lindholm D, Korhonen L (2008). Inhibition of endoplasmic reticulum stress counteracts neuronal cell death and protein aggregation caused by N-terminal mutant huntingtin proteins. *Exp Cell Res*. **314**, 950-960.

Reisberg B, Doody R, Stoffler A, Schmitt F, Ferris S, Mobius HJ (2003). Memantine in moderate-to-severe Alzheimer's disease. *N Engl J Med*. **348**, 1333-1341.

Renner M, Lacor PN, Velasco PT, Xu J, Contractor A, Klein WL, Triller A (2010). Deleterious effects of amyloid beta oligomers acting as an extracellular scaffold for mGluR5. *Neuron*. **66**, 739-754.

Resende R, Ferreiro E, Pereira C, Oliveira CR (2008a). ER stress is involved in Abeta-induced GSK-3beta activation and tau phosphorylation. *J Neurosci Res*. **86**, 2091-2099.

Resende R, Ferreiro E, Pereira C, Resende de Oliveira C (2008b). Neurotoxic effect of oligomeric and fibrillar species of amyloid-beta peptide 1-42: involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. *Neuroscience*. **155**, 725-737.

Reynolds IJ, Miller RJ (1989). Ifenprodil is a novel type of N-methyl-D-aspartate receptor antagonist: interaction with polyamines. *Mol Pharmacol*. **36**, 758-765.

Robinson DM, Keating GM (2006). Memantine: a review of its use in Alzheimer's disease. *Drugs*. **66**, 1515-1534.

Rogers TB, Inesi G, Wade R, Lederer WJ (1995). Use of thapsigargin to study Ca^{2+} homeostasis in cardiac cells. *Biosci Rep*. **15**, 341-349.

Ron D, Walter P (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol*. **8**, 519-529.

Sakono M, Zako T (2010). Amyloid oligomers: formation and toxicity of Abeta oligomers. *FEBS J*. **277**, 1348-1358.

Salter MW, Kalia LV (2004). Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci*. **5**, 317-328.

Santoro A, Balbi V, Balducci E, Pirazzini C, Rosini F, Tavano F, Achilli A, Siviero P, Minicuci N, Bellavista E, Mishto M, Salvioli S, Marchegiani F, Cardelli M, Olivieri F, Nacmias B, Chiamenti AM, Benussi L, Ghidoni R, Rose G, Gabelli C, Binetti G, Sorbi S, Crepaldi G, Passarino G, Torroni A, Franceschi C (2010). Evidence for sub-haplogroup h5 of mitochondrial DNA as a risk factor for late onset Alzheimer's disease. *PLoS One*. **5**, e12037.

Santos CX, Tanaka LY, Wosniak J, Laurindo FR (2009). Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Antioxid Redox Signal*. **11**, 2409-2427.

Sato N, Imaizumi K, Manabe T, Taniguchi M, Hitomi J, Katayama T, Yoneda T, Morihara T, Yasuda Y, Takagi T, Kudo T, Tsuda T, Itoyama Y, Makifuchi T, Fraser PE, St George-Hyslop P, Tohyama M (2001). Increased production of beta-amyloid and vulnerability to endoplasmic reticulum stress by an aberrant spliced form of presenilin 2. *J Biol Chem*. **276**, 2108-2114.

Satoh K, Matsu-Ura T, Enomoto M, Nakamura H, Michikawa T, Mikoshiba K (2011). Highly cooperative dependence of sarco/endoplasmic reticulum calcium ATPase (SERCA) 2a pump activity on cytosolic calcium in living cells. *J Biol Chem*.

Schapansky J, Olson K, Van Der Ploeg R, Glazner G (2007). NF-kappaB activated by ER calcium release inhibits Abeta-mediated expression of CHOP protein: enhancement by AD-linked mutant presenilin 1. *Exp Neurol*. **208**, 169-176.

Scheff SW, Price DA, Schmitt FA, DeKosky ST, Mufson EJ (2007). Synaptic alterations in CA1 in mild Alzheimer disease and mild cognitive impairment. *Neurology*. **68**, 1501-1508.

Scheper W, Nijholt DA, Hoozemans JJ (2011). The unfolded protein response and proteostasis in Alzheimer disease: Preferential activation of autophagy by endoplasmic reticulum stress. *Autophagy*. **7**.

Schroder M (2006). The unfolded protein response. *Mol Biotechnol*. **34**, 279-290.

Schroder M, Kaufman RJ (2005). ER stress and the unfolded protein response. *Mutat Res*. **569**, 29-63.

Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, Korsmeyer SJ (2003). BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. *Science*. **300**, 135-139.

Scorziello A, Meucci O, Florio T, Fattore M, Forloni G, Salmona M, Schettini G (1996). beta 25-35 alters calcium homeostasis and induces neurotoxicity in cerebellar granule cells. *J Neurochem*. **66**, 1995-2003.

Selkoe DJ (1993). Physiological production of the beta-amyloid protein and the mechanism of Alzheimer's disease. *Trends Neurosci*. **16**, 403-409.

Selkoe DJ (2001). Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev*. **81**, 741-766.

Selwood SP, Parvathy S, Cordell B, Ryan HS, Oshidari F, Vincent V, Yesavage J, Lazzeroni LC, Murphy GM, Jr. (2009). Gene expression profile of the PDAPP mouse model for Alzheimer's disease with and without Apolipoprotein E. *Neurobiol Aging*. **30**, 574-590.

Shankar GM, Walsh DM (2009). Alzheimer's disease: synaptic dysfunction and Abeta. *Mol Neurodegener*. **4**, 48.

Sharaf El Dein O, Gallerne C, Deniaud A, Brenner C, Lemaire C (2009). Role of the permeability transition pore complex in lethal inter-organelle crosstalk. *Front Biosci*. **14**, 3465-3482.

Sheehan JP, Swerdlow RH, Miller SW, Davis RE, Parks JK, Parker WD, Tuttle JB (1997). Calcium homeostasis and reactive oxygen species

production in cells transformed by mitochondria from individuals with sporadic Alzheimer's disease. *J Neurosci*. **17**, 4612-4622.

Shelat PB, Chalimoniuk M, Wang JH, Strosznajder JB, Lee JC, Sun AY, Simonyi A, Sun GY (2008). Amyloid beta peptide and NMDA induce ROS from NADPH oxidase and AA release from cytosolic phospholipase A2 in cortical neurons. *J Neurochem*. **106**, 45-55.

Shoffner JM, Brown MD, Torroni A, Lott MT, Cabell MF, Mirra SS, Beal MF, Yang CC, Gearing M, Salvo R, et al. (1993). Mitochondrial DNA variants observed in Alzheimer disease and Parkinson disease patients. *Genomics*. **17**, 171-184.

Sisodia SS , St George-Hyslop PH (2002). gamma-Secretase, Notch, Abeta and Alzheimer's disease: where do the presenilins fit in? *Nat Rev Neurosci*. **3**, 281-290.

Small GW, Mazziotta JC, Collins MT, Baxter LR, Phelps ME, Mandelkern MA, Kaplan A, La Rue A, Adamson CF, Chang L, et al. (1995). Apolipoprotein E type 4 allele and cerebral glucose metabolism in relatives at risk for familial Alzheimer disease. *JAMA*. **273**, 942-947.

Smith IF, Hitt B, Green KN, Oddo S, LaFerla FM (2005). Enhanced caffeine-induced Ca^{2+} release in the 3xTg-AD mouse model of Alzheimer's disease. *J Neurochem*. **94**, 1711-1718.

Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, Greengard P (2005). Regulation of NMDA receptor trafficking by amyloid-beta. *Nat Neurosci*. **8**, 1051-1058.

Song S, Lee H, Kam TI, Tai ML, Lee JY, Noh JY, Shim SM, Seo SJ, Kong YY, Nakagawa T, Chung CW, Choi DY, Oubrahim H, Jung YK (2008). E2-25K/Hip-2 regulates caspase-12 in ER stress-mediated Abeta neurotoxicity. *J Cell Biol*. **182**, 675-684.

Stutzmann GE, Caccamo A, LaFerla FM, Parker I (2004). Dysregulated IP3 signaling in cortical neurons of knock-in mice expressing an Alzheimer's-linked mutation in presenilin1 results in exaggerated Ca^{2+} signals and altered membrane excitability. *J Neurosci*. **24**, 508-513.

Stutzmann GE, LaFerla FM, Parker I (2003). Ca2+ signaling in mouse cortical neurons studied by two-photon imaging and photoreleased inositol triphosphate. *J Neurosci*. **23**, 758-765.

Stutzmann GE, Smith I, Caccamo A, Oddo S, Laferla FM, Parker I (2006). Enhanced ryanodine receptor recruitment contributes to Ca^{2+}

disruptions in young, adult, and aged Alzheimer's disease mice. *J Neurosci*. **26**, 5180-5189.

Stutzmann GE, Smith I, Caccamo A, Oddo S, Parker I, Laferla F (2007). Enhanced ryanodine-mediated calcium release in mutant PS1-expressing Alzheimer's mouse models. *Ann N Y Acad Sci*. **1097**, 265-277.

Supnet C, Grant J, Kong H, Westaway D, Mayne M (2006). Amyloid-beta-(1-42) increases ryanodine receptor-3 expression and function in neurons of TgCRND8 mice. *J Biol Chem*. **281**, 38440-38447.

Swerdlow RH, Burns JM, Khan SM (2010). The Alzheimer's disease mitochondrial cascade hypothesis. *J Alzheimers Dis*. **20 Suppl 2**, S265-279.

Swerdlow RH, Khan SM (2009). The Alzheimer's disease mitochondrial cascade hypothesis: an update. *Exp Neurol*. **218**, 308-315.

Swerdlow RH, Parks JK, Cassarino DS, Maguire DJ, Maguire RS, Bennett JP, Jr., Davis RE, Parker WD, Jr. (1997). Cybrids in Alzheimer's disease: a cellular model of the disease? *Neurology*. **49**, 918-925.

Szado T, Vanderheyden V, Parys JB, De Smedt H, Rietdorf K, Kotelevets L, Chastre E, Khan F, Landegren U, Soderberg O, Bootman MD, Roderick HL (2008). Phosphorylation of inositol 1,4,5-trisphosphate receptors by protein kinase B/Akt inhibits Ca^{2+} release and apoptosis. *Proc Natl Acad Sci U S A*. **105**, 2427-2432.

Szalai G, Krishnamurthy R, Hajnoczky G (1999). Apoptosis driven by IP(3)-linked mitochondrial calcium signals. *EMBO J*. **18**, 6349-6361.

Szegedi V, Juhasz G, Parsons CG, Budai D (2010). In vivo evidence for functional NMDA receptor blockade by memantine in rat hippocampal neurons. *J Neural Transm*. **117**, 1189-1194.

Szegezdi E, Macdonald DC, Ni Chonghaile T, Gupta S, Samali A (2009). Bcl-2 family on guard at the ER. *Am J Physiol Cell Physiol*. **296**, C941-953.

Tabas I, Ron D (2011). Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol*. **13**, 184-190.

Tajiri S, Yano S, Morioka M, Kuratsu J, Mori M, Gotoh T (2006). CHOP is involved in neuronal apoptosis induced by neurotrophic factor deprivation. *FEBS Lett*. **580**, 3462-3468.

Takahashi K, Niidome T, Akaike A, Kihara T, Sugimoto H (2009). Amyloid precursor protein promotes endoplasmic reticulum stress-induced cell death via C/EBP homologous protein-mediated pathway. *J Neurochem*. **109**, 1324-1337.

Takuma K, Fang F, Zhang W, Yan S, Fukuzaki E, Du H, Sosunov A, McKhann G, Funatsu Y, Nakamichi N, Nagai T, Mizoguchi H, Ibi D, Hori O, Ogawa S, Stern DM, Yamada K, Yan SS (2009). RAGE-mediated signaling contributes to intraneuronal transport of amyloid-beta and neuronal dysfunction. *Proc Natl Acad Sci U S A*. **106**, 20021-20026.

Takuma K, Yao J, Huang J, Xu H, Chen X, Luddy J, Trillat AC, Stern DM, Arancio O, Yan SS (2005). ABAD enhances Abeta-induced cell stress via mitochondrial dysfunction. *FASEB J*. **19**, 597-598.

Tardif KD, Waris G, Siddiqui A (2005). Hepatitis C virus, ER stress, and oxidative stress. *Trends Microbiol*. **13**, 159-163.

Thinakaran G, Koo EH (2008). Amyloid precursor protein trafficking, processing, and function. *J Biol Chem*. **283**, 29615-29619.

Thornberry NA, Lazebnik Y (1998). Caspases: enemies within. *Science*. **281**, 1312-1316.

Tilg H, Moschen AR (2010). Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology*. **52**, 1836-1846.

Tomiyama T (2010). [Involvement of beta-amyloid in the etiology of Alzheimer's disease]. *Brain Nerve*. **62**, 691-699.

Tomiyama T, Matsuyama S, Iso H, Umeda T, Takuma H, Ohnishi K, Ishibashi K, Teraoka R, Sakama N, Yamashita T, Nishitsuji K, Ito K, Shimada H, Lambert MP, Klein WL, Mori H (2010). A mouse model of amyloid beta oligomers: their contribution to synaptic alteration, abnormal tau phosphorylation, glial activation, and neuronal loss in vivo. *J Neurosci*. **30**, 4845-4856.

Toyoshima C, Nakasako M, Nomura H, Ogawa H (2000). Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. *Nature*. **405**, 647-655.

Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell*. **101**, 249-258.

Trusina A, Papa FR, Tang C (2008). Rationalizing translation attenuation in the network architecture of the unfolded protein response. *Proc Natl Acad Sci U S A*. **105**, 20280-20285.

Valla J, Schneider L, Niedzielko T, Coon KD, Caselli R, Sabbagh MN, Ahern GL, Baxter L, Alexander G, Walker DG, Reiman EM (2006). Impaired

platelet mitochondrial activity in Alzheimer's disease and mild cognitive impairment. *Mitochondrion*. **6**, 323-330.

van der Walt JM, Dementieva YA, Martin ER, Scott WK, Nicodemus KK, Kroner CC, Welsh-Bohmer KA, Saunders AM, Roses AD, Small GW, Schmechel DE, Murali Doraiswamy P, Gilbert JR, Haines JL, Vance JM, Pericak-Vance MA (2004). Analysis of European mitochondrial haplogroups with Alzheimer disease risk. *Neurosci Lett*. **365**, 28-32.

Velliquette RA, O'Connor T, Vassar R (2005). Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. *J Neurosci*. **25**, 10874-10883.

Vembar SS, Brodsky JL (2008). One step at a time: endoplasmic reticulum-associated degradation. *Nat Rev Mol Cell Biol*. **9**, 944-957.

Verkhratsky A (2002). The endoplasmic reticulum and neuronal calcium signalling. *Cell Calcium*. **32**, 393-404.

Vitiello PF, Wu YC, Staversky RJ, O'Reilly MA (2009). p21(Cip1) protects against oxidative stress by suppressing ER-dependent activation of mitochondrial death pathways. *Free Radic Biol Med*. **46**, 33-41.

Wallace DC (1994). Mitochondrial DNA mutations in diseases of energy metabolism. *J Bioenerg Biomembr*. **26**, 241-250.

Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM, Ron D (1996). Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). *Mol Cell Biol*. **16**, 4273-4280.

Warton DC, Tzagoloff A (1967). Cytochrome oxidase from beef heart mitochondria. *Methods Enzymol.***10**, 245-250.

Winton MJ, Lee EB, Sun E, Wong MM, Leight S, Zhang B, Trojanowski JQ, Lee VM (2011). Intraneuronal APP, Not Free A{beta} Peptides in 3xTg-AD Mice: Implications for Tau versus A{beta}-Mediated Alzheimer Neurodegeneration. *J Neurosci*. **31**, 7691-7699.

Wootz H, Hansson I, Korhonen L, Napankangas U, Lindholm D (2004). Caspase-12 cleavage and increased oxidative stress during motoneuron degeneration in transgenic mouse model of ALS. *Biochem Biophys Res Commun*. **322**, 281-286.

Xu C, Bailly-Maitre B, Reed JC (2005). Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest*. **115**, 2656-2664.

Yan M, Shen J, Person MD, Kuang X, Lynn WS, Atlas D, Wong PK (2008). Endoplasmic reticulum stress and unfolded protein response in

Atm-deficient thymocytes and thymic lymphoma cells are attributable to oxidative stress. *Neoplasia*. **10**, 160-167.

Yang LB, Lindholm K, Yan R, Citron M, Xia W, Yang XL, Beach T, Sue L, Wong P, Price D, Li R, Shen Y (2003). Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nat Med*. **9**, 3-4.

Yao Y, Parker I (1992). Potentiation of inositol trisphosphate-induced Ca^{2+} mobilization in Xenopus oocytes by cytosolic Ca2+. *J Physiol*. **458**, 319-338.

Yoshida H (2007). ER stress and diseases. *FEBS J*. **274**, 630-658.

Yoshida H (2009). ER stress response, peroxisome proliferation, mitochondrial unfolded protein response and Golgi stress response. *IUBMB Life*. **61**, 871-879.

Yu XM, Askalan R, Keil GJ, 2nd, Salter MW (1997). NMDA channel regulation by channel-associated protein tyrosine kinase Src. *Science*. **275**, 674-678.

Zhang C, Wu B, Beglopoulos V, Wines-Samuelson M, Zhang D, Dragatsis I, Sudhof TC, Shen J (2009). Presenilins are essential for regulating neurotransmitter release. *Nature*. **460**, 632-636.

Zhang D, Armstrong JS (2007). Bax and the mitochondrial permeability transition cooperate in the release of cytochrome c during endoplasmic reticulum-stress-induced apoptosis. *Cell Death Differ*. **14**, 703-715.

Zhang H, Li Q, Li Z, Mei Y, Guo Y (2008). The protection of Bcl-2 overexpression on rat cortical neuronal injury caused by analogous ischemia/reperfusion in vitro. *Neurosci Res*. **62**, 140-146.

Zhang J, Diamond JS (2006). Distinct perisynaptic and synaptic localization of NMDA and AMPA receptors on ganglion cells in rat retina. *J Comp Neurol*. **498**, 810-820.

Zhang K, Kaufman RJ (2006). The unfolded protein response: a stress signaling pathway critical for health and disease. *Neurology*. **66**, S102-109.

Zhao L, Ackerman SL (2006). Endoplasmic reticulum stress in health and disease. *Curr Opin Cell Biol*. **18**, 444-452.