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DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Changes in Transcription Factors Related to Mitochondrial Biogenesis and Antioxidant Defenses in Alzheimer's disease models

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutor Ana Cristina Rego (Universidade de Coimbra) e sob co-orientação do Professor Carlos B. Duarte (Universidade de Coimbra)

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INDEX

ABBREVIATIONS

α7nAChRs - Nicotinic Acetylcholine Receptors α 7 receptors

ABAD - Aβ-binding alcohol dehydrogenase

AChE - Acetylcholinesterase

AD - Alzheimer's disease

ADAM - A disintegrin and metalloproteinase

AICD - Intracellular domain of APP

AMPAR - 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid receptors

APH - Anterior-pharynx-defective-1

Apo E - Apoliprotein E

APP - Amyloid precursor protein

ARE - Antioxidant response element

Aβ - Amyloid beta peptide

AβID - Aβ interacting domain

BACE - β-site APP-cleaving enzyme

BIN - Bridging integrator

CBP - CREB binding protein

CLU - Clusterin

CR - Complement component receptor

CREB - cAMP responde-element binding protein

CSF – Cerebralspinal fluid

CTF - Carboxyl terminal fragment

DCF - 2,7-dichlorofluorescein

DRP - Dynamin-related protein

EMSA - Electrophoretic mobility shift assay

ER - Endoplasmic reticulum

ERK - Extracellular signal-regulated protein kinase

FOXO - Forkhead transcription factor

GCLc - Glutamylcysteine-light chain synthase

GCN5 - General Control Non-Repressed Protein 5

GPx - Glutathione peroxidases

HAT - Histone acetyltransferases HAT

HO - Heme oxygenase

IDE - Insulin degrading enzyme

JNK - c-Jun N-terminal kinase

Keap - Kelch-like ECH-associated protein

KO - Knockout

LRP - Lipoprotein receptor-related protein

LTP - Long-term potentiation

MCI - Mild cognitive impairment

MMSE - Mini mental state examination

MnSOD - Manganese superoxide dismutase

NEP - Neural endopeptidase

NFT- Neurofibrillary tangles

NMDAR - N-methyl-D-aspartate receptors

NQO - (NAD(P)H quinone oxidoreductase

NRF - Nuclear respiratory factor

Nrf2 - Nuclear factor erythroid derived 2-related factors

PEN - Presenilin-enhancer

PGC-1α - Peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1 α

PICALM - Phosphatidylinositol-binding clathrin assembly protein

PKC - Protein kinase C

PSD-95 - Post-synaptic density protein 95

PSEN - Presenilins

RA - Retinoic acid (RA

RAGE - Advanced glycation end-products (AGE) receptors

ROS - Reactive oxygen species

SAP 97 - Synapse associated protein 97

sAPP - Soluble ectodomain of APP

SIRT1 - Sirtuin 1

SNP - Single nucleotide substitution

SOD - Superoxide dismutase

Tbhq - Tert-butylhydroquinone

Tfam - Transcription factor A

ABSTRACT

Alzheimer's disease (AD) is the major cause of dementia in the elderly population. Abnormal accumulation of amyloid beta peptide (Aβ) in the brain is one of the hallmarks of the disease and oxidative stress and mitochondrial and synaptic dysfunction have also been observed in AD. In addition, several transcription factors that regulate the transcription of genes encoding proteins involved in antioxidant defenses and mitochondrial biogenesis are also altered in AD, pointing out to a possible role of Aβ in the regulation of the transcription of some genes. In this work, we evaluated the alterations in the nuclear levels of transcription factors related to mitochondrial biogenesis (PGC-1α and Tfam), antioxidant defense (Nrf2 and PGC-1α) and N-methyl-D-aspartate receptor (NMDAR)-linked cAMP signaling (CREB) and some of their target proteins (SOD1, GLCc and Tfam) in mature rat cortical cells (15 DIV) exposed to $A\beta_{1-42}$ oligomer-enriched population and also in the cortex of 3 and >15 month-old (mo) 3xTg-AD mice. We also evaluated the relationship between the changes in transcription factors and ROS production, as well as the role of NMDAR in $A\beta_{1-42}$-mediated effects.

Our data showed increased nuclear PGC-1α levels in 3 and 15 mo 3xTg-AD mice and in cortical cells exposed to $A\beta_{1-42}$ for 2 h. The latter decreased to values similar to the control in the presence of ifenprodil and memantine, suggesting an important role for GluN2B subunits in $A\beta_{1-42}$-mediated rise in PGC-1α levels. Nuclear Nrf2 protein levels were reduced in 15 mo 3xTg-AD male mice cortices; nevertheless, decreased SOD1 levels was observed in 3 mo 3xTg-AD and in both 15 mo WT and 3xTg-AD mice. Nrf2 protein and phosphorylation levels also decreased in cortical cells exposed to $A\beta_{1-42}$ for 24 h, which correlated with enhanced ROS production. NMDAR antagonists did not prevent the effects of $A\beta_{1-42}$ on the nuclear levels of pNrf2 and Nrf2, suggesting that Aβ-mediated effects may implicate other receptor(s) or a direct impairment by $A\beta_{1-42}$ in the phosphorylation of Nrf2 at Ser 40, thus preventing Nrf2 translocation to the nucleus.

Similarly to Nrf2, nuclear CREB protein levels were reduced in cortical cells exposed to $A\beta_{1-42}$ for 24 h and in the cortex of 15 mo 3xTg-AD mice. In contrast, nuclear pCREB levels were increased in cortical cells exposed to $A\beta_{1-42}$ for 24 h. Aβ-mediated effects in CREBwere largely prevented by memantine, a selective NMDAR antagonists used in the treatment of moderate-severe cases of AD.

In conclusion, PGC-1α levels are enhanced in early phases of Aβ exposure, whereas CREB and Nrf2 decrease in later phases. Reduced Nrf2 may contribute for increased production of ROS and consequently to the worsening of cognitive deficits.

Keywords: Nrf2, PGC-1α, CREB, oxidative stress, mitochondrial biogenesis, NMDA receptor, amyloid-beta peptide, ROS.

RESUMO

A doença de Alzheimer (AD, do inglês 'Alzheimer's disease') é a maior causa de demência na população idosa. A acumulação anormal do péptido β-amilóide (Aβ) é uma das principais características da doença e o estresse oxidativo, assim como a disfunção mitocondrial e sináptica foram anteriormente descritos em doentes e modelos da AD. Vários fatores de transcrição regulam a transcrição de genes que codificam proteínas envolvidas nos mecanismos das defesas antioxidantes e na biogénese mitocondrial e alguns desses fatores estão alterados na AD, apontando para um possível papel do Aβ na regulação da transcrição de alguns genes. Neste trabalho avaliámos as alterações nos níveis nucleares de fatores de transcrição relacionados com a biogénese mitocondrial (PGC-1α e Tfam), defesas antioxidantes (Nrf2) e sinalização de cAMP ligada aos receptores NMDA (CREB) assim como proteínas reguladas por eles (SOD1, GCLc and Tfam) em células corticais de rato expostas a $A\beta_{1-42}$. Avaliámos também a relação entre as alterações nos fatores de transcrição e os níveis de produção de ROS, assim como o envolvimento dos receptores NMDA nos efeitos mediados por $A\beta_{1-42}$.

Os nossos dados mostraram um aumento do PGC-1α nuclear em murganhos 3xTg-AD e em células corticais expostas a $A\beta_{1-42}$ por 2 horas, um efeito prevenido por ifenprodil e memantina, o que sugere um envolvimento da subunidade GluN2B nos efeitos mediados por $A\beta_{1-42}$ nos níveis de PGC-1α. Os níveis nucleares de Nrf2 diminuíram em murganhos 3xTg-AD de 15 meses; não obstante, foram observados níveis menores de SOD1 nos murganhos 3xTg-AD de 3 meses e tanto nos murganhos WT como 3xTg-AD de 15 meses. Os níveis de Nrf2 e pNrf2 também diminuíram em células corticais expostas a $A\beta_{1-42}$ por 24 h. Os antagonistas dos receptores NMDA não preveniram os efeitos do $A\beta_{1-42}$ nos níveis nucleares de Nrf2 e pNrf2, sugerindo que os efeitos mediados por $A\beta_{1-42}$ neste caso, deverão envolver outro receptor ou então uma diminuição da fosforilação da serina 40 do Nrf2 por interacção directa do $A\beta_{1-42}$ com cinases de Nrf2, impedindo a translocação do Nrf2 para o núcleo. Assim como para o Nrf2, os níveis de CREB nuclear diminuíram nas células corticais de rato, expostos a $A\beta_{1-42}$ por 24 h e no

córtex de murganhos 3xTg-AD de 15 meses. Em contraste, os níveis de pCREB nuclear aumentaram nas células corticais expostas a $A\beta_{1-42}$ por 24 horas, efeito prevenido pela memantina, um antagonista usado no tratamento de AD.

Em conclusão, os níveis de PGC-1α aumentam em fases precoces da exposição a $A\beta_{1-42}$ enquanto os níveis de CREB e Nrf2 decrescem em fases mais tardias. Por sua vez, a redução dos níveis de Nrf2 nuclear podem contribuir para o aumento da produção de ROS e consequentemente para o agravamento dos défices cognitivos.

Palavras-chave: Nrf2, PGC-1α, CREB, estresse oxidativo, biogénese mitocondrial, receptores NMDA, péptido beta-amilóide, ROS

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CHAPTER 1 - INTRODUCTION

1.1. General features of Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most prevalent cause of dementia in the world. Advanced age is the major risk for the development of AD. In the initial stages, disease symptoms include memory loss, especially of recent events, along with impairment in other cognitive functions, altered mood, judgment and language in later stages that rapidly deteriorates, leading to a complete dependence on caregivers (LaFerla and Oddo, 2005).

Hallmarks of the disease include the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFT) in both cortex and hippocampus (Figure1.1.)

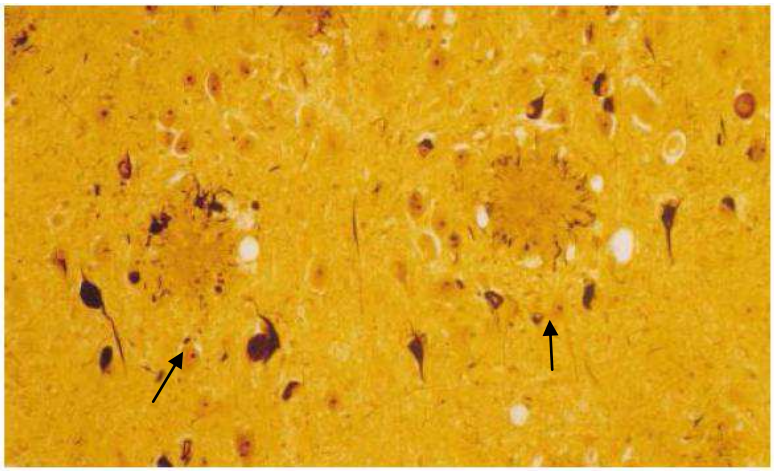


Figure 1.1 Classic neuropathological lesions present in AD patient brains. The two senile plaques (arrows) are surrounded by neurofibrillary tangles containing neurons (dark spots) from (Selkoe, 1999).

Amyloid plaques are compact and spherical extracellular deposits of amyloid beta peptide (Aβ) (Selkoe and Schenk, 2003; LaFerla et al., 2007) whereas NFT consists of hyperphosphorylated twisted filaments of the microtubule-associated protein Tau (Selkoe, 2001; Lambert and Amouyel, 2011; Zhang et al., 2011), which lead to neuronal degeneration due to deleterious effects on axonal transport mechanisms (Lambert and Amouyel, 2011),

Only about 10% of AD patients inherit the disease in a pattern of autosomal dominant transmission (Masters et al., 1981). In these cases, the disease is caused by inherited mutations

in a few genes (Bolanos et al., 2009; Zhang et al., 2011), such as the amyloid precursor protein (APP) and presenilins (PSEN) 1 and 2 genes (Tanzi and Bertram, 2001; LaFerla and Oddo, 2005; Zhang et al., 2011). The remaining 90% are sporadic forms and for a long time, only the apoliprotein E (Apo E) ε4 gene polymorphism was thought to be a risk factor for sporadic AD (Risner et al., 2006; Lambert and Amouyel, 2011) since ApoE regulates both intracellular and extracellular clearance of Aβ; indeed, the ε4 variant leads to a less efficient clearance than the other variants (ε1, ε2 and ε3) (Laws et al., 2003). In caucasians the prevalence of AD is higher than in other ethnicities; the risk increases from 20% when no ApoE ε4 is present to 90% when two copies of the ε4 allele are present. Moreover, the age of onset of clinical disease symptoms varies depending on the number of ε4 alleles, for non ε4 carriers the mean was found to be 84.5 years of age, whereas in heterozygous and homozygous carriers for ε4 the mean was 75.5 and 68.8 years of age, respectively (Corder et al., 1993). Recently, other genes have been suggested as risk factors for AD development. Studies in a high number of AD patients and control individuals, performed by genome-wide association studies, demonstrated that Clusterin (CLU), complement component receptor 1 (CR1) (Lambert et al., 2009) phosphatidylinositol-binding clathrin assembly protein (PICALM) (Harold et al., 2009) and bridging integrator 1 (BIN 1) (Seshadri et al., 2010) were related to the development of the disease. As one of the most abundant apolipoproteins in the central nervous system, CLU has been suggested as a participant in the Aβ clearance while the complement pathway, involving CR1, may favor amyloid fibrils and the clearance of apoptotic cells. PICALM is mainly expressed in the endothelium of blood vessel walls and may be involved in the transport of Aβ across the blood brain barrier and into the bloodstream (Lambert and Amouyel, 2011).

1.1.1. Stages of AD in human patients

AD symptoms begin as occasional minor lapses for recalling recent events of daily life and failing in remember a conversation or activity or even be confused about information recently received, but the confirmation of the disease requires postmortem observation of Aβ plaques and tau tangles. The AD diagnosis is made by neuropsychological tests, PET scans and

biomarkers such as Aβ, tau and P-tau (phosphorylated tau) levels in the cerebralspinal fluid (CSF) (Blennow, 2004; Hampel et al., 2004) Regarding the results obtained in the neuropsychological tests, neuroimaging tests and biomarker levels, AD patients are subdivided in three different stages, mild, moderate and severe although sometimes is difficult to differentiate these stages.

Mild cognitive impairment (MCI) begins with amnestic symptoms, but do not have other cognitive impairment (Selkoe and Schenk, 2003). MCI is often referred as an early stage of AD but there is evidence that not all MCI patients develop AD (Visser et al., 2005) and it is estimated that 10 to 15% of patients with MCI develop AD within 1 year (Hampel et al., 2004).

Patients with MCI or mild AD have fully preserved alertness, no language disturbance and normal motor and sensory function. After the first couple of years additional minor problems arise. As deficits increase, patients might disinterest in hobbies, show apathy and language and mathematical problems. After few years of progressive memory decline, patients begin to experience deficits in motor function, like balance and walking. Over several years to a decade AD patients gradually deteriorate into a marked dementia, full disorientation, memory impairment and global cognitive deficits. Many patients become confined to a bed and die because of respiratory difficulties such as aspiration or pneumonia (Selkoe and Schenk, 2003)

As the severity of the disease increase, Aβ levels in the CSF decrease, as a result of aggregation of the peptide in the brain, while tau and P-tau levels increase in the CSF. The mini mental state examination (MMSE) is a test that evaluates the mental state and grades from 0 to 30. The baseline MMSE for mild to moderate AD patients is about 16 to 26 and severe AD patients usually have a MMSE value below 16 (Ito et al., 2011).

1.2. APP processing and Aβ production

Aβ results from the processing of APP, a transmembrane protein that acquires N- and O-linked sugars immediately after its biosynthesis and has a half-life of about 45–60 minutes in most cell types (Weidemann et al., 1989). Full-length APP is synthesized in endoplasmic reticulum (ER) and then transported to the Golgi apparatus (Sisodia et al., 1993) and to membrane cell surface

where it is first cleaved by α-secretase (Parvathy et al., 1999) that prevents the formation of Aβ peptide since the cleavage site is within Aβ domain (Qin et al., 2006) (Figure 1.2).

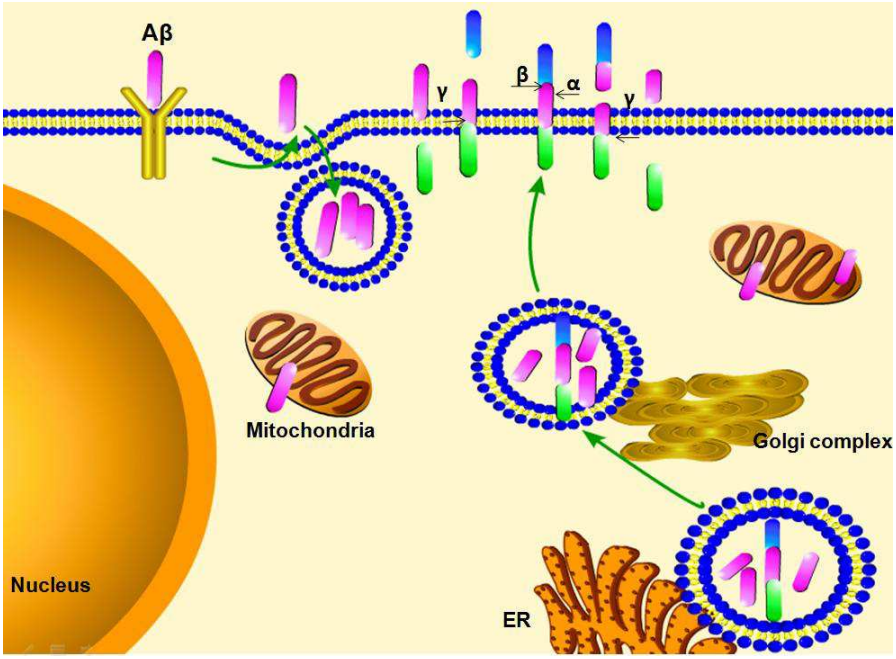


Figure. 1.2. Schematic representation of APP cleavage. Non-amyloidogenic cleavage of APP requires α and γ secretase and originates sAPPα, α-CTF, p3 and AICD. On the other hand, the amyloidogenic pathway involves cleavage of APP by β and γ secretase, originating sAPPβ, βCTF, Aβ, AICD, Jcasp and C31. APP- amyloid precursor protein, AICD- β amyloid precursor intracellular domain, CTF-C-terminal fragment, Aβ – amyloid β, α – α cleavage, β- β cleavage, γ- cleavage.

Thus, in the non amyloidogenic pathway, APP is first cleaved by α-secretase leading to the production of α carboxyl terminal fragment (α-CTF) and a soluble ectodomain of APP (sAPPα). The α-CTF fragment is then cleaved by γ-secretase, generating P3 and the intracellular domain of APP (AICD) (Zhang et al., 2011) (Figure 1.2). Three members of the a disintegrin and metalloproteinase (ADAM) family (9,10 and 17) have been suggested as α-secretase; (Haass and Selkoe, 2007; LaFerla et al., 2007; Zhang et al., 2011) and there is evidence that ADAM10 is reduced in platelets and CSF of AD patients (Colciaghi et al., 2002).

In the amyloidogenic pathway, the first step in Aβ generation consists on APP cleavage by β-site APP-cleaving enzyme (BACE) (Vassar et al., 1999), a membrane-bound aspartyl protease with a characteristic type I transmembrane domain near the C-terminus. In fact, the major β-

secretase BACE1 deficient AD mice model showed the rescue of cholinergic dysfunction, neuronal loss and memory deficits, and marked reduction in $A\beta_{40}/A\beta_{1-42}$ levels. However, BACE1 knockout (KO) mice have phenotypic abnormalities. In fact, a significant number of BACE1 null mice die in the first weeks post-birth, suggesting an important function for BACE1 in development (Dominguez et al., 2005).

After β cleavage, the β-CTF fragment remains associated to the membrane, being then cleaved by γ-secretase, generating Aβ and the AICD fragment (Selkoe, 2001; LaFerla et al., 2007) (Figure 1.2). The exact site in which the γ-secretase cleaves the β-CTF can vary, yielding Aβ of different lengths. $A\beta_{1-40}$ and $A\beta_{1-42}$ are the main toxic species and despite the fact that $A\beta_{1-42}$ peptides make just about 10% of the total Aβ produced (Zhang et al., 2011) they are the pathologically most relevant forms as they form the core of amyloid plaques and are more prone to aggregation (Munter et al., 2007). The γ-secretase activity resides in a complex of four components, PSEN1 or PSEN2, nicastrin, anterior-pharynx-defective-1 (APH-1) and presenilin-enhancer 2 (PEN-2) (Haass and Selkoe, 2007). Presenilins are multi-transmembrane proteins that form heterodimers and nicastrin is a type-I transmembrane glycoprotein that acts as a cofactor of presenilins being the scaffold protein within the γ-secretase complex, recruiting Notch and APP to the γ-secretase complex. APH-1 interacts with nicastrin to form a stable intermediate and PEN-2 regulates presenilin endoproteolysis (Kimberly and Wolfe, 2003).

Both amyloid fibrils and soluble oligomeric species of Aβ exhibited neurotoxicity, contributing for neurodegeneration in AD. Aβ fibrils were shown to be toxic in NIH-3T3, SH-SY5Y, HTB186 and M059K cells, whereas Aβ oligomers shown to be deleterious in NT-2 cells and specific regions of organotypic slices from hippocampus and cerebellum. Although the Aβ fibrils are neurotoxic, they were found to be spread throughout the brain. In this way, they would cause vast neuronal death if they were the form causative of AD; thus, soluble oligomeric Aβ provides a possible explanation for the selective initial regional neurodegeneration that characterizes AD (Kim et al., 2003). Accordingly, compounds that block oligomerization of Aβ completely blocked the neurotoxicity of Aβ in rat hippocampal neurons in culture (De Felice et al., 2004). Aβ oligomers were shown to be more toxic than fibrils in cortical neuronal cultures

since oligomeric $A\beta_{1-42}$ depleted ER Ca^{2+} levels leading to intracellular dyshomeostasis (Resende et al., 2008). Moreover, oligomeric Aβ inhibited bidirectional axonal transport as a consequence of casein kinase 2 activation, which leads to phosphorylation of kinesin-1 light chain and subsequent release from its cargoes, in isolated axoplasms (Pigino et al., 2009). Aβ senile plaques are present not only in AD but also in elderly non-demented individuals; however, senile plaques in non-demented individuals are similar in composition to those in AD patients, suggesting that other factors may play a role, along with Aβ peptide in the development of AD (Fukumoto et al., 1996). Despite the well described toxic properties of Aβ, non pathological functions have been also described, namely those related with cholesterol metabolism. $A\beta_{1-40}$ was previously shown to reduce cholesterol levels through the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a key enzyme in cholesterol synthesis (Grimm et al., 2005) and $A\beta_{1-1-42}$ promotes cholesterol trafficking to Golgi complex of astrocytes in a process dependent on caveolin-1 (Igbavboa et al., 2009). Furthermore, oligomeric Aβ40 was shown to prevent the death of neurons cultured in medium containing Fe (II) in the absence of antioxidants (Zou et al., 2002).

1.3. Possible intracellular role of Aβ

There are currently several evidences that Aβ accumulates intracellularly; (Grundke-Iqbal et al., 1989; D'Andrea et al., 2002; Takahashi et al., 2002) and that the accumulation occur early in the neuropathological phenotype of AD (Gouras et al., 2000).

Several authors have observed intracellular Aβ in several regions of the brain (D'Andrea et al., 2002; Oddo et al., 2003), frequently in neurofibrillary tangle-containing neurons (Grundke-Iqbal et al., 1989), but the accumulation of $A\beta_{1-42}$ in AD-vulnerable regions seems to occur before NFT and amyloid plaque deposition (Gouras et al., 2010). Within the cells, aggregated $A\beta_{1-42}$ appear as dense packed granules, often denominated the perikaryal cytoplasm (Nagele et al., 2002). As described before, Aβ peptide is formed within the ER, Golgi apparatus and also in the endosomal/lysosomal system (Pagani and Eckert, 2011). However, intracellular Aβ may occur due to internalization of Aβ into intracellular pools after the interaction of pre secreted Aβ

with membrane transporters and receptors, nicotinic acetylcholine receptors α 7 receprotes (α7nAChRs), N-methyl-D-aspartate receptors (NMDARs) and advanced glycation end-products (AGE) receptors (RAGE) (Sasaki et al., 2001; Nagele et al., 2002; Snyder et al., 2005).

Nagele et al., (2002) showed that the α7nAChRs, highly permeable to Ca^{2+}, co-localize with $A\beta_{1-42}$ within neurons of AD brains and the rate and extent of $A\beta_{1-42}$ internalization is directly related to the α7nAChRs protein levels. Furthermore, internalization is effectively blocked by α-bungarotoxin, an α7nAChR receptor antagonist, and by phenylarsine oxide, an inhibitor of endocytosis, suggesting that intraneuronal accumulation of $A\beta_{1-42}$ occurs predominantly in neurons expressing α7nAChRs and is mediated by endocytosis (Nagele et al., 2002). During normal aging, human brains accumulate AGEs within neurons and senile plaques, thus RAGE has been also implicated in the pathogenesis of AD (Li et al., 1998). In fact, Aβ and RAGE were shown to co-localize in astrocytes of AD brains and there is also evidence that glycated Aβ is taken up via RAGE being degraded through the lysosomal pathway in astrocytes (Sasaki et al., 2001). Furthermore, binding of Aβ to RAGE in neurons sets off a cascade of events that result in oxidative stress and NF-kB activation (Du Yan et al., 1997).

Snyder et al. (2005) also reported that Aβ promotes NMDAR endocytosis in cortical neurons, leading to the reduction in the amount of surface NMDARs, which was prevented following treatment with γ-secretase inhibitors. Interestingly, Aβ-dependent NMDAR endocytosis was shown to be dependent on the α7nAChRs (Snyder et al., 2005). These results suggest that Aβ may impair NMDAR-mediated signaling, contributing to the synaptic dysfunction observed in AD. Furthermore, $A\beta_{1-42}$ uptake is completely blocked by NMDAR antagonists, suggesting an involvement of this receptor in the re-uptake of the peptide (Bi et al., 2002). Accordingly, we recently showed NMDAR-dependent Ca^{2+} rise evoked by $A\beta_{1-42}$ in cortical cells (Ferreira et al., 2012). Also, ApoE KO PDAPP transgenic mice showed a dramatic decrease in intraneuronal Aβ, suggesting that ApoE might modulate the internalization of Aβ, possibly by the interaction with low density lipoprotein receptor-related protein (LRP) (Zerbinatti et al., 2006). Finally, in vitro studies performed by Yu et al. (2010) showed that endocytosis of oligomeric Aβ is linked to neurotoxicity via a dynamin-dependent and RhoA-mediated endocytic pathway in Neuro-2A

cells (Yu et al., 2010). In contrast with these findings, Small et al. (2007) concluded that Aβ binds to membrane lipid rather than to a protein component, indicating that Aβ may exert its effect by altering membrane lipid composition or fluidity which could influence the receptors distribution or lipid raft components (Small et al., 2007). It was also demonstrated that in organotypic hippocampal slice cultures, $A\beta_{1-42}$ is internalized by CA1 hippocampal neurons but is not retained by other hippocampal subdivisions such as CA3 and dentate gyrus, leading to enhanced production of amyloidogenic APP fragments and deterioration of central synapses in a selective way (Bahr et al., 1998). Intracellular role of Aβ has been described in several AD models. In the triple transgenic mice (3xTg-AD), intraneuronal accumulation of Aβ, first detectable in neocortex regions, appears to cause the onset of early AD-related cognitive deficits; in this context, the clearance of Aβ by immunotherapy was shown to rescue early cognitive deficits (Billings et al., 2005).

3xTg-AD mice present extracellular Aβ deposit by 6 months of age, but exhibit synaptic dysfunction and deficits in long-term potentiation (LTP), a form synaptic plasticity thought to underlie memory and learning, before the extracellular deposition of Aβ. However, these deficits, occurring between 3 and 4 months of age, are associated with intracellular Aβ as evaluated by immunoreactivity using end-specific antibodies that selectively recognize $A\beta_{1-42}$. Furthermore, the 2xTg mice, which do not overexpress APP or present intracellular accumulation of Aβ, do not show pronounced LTP deficits, again suggesting that synaptic dysfunction is related to intracellular accumulation of Aβ (Oddo et al., 2003).

In both AD human brain cortical tissue and Tg2576 transgenic mice, intraneuronal $A\beta_{1-42}$ increases with age and accumulates in multivesicular bodies in both pre- and postsynaptic compartments which may lead to abnormal synaptic morphology (Takahashi et al., 2002); moreover, Aβ led to inhibition of ubiquitin-dependent protein degradation in rabbit reticulocytes (Gregori et al., 1995). In addition, in the double APP and PS1 mutant transgenic rat, intracellular Aβ expression led to increased number of Golgi apparatus elements, lysosomes and lipofuscin bodies in the hippocampal area (Li et al., 2007), again suggesting the inhibition of protein degradation pathways such as autophagy.

Furthermore, there is evidence that $A\beta_{1-42}$ is selectively toxic to human neurons through activation of the p53 and Bax proteins-associated pro-apoptotic pathways (Zhang et al., 2002). Intriguingly, levels of intraneuronal non-oligomeric $A\beta_{1-42}$ and intraneuronal 8-hydroxyguanosine, an oxidized nucleoside, were inversely correlated in postmortem brain tissue of the hippocampus of AD patients, which suggests that intraneuronal accumulation of non-oligomeric Aβ might be a compensatory response towards oxidative stress in AD neurons (Nunomura et al., 2010).

1.4. Aβ and mitochondria – effects on mitochondrial function and oxidative stress

There is evidence that Aβ accumulates in intracellular organelles, namely in the mitochondria, before extracellular Aβ deposition. Mitochondria are dynamic organelles responsible for the maintenance of the bioenergetic state of the cell; therefore any alteration in the correct function, biogenesis, morphology and/or dynamic could be harmful for the cell. In fact, big efforts have been made to understand in which extent mitochondrial damage is related to the disease development.

Mitochondria generate energy through the activity of the tricarboxylic acid cycle and the oxidative phosphorylation via the electron transport chain, consisting of four different complexes, complex I or NADH-ubiquinone oxidoreductase, complex II or succinate-ubiquinone oxidoreductase, complex III or ubiquinol-cytochrome c oxidoreductase and complex IV or cytochrome c oxidase. The electrochemical gradient is achieved through the membrane ATP synthase (Complex V) (Bolanos et al., 2009). Mitochondria are dynamic organelles that are continuously subjected to fission, required for mitochondrial renewal, redistribution, and transport into synapses maintaining a pool of healthy mitochondria, and fusion, which facilitates communication with each other and their distribution across long distances and to synapses, suggesting a protective mechanism in helping the maintenance of sufficient bioenergetic levels adjusted to situations with high-energy demands (Pagani and Eckert, 2011). Unbalanced fission

leads to mitochondrial elongation, and unbalanced fusion leads to excessive mitochondrial fragmentation, both of which impair the mitochondrial function (Bolanos et al., 2009).

Human mtDNA is a small circular genome encoding only 13 proteins, including essential subunits of the mitochondria respiratory chain, but the majority of mitochondrial proteins are encoded by nuclear DNA (Scarpulla, 2011) (Figure 1.3). Tfam (mitochondrial transcription factor) is an activator factor essential for the initiation of the transcription of mitochondrial genome (Shoubridge, 2002).

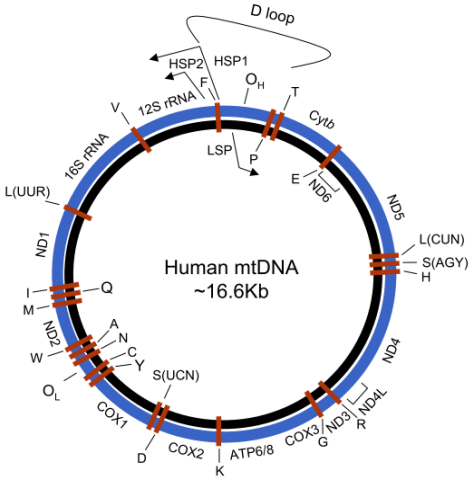


Figure. 1.3 Schematic representation of the human mitochondrial genome. Heavy (blue) and light (black) strands. Protein coding and rRNA genes are interspersed with 22 tRNA genes (red bars denoted by the single-letter amino acid code). Duplicate tRNA genes for leucine (L) and serine (S) are distinguished by their codon recognition (parentheses). The D-loop regulatory region contains the L- and H-strand promoters (LSP, HSP1, and HSP2), with arrows showing the direction of transcription. Protein coding genes include the following: cytochrome oxidase (COX) subunits 1, 2, and 3; NADH dehydrogenase (ND) subunits 1, 2, 3, 4, 4L, 5, and 6; ATP synthase (ATPS) subunits 6 and 8; cytochrome b (Cyt b) (Scarpulla, 2008).

AD mice models showed differences between synaptic and non-synaptic mitochondria. Synaptic mitochondria accumulate more Aβ and show early deficits in mitochondrial function, as shown by increased mitochondrial permeability, decline in both respiratory function and in the activity of cytochrome c oxidase, increased mitochondrial oxidative stress and altered mitochondrial distribution and trafficking in axons (Du et al., 2010). Moreover, synaptosomes-derived synaptic mitochondria have high levels of cyclophilin D, involved in the mitochondrial

permeability transition pore formation, which makes them more susceptible to calcium changes (Naga et al., 2007).

Using immunobloting, digitonin fractionation and electron microscopy techniques, Manczak et al. (2006) found a relationship between APP derivatives and mitochondria in brain slices from T2576 mice. Levels of soluble Aβ were correlated with increased levels of hydrogen peroxide (H_2O_2) production and decreased cytochrome c oxidase activity in Tg2576 mice, before the appearance of Aβ plaques (Manczak et al., 2006); also, cultured cortical neurons from transgenic mice expressing human mutant APP (Tg mAPP) demonstrated mitochondrial deposition of Aβ (Caspersen et al., 2005). Moreover, overexpression of Aβ in Drosophila results in Aβ accumulation in soma and axons of neurons leading to depletion of presynaptic mitochondria, decreased mitochondrial axonal transport and changes in mitochondrial number and morphology (Zhao et al., 2010). Aβ may be taken up by mitochondria through TOM import machinery although it has been also suggested that Aβ can be produced locally at the mitochondria. Mitochondrial γ-secretase processes different substrates including APP; also, AICD can be produced inside mitochondria further indicating that Aβ can also be produced inside mitochondria (Pavlov et al., 2011).

Several enzymes were shown to degrade Aβ namely neural endopeptidase (NEP) that degrade extracellular Aβ, insulin degrading enzyme (IDE) (Sudoh et al., 2002) and presequence protease (Prep) that degrade intracellular Aβ. PreP can be inactivated under oxidizing conditions which occurs when there are high amounts of Aβ in mitochondria, thus preventing Aβ clearance (Pagani and Eckert, 2011). Furthermore, Aβ binds to the mitochondrial Aβ-binding alcohol dehydrogenase (ABAD), a member of the short chain dehydrogenase reductase family, present in mitochondria matrix (Takuma et al., 2005). It was demonstrated that ABAD is upregulated in AD patient brains(Wen et al., 2002) and in double transgenic mice (He et al., 2002) and that ABAD inhibitors prevent Aβ-induced apoptosis and reactive oxygen species (ROS) production in neurons (Lustbader et al., 2004).

Thus, $A\beta_{1-42}$ can interfere with several components of mitochondria including proteins of the import machinery, fusion and fission proteins, leading to mitochondrial dysfunction (Sirk et al.,

2007); this favors mitochondrial fission, which leads to mitochondrial abnormal distribution followed by mitochondrial depletion from axons and dendrites, and subsequent synaptic loss (Wang et al., 2009). Accordingly, exposure to Aβ increased the protein levels of fission 1, decreased the levels of optic atrophy protein 1 and caused oxidative damage to dynamin-related protein 1 (Drp1). In addition, Drp1, a regulator of mitochondrial fission, protein levels and distribution were reduced in sporadic AD fibroblasts, which is related to abnormal mitochondrial distribution (Wang et al., 2008).

Intracellular Aβ also interferes with the oxidative phosphorylation and ROS production within mitochondria, leading to decreased mitochondrial membrane potential, complex IV (cytochrome c oxidase) activity and ATP production (Hauptmann et al., 2009).

In previous studies, other functional and morphological changes associated with increased Aβ production have been observed in AD models. Manczak et al. (2006) observed that APP transgenic mice presented increased expression of mitochondrial genes which might be a compensatory response to mitochondrial oxidative damage caused by the elevated levels of APP and Aβ (Manczak et al., 2006). Also, brain tissue from AD patients exhibited reduced complex IV activity (Mutisya et al., 1994), and deficient microtubule metabolism resulting in the accumulation of mitochondrial debris in the neuron perikaryon (Castellani et al., 2002). An immediate consequence of mitochondrial dysfunction is the rising of ROS production; furthermore, there is evidence that the antioxidant defenses and repair systems are not fully functioning in AD, also contributing to oxidative stress. In fact, antioxidants such as carotene, lycopene, vitamin A, C and E are reduced in AD brains (Baldeiras et al., 2010). Also, in Tg19959 transgenic mice there is a partial deficiency in the mitochondrial antioxidant manganese superoxide dismutase (MnSOD) which exacerbates amyloid pathology. Accordingly, overexpression of MnSOD improved resistance to Aβ, slowed plaque formation and attenuated the phenotype in a transgenic AD mouse model (Dumont et al., 2009).

1.5. Is Aβ transcriptionally active?

Aβ oligomers were recently shown to cause changes in gene expression in human adult cortical slices. Twenty seven genes implicated in vesicle trafficking, cell adhesion, actin cytoskeleton dynamics and insulin signaling, among other pathways, were found to be differentially expressed; in fact, most genes (70%) were shown to be downregulated by Aβ oligomers (Sebollela et al., 2012). Interestingly, Maloney & Lahiri (2011) demonstrated that Aβ could potentially act as a transcription factor upon AD-associated genes. Using electrophoretic mobility shift assay (EMSA) those authors demonstrated that Aβ binds to an Aβ interacting domain (AβID) with a consensus sequence (KGGRKTGGGG) (Maloney and Lahiri, 2011). Interestingly, a single nucleotide substitution (SNP) (G→A) in the seventh nucleotide of the consensus sequence eliminates the Aβ-DNA interaction capacity and corresponds to a SNP associated with increased AD risk (Lahiri et al., 2005; Bailey et al., 2011).

Amongst several fragments of Aβ, the cytotoxic $A\beta_{25-35}$ had greatest DNA affinity (Maloney and Lahiri, 2011). Accordingly, Aβ peptides with alanine or isoleucine substitutions of glycine 33 in GxxxG motifs shown increased propensity to form bigger oligomers (Harmeier et al., 2009), suggesting that Aβ might have a novel function in the pathogenesis of AD. As a transcription factor, Aβ may directly influence the expression of disease-modifying genes.

Using a bioinformatics approach, Augustin et al., (2011) found that distinct AD-related genes share modules (combination of transcription factors in a defined order, distance range and orientation), suggesting a transcriptional co-regulation (Augustin et al., 2011)

Different expression levels of APOE gene splice variants, which are under the control of different promoters in normal and AD brain tissue, were observed in AD temporal lobe, indicating that alternative splicing and promoter usage of the APOE gene in AD brain tissue could reflect the progression of the neurodegeneration (Twine et al., 2011). Hydrogen peroxide promoted the uptake of Aβ into nuclei associated along with increased activation of the p53 promoter, resulting in p53-dependent apoptosis in neuroblastoma cells and in guinea pig mixed primary cell brain cultures treated with Aβ (Bailey et al., 2011). Accordingly, p53 expression was shown to be elevated in frontal cortex of human sporadic AD brains (Ohyagi et al., 2005).

Oxidative stress and Aβ treatment also lead to increased activity of BACE1 gene promoter, possibly meaning that under cytotoxic condition, Aβ activity may enhance Aβ levels that would then cross a pathogenic threshold leading to amyloidogenesis in AD (Bailey et al., 2011).

1.6. Synaptic Dysfunction

AD is characterized by synaptic degeneration with loss of a large number of neurons in several brain regions, namely in the hippocampus and cortex (Correia et al., 2011; Lambert and Amouyel, 2011). In fact, there is evidence that the major presynaptic vesicle protein, synaptophysin and the postsynaptic synaptopodin (a proline rich protein intimately associated with actin microfilaments) and post-synaptic density protein 95 (PSD-95) levels are decreased in AD patients (Reddy et al., 2010).

In AD, the cholinergic neurons are more affected than other types of neurons (Ferrari and Greene, 1998; Selkoe, 2001); thus, AChE (acetylcholinesterase) inhibitors have been used as agents for the therapy of AD (Akasofu et al., 2008). However, AChE inhibitors are not efficient in all patients, lose efficacy over time and usually have unpleasant side effects (Risner et al., 2006).

Altered glutamatergic circuits are also implicated in the early phases of AD (Marcello et al., 2007). Glutamate-mediated neurotransmission involves two types of receptors, metabotropic and ionotropic receptors. Metabotropic glutamate receptors are coupled to G-proteins that when activated generate intracellular secondary messengers. On the other hand, ionotropic glutamate receptors, are associated with ion channels that open when activated by their ligands, including kainite, 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid receptors (AMPAR) and NMDAR. AMPA receptors are constituted by four different subunits (GluA1-GluA4) which assemble in different combinations, leading to the formation of receptors with different properties (Santos et al., 2009). AMPA receptors containing GluA1, GluA2 and GluA2/3 subunits were shown to be reduced in vulnerable regions of the AD brain (Aronica et al., 1998; Carter et al., 2004). Synaptic NMDA receptors (NMDARs) are multisubunit complexes

associating two GluN1 and two GluN2 subunits that exist as seven and four subtypes respectively (GluN1a-g and GluN2A-D). NMDAR more rarely associate GluN3 subunits which exist as two subtypes (GluN3A-B) (Mony et al., 2009). NMDARs activation seems to have an inhibitory effect on amyloidogenic processing in AD (Bell and Hardingham, 2011). In fact, in hippocampal synapses, NMDARs activation lead to the recruitment of α-secretase (ADAM10) which requires synapse associated protein 97 (SAP97) (Marcello et al., 2007) increase APP transport to synapses and processing to non-amyloidogenic products, reducing intraneuronal Aβ and the action of the Aβ-degrading protease, neprilysin (Tampellini et al., 2009). In addition, synaptic activity increases local mitochondrial function, enhances antioxidant defenses and trophic support and suppresses apoptotic pathways (Bell and Hardingham, 2011).

Apparently, GluN2B subunit-containing NMDARs make the major contribution to neurodegeneration, while those containing GluN2A subunits seem to have a protective role in AD. Stimulation of synaptic NMDA receptors promotes transcriptional activation through phosphorylation of cAMP responde-element binding protein (CREB) at Ser133 by PKA (Snyder et al., 2005). Thus, a decrease in GluN2A-containing NMDARs reduce the transcription of pro-survival genes by down-regulating CREB signaling (Liu et al., 2004; Chen et al., 2008). Indeed, the loss of NMDARs may be triggered by Aβ which co-localizes with PSD-95(Dewachter et al., 2009). In fact, Aβ decreases surface and synaptic expression of NMDARs either by preventing surface delivery or by endocytosis leading to decreased synaptic strength and disruption of synaptic plasticity mechanisms (Snyder et al., 2005; Proctor et al., 2011).

The activation of NMDAR allows calcium influx and facilitates internalization of membrane proteins and there is evidence that NMDAR antagonists prevent the uptake and effects of $A\beta_{1-42}$ (Bi et al., 2002).

On the other hand, overstimulation of the NMDARs by glutamate could also potentiate neurodegeneration since it ultimately leads to calcium overload that may disturb organelle functioning and damage neurons (Ferreira et al., 2012). In addition, while synaptic NMDAR activation has been shown to be neuroprotective, since it leads to the activation of

neuroprotective pathways, namely PI3K, Akt and CREB pathways (Papadia et al., 2005) and also stimulates antioxidant defense protein such as peroxiredoxin and thioredoxin (Papadia et al., 2008), the activation of extrasynaptic NMDAR leads to cell death. This location-dependence pattern results from opposing actions on intracellular signaling pathways (Hardingham and Bading, 2010). Therefore, memantine, an uncompetitive NMDA receptor antagonist has been used in moderate to severe cases of AD and showed good results in the reduction of clinical deterioration (Reisberg et al., 2003).

1.7. Transcription factors related with mitochondrial biogenesis and antioxidant defenses

Recent evidence point out for modified levels of several transcription factors in human postmortem brain samples and cellular and animal models of AD. In this section, we describe some of these changes focusing on transcription factors that regulate mitochondrial biogenesis, such as mitochondrial transcription factor A (Tfam) and Peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1 α- (PGC-1α), and antioxidant defense, namely nuclear factor erythroid derived 2-related factors (Nrf) 1/2 and their link to changes in CREB, and CREB binding protein (CBP).

1.7.1. Nuclear factor erythroid derived 2-related factors (Nrfs)

The CNC (name derived from the cap-on-colar protein in Drosophila) family includes four closely related transcription factors, p45-NFE2, Nrf1, Nrf2 and Nrf3. These members have a CNC domain next to a bZip (basic region leucine zipper) domain (Ohtsuji et al., 2008).

Nrf1 is widely expressed in lung, kidney, liver heart and muscle (Kwong et al., 1999), and has an N-terminal domain that is responsible for anchorage to the ER which is necessary to generate the 120 kDa glycosylated protein (Zhang et al., 2007). Following ER stress, Nrf1 is translocated to the nucleus, where it can act as a transcriptional activator (Wang and Chan, 2006). Nrf1 can bind to antioxidant response element (ARE) in the promoter of antioxidant protein coding genes. In fact, Nrf1 knockout fibroblasts have decreased levels of glutathione and are sensitive to the toxic effects of oxidants. Glutamylcysteine-light chain synthase (GCLc) and glutathione

synthase are downregulated in Nrf1-deficient fibroblasts (Kwong et al., 1999). Additionally, the expression of metallothionein 1 and 2, which protect cells from metal-induced damage, is decreased in Nrf1 knockout mice. Although both Nrf1 and Nrf2 bind to metallothionein 1 ARE with similar affinity, the gene expression was preferentially activated by Nrf1 (Ohtsuji et al., 2008).

Nrf2 is a 66- to 68 kDa protein (Jaiswal, 2004) that plays a central role in regulation of the cellular redox state through the transcription of cytoprotective proteins (Nguyen et al., 2004). Nuclear Nrf2 is significantly reduced in AD, even in the presence of oxidative stress (Ramsey et al., 2007). Furthermore, common variants of NFE2L2 gene may affect disease progression, potentially altering clinically recognized disease onset (Otter et al., 2010).

Nrf2 contains two transcription activation domains, Neh4 and Neh5, which individually and cooperatively bind to CBP; in accordance, specific inhibitors of CBP significantly reduced Nrf2 activity (Katoh et al., 2001).

Under normal conditions, Nrf2 transcription is repressed by negative regulator Kelch-like ECH-associated protein 1 (Keap1) but when exposed to ROS, Nrf2 dissociates from cytosolic Keap1 and translocates to the nucleus where it binds to ARE in the promoter of genes of antioxidant enzymes (Itoh et al., 1999). Nrf2 regulates GCLc, the catalytic subunit of glutamate cysteine ligase, the rate limiting enzyme for the synthesis of glutathione (Sekhar et al., 2003), superoxide dismutase 1 (SOD1) (Park and Rho, 2002), glutathione peroxidases (GPx) (Banning et al., 2005) and HO-1 (heme oxygenase 1) (Alam et al., 1999).

In addition, there is evidence that histone deacetylase 2 (HDAC2) may control Nrf2 activity via deacetylation and that a decrease in HDAC may cause impaired function of Nrf2, leading to the downregulation of antioxidant responsive genes such as heme oxygenase-1 (HO-1) (Mercado et al., 2011). On the other hand, the blockage of Nrf2 degradation, increases the production of (NAD(P)H quinone oxidoreductase 1 (NQO1) in the presence of oxidative stressors (Ma et al., 2004). Furthermore, retinoic acid (RA) and 12-O-tetradecanoylphorbol 13-acetate, two inducers of neuronal differentiation, are able to induce Nrf2 and NQO1 in a dose and time-dependent manner. In fact, RA-induced Nrf2 up-regulation is associated with neurite growth and induced

the two neuronal differentiation markers, neurofilament-M and microtubule-associated protein 2 (MAP2), suggesting a therapeutic potential for Nrf2 activators in patients with neurodegenerative diseases (Zhao et al., 2009).

Activation of protein kinases, such as protein kinase C (PKC) (Huang et al., 2000), c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK) induces Nrf2 phosphorylation, which may stimulate the dissociation of Nrf2 from its repressor Keap1 and subsequent translocation into nucleus (Xu et al., 2006). Phosphorylation of Nrf2 is also considered to facilitate its interaction with the transcriptional coactivator CBP/p300 and recruitment of components of transcription initiation machinery (Surh et al., 2008). In contrast, the phosphorylation of Nrf2 by GSK-3β leads to its inactivation since it is translocated from the nucleus to the cytosol (Salazar et al., 2006). There is evidence that GSK-3β phosphorylates the tyrosine kinase Fyn, leading to nuclear localization of Fyn, which phosphorylates tyrosine 568 of Nrf2 in the nucleus leading to Nrf2 export to the cytosol (Jain and Jaiswal, 2006). Interestingly, it was demonstrated that in normal brain, Nrf2 localizes both in the cytoplasm and nucleus, although in AD brains it localizes preferentially in cytoplasm of hippocampal neurons(Ramsey et al., 2007) .

Several compounds may exert protective effects through activation of Nrf2 transcription. Neurons within CA1 region of hippocampus were largely protected against neurodegeneration induced by Aβ in the presence of carnosic acid, a potent antioxidant that activates the Keap1/Nrf2 transcription pathway (Azad et al., 2011) by binding to specific Keap1 cysteine residues and activating phase 2 enzymes (Satoh et al., 2008). Tert-butylhydroquinone (tBHQ), a phenolic antioxidant, is a known inducer of Nrf2 in NT2N neurons not only by protecting neurons against oxidative stress, but also decreasing Aβ formation (Eftekharzadeh et al., 2010). Oxazine derivatives can also attenuate the extent of apoptosis known to occur in AD, by stabilizing Nrf2 in the nucleus and upregulating HO-1 in PC12 cells (Ansari et al., 2011).

1.7.2. CREB/CBP

CREB is a constitutively expressed nuclear transcription factor of the family of dimerizing leucine zipper transcription factors (Pugazhenthi et al., 2011) and regulates the expression of genes involved in neuronal survival and function (Lonze and Ginty, 2002).

Several genes important for neuronal function contain a CRE in their promoter region which makes CREB a widely studied transcription factor (Chu et al., 2007). For instance, the regulation of GluN1 and GluN2B subunits of the NMDARs, which are involved in the processes of memory and learning, are regulated by CREB (Lau et al., 2004; Rani et al., 2005).

Serine 133 in the KID domain of CREB is a key regulatory site that must be phosphorylated in order to activate CREB. Several stimuli have been proven to promote its activation through phosphorylation (Johannessen et al., 2004) including cAMP-dependent protein kinase, PKC, ERK and Ca^{2+}/calmodulin-dependent protein kinase, (Gonzalez et al., 1989; Watson et al., 2001; Chong et al., 2003). Once phosphorylated, CREB binds to its activators CBP and p300, facilitating the expression of target genes (Kwok et al., 1994). Transcriptional co-activators CBP and p300 have high degree of homology and similar pattern of expression and stimulate CREB by modifying transcription factors and histone acetylation (Liu et al., 2012).

In the context of AD, previous studies showed that mRNA levels of CREB and its target BDNF are reduced in hippocampal and cortical neurons of Tg2576 mice model. An inverse correlation between SDS-extracted Aβ soluble form and CREB protein levels was also found in AD postmortem hippocampal samples (Pugazhenthi et al., 2011). Pretreatment with $A\beta_{1-42}$ at sublethal concentrations, resulted in a suppression of CREB phosphorylation induced by exposure to 10 µM NMDA and suppressed the activation of BDNF promoter in rat cortical neurons (Tong et al., 2001). Thus, Aβ was suggested to alter hippocampal dependent synaptic plasticity and memory and to mediate synaptic loss through the CREB signaling pathway (Saura and Valero, 2011).

Loss of function of CBP/p300 was also associated with familial AD (Francis et al., 2007). Mutations in PS1 and APP were shown to alter CBP and CREB function (Vitolo et al., 2002). In fact, wild-type PS1 stimulates the transcriptional activity of CBP whereas PS1 and PS2

knockout mice were shown to have a reduction of CBP levels, which probably leads to neuronal degeneration (Francis et al., 2006). Interestingly, recent work showed that restoring CREB function via viral brain delivery of CBP improves learning and memory deficits in 3xTg-AD mice (Caccamo et al., 2010), revealing an important role of CREB/CBP nuclear signaling.

1.7.3. Mitochondrial transcription factor A (Tfam)

There is evidence that the genetic variant rs2306604 A-allele of Tfam can be a moderate risk factor for AD in European subjects (Belin et al., 2007). On the other hand, a study performed in a large Chinese cohort consisting of 394 patients and 390 healthy controls showed that there were significant differences in genotype and allele frequencies of the SNP rs1937 between AD patients and controls and that the minor C allele of rs1937 variant acted as a moderate protective factor for sporadic AD (Zhang et al., 2011). In contrast, it was reported that Tfam haplotype containing rs1937 G may be a moderate risk factor for AD in German, Swiss and Italian samples of AD subjects (Gunther et al., 2004).

Tfam is a nuclear-encoded transcription factor that is imported into mitochondria where it is essential for mtDNA maintenance and transcription (Belin et al., 2007). Tfam is a highly conserved 25 kDa protein (Reyes et al., 2002) that interacts with light and heavy strand promoters in mitochondrial DNA, bending and unwinding mitochondrial transcription promoters (D'Errico et al., 2005). Accordingly, the amount of human Tfam in human HeLa cells, but not the transcription level is directly correlated with the amount of mtDNA (Kanki et al., 2004). Furthermore, Tfam knockdown induces asymmetric segregation of mtDNA between dividing daughter cells, suggesting an essential role for human Tfam in symmetric segregation of mtDNA (Kasashima et al., 2011).

To exert its function, Tfam binds to RNA polymerase and to mitochondrial transcription factor B (Tfbm) 1 and 2 which are ubiquitously expressed nuclear encoded transcription factors that are transported to mitochondria where they can support mtDNA transcription, from both heavy and light strands of mitochondrial DNA (Falkenberg et al., 2002; Ramachandran et al., 2008).

1.7.4. Peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1 α- (PGC-1α)

PGC-1α has been reported as a critical regulator of mitochondrial energy metabolism and biogenesis (Anderson and Prolla, 2009; Wareski et al., 2009). PGC-1-α protein content is negatively associated with both AD-type neuritic plaques and Aβ content in human postmortem brains of AD patients (Qin et al., 2009). Furthermore, PGC-1α nuclear protein levels as well as NRF-1, NRF-2β and Tfam protein levels significantly decrease in hippocampal tissue from AD patients comparatively to age-matched controls and also in APP_{swe} M17 neuroblastoma cells. Also, PGC-1α knockdown reduced mtDNA/nDNA and ATP levels whereas PGC-1α overexpression restores target protein expression and ATP levels to the level comparable to WT M17 cells (Sheng et al., 2012).

PGC-1α is part of a family of proteins that includes PGC 1-β and PRC (PGC-1-related coactivator) and share a high homology at the N and C terminus (Puigserver and Spiegelman, 2003). PGC-1α is a protein with 798 amino acids that binds several histone acetyltransferases (HAT)-containing proteins at their N-terminal regions including CBP and steroid receptor coactivator 1 (Puigserver et al., 1999) remodeling histones within chromatin and increasing the access of the transcriptional machinery to target genes. PGC-1α contains three LXXLL (L1-L3) domains and downstream the L3 motif, there is a negative regulatory region that aids the docking of PPARγ and NRF (nuclear respiratory factor) -1 and -2 (Soyal et al., 2006). PGC-1α requires NRF-2 binding sites for maximal activation of target promoters; however there is no direct binding of the NRF-2 and PGC-1α (Scarpulla, 2011) (Figure 1.4)

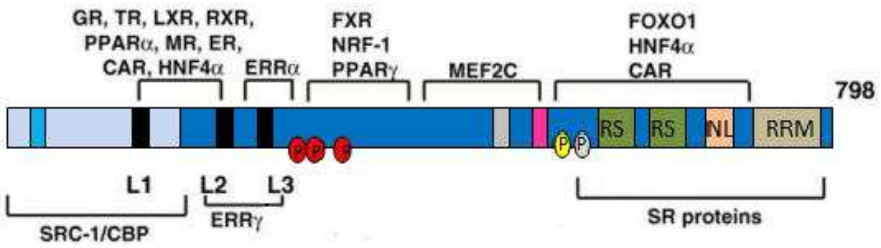


Figure. 1.4 Schematic representation of PGC-1α protein binding domains and interactors. Three LXXLL motifs (L1-L3) are within a region shown to bind MEF2. Three p38 MAPK phosphorylation sites are located within a negative regulatory region. Casein kinase 1 and 2 phosphorylation sites are represented in yellow and grey, respectively. NL- nuclear localization signal; RRM – RNA recognition motif; CAR constitutive androstane receptor; ER – estrogen receptor; RXR – Retinoid X receptor; Src-1 – steroid receptor coactivator. Adapted from (Soyal et al., 2006).

Ectopic expression of PGC-1α increases the activity of Tfam. However, TFAM promoter activation is diminished when the NRF-1 and NRF-2 binding sites are mutated (Wu et al., 1999). PGC-1α has also a region required for the co-activation of the GLUT4 via MADS box transcription enhancer factor (Michael et al., 2001) and also interacts and co-activates forkhead transcription factor (FOXO) 1 in a process inhibited by Akt-mediated phosphorylation (Puigserver et al., 2003).

PGC-1α is activated by phosphorylation of three conserved threonine and serine residues by the p38 mitogen-activated protein kinase (Puigserver et al., 2001) and by AMP activated protein kinase direct phosphorylation (Terada et al., 2002). Additionally, sirtuin 1 (SIRT1) (homologue of the Sacharomyces cerevisiae silencing information regulater 2 (Sir2)) activates PGC-1α by deacetylation (Nemoto et al., 2005). In contrast, General Control Non-Repressed Protein 5 (GCN5), a histone acetyl transferase, acetylates and thus inhibits PGC-1α activity (Lerin et al., 2006).

PGC-1-α regulates adaptative thermogenesis (Puigserver et al., 1998) fiber switching in skeletal muscle (Lin et al., 2002) and neoglucogenesis in liver (Puigserver et al., 2003). In myotubes, overexpression of PGC-1α leads to an increase in the mtDNA content and in the mRNA of nuclearly encoded COX IV, the mitochondrially encoded COX II and cytochrome c (Wu et al., 1999).

Concordantly, decrease of about 30-50% in expression of genes of oxidative phosphorylation, fatty acid oxidation and ATP synthesis were observed in PGC-1α KO mice, despite the normal volume of mitochondria observed in these animal muscle beds (Arany et al., 2005). Overexpression of PGC-1α in cortical neurons led to an increase of 13% while PGC-1α knock-down by shRNA caused a decrease in 17% in mitochondrial density in axons of rat cortical neurons. Also, neuronal overexpression of PGC-1α increased ATP levels by about 30% (Wareski et al., 2009).

Analysis of the PGC-1α promoter revealed binding sites for CREB, Nrf2, Mef2 and FoxO3a. Interestingly, in the presence of H_2O_2 the activity of this promoter increased 4-fold, which was maintained even when Mef2, Nrf2 and FoxO3a binding sites were mutated. In contrast, a

mutation in CREB binding site reduced the basal activity of the PGC-1α promoter and the effect induced by H_2O_2, suggesting that CREB plays an important role in the regulation of the PGC-1α promoter. In addition, the binding of phospho-CREB to the PGC-1α promoter increased during oxidative stress in cells. PGC-1α is co-induced with several key ROS-detoxifying enzymes, such as GPx and SOD2, upon treatment of cells with oxidative stressors being PGC-1α KO mice much more sensitive to the neurodegenerative effects of oxidative stressors, including to those that induce degeneration of dopaminergic and glutamatergic neurons in the brain (St-Pierre et al., 2006). PGC-1α KO mice displayed neurodegenerative lesions in the brain, particularly in the striatum, and showed behavioral abnormalities (Lin et al., 2004). Thus, PGC-1α may provide an accurate balance between metabolic requirements and cytotoxic protection.

1.8. AD models

1.8.1. Animal Models – the case of 3xTg-AD mice

There are several mouse models that mimic what happens in AD human patients. The transgenic mice, express mutant forms or overexpress some genes that are altered in AD. Despite the efforts, most of the transgenic mice do not exhibit the abnormalities that are characteristic of AD patients. Transgenic mice vary in the genes that are mutated and in the form by which this mutation was caused. They can be created by using yeast artificial chromosomes, embryonic stem cells, microinjection of complementary DNA (cDNA) constructs and gene targeting (Price and Sisodia, 1998). Table 1.1. shows some examples of transgenic animals that were developed to model AD and that present different characteristics.

Table1.1. Transgenic Rodent models of AD pathology. (Spires and Hyman, 2005).

Name	Gene(s) Overexpressed	Neuropathology Plaques	P-tau	NFT	Cell Loss	Memory Deficits	Age of Onset (of Pathology)
PDAPP mice	APP minigene, V717F mutation	Yes	Yes	No	No	Yes	6–8 months
Tg2576 mice	APP Swe cDNA (695)	Yes	Yes	No	No	Yes	9–11 Months
APP23 mice	APP Swe cDNA (751)	Yes	Yes	No	Yes (CA1)	Yes	6 Months
TgCRND8 mice	APP cDNA Swe and V717F mutations	Yes	nr	No	nr	Yes	3 Months
APPSwe TgC3-3 mice	APP cDNA (695) Swe	Yes	nr	nr	nr	nr	18 Months
PSAPP mice	Tg2576 and PSI M146L	Yes	Yes	nr	Minor	Yes	6 Months
Tg478/1116/11587 rat	APP Swe, APP Swe and V717F, PS1, M146V	Yes	nr	nr	nr	nr	9 Months
ALZ7 mice	4R tau	No	Yes	No	No	nr	-
ALZ17 mice	4R tau	No	Yes	No	No	nr	-
7TauTg mice	3R tau	No	Yes	Yes	nr	nr	18–20 Months
JNPL3 mice	4R tau P301L	No	Yes	Yes	Yes	Yes	5 Months
pR5 mice	4R tau P301L	No	Yes	Yes	Yes	nr	8 Months
TAPP mice	Tg2576x JNPL3	Yes	Yes	Yes	nr	nr	6 Months
3xTg-AD	APP (Swe), PS1 (M146V), tau (P301L)	Yes	Yes	Yes	nr	nr	3 Months

nr = not reported; Swe = Swedish mutation; P-tau = phosphorylated tau immunoreactivity.

Due to the fact of developing both tau pathology and Aβ aggregates, 3xTg-AD mice are a good model to study the pathways involved in AD. In addition, is currently available at CNC, University of Coimbra.

The transgenic mice 3xTg-AD was first described by Oddo et al. (2003). This transgenic line was created by the microinjection of two transgenes, APPSwe and TauP301L into single-cell embryos from homozygous PS1M146V knockin mice.

Importantly, the 3xTg-AD mouse was the first model shown to develop plaques and tangles in relevant AD brain regions. They show synaptic dysfunction as well as LTP deficits before plaques and tangles arise. This model has been useful to study the impact of Aβ and tau on synaptic plasticity and to evaluate the efficacy of anti-AD therapies. In this model, Aβ formation precedes tangle formation. Intraneuronal Aβ is one of the earliest pathological manifestations and is apparent between 3 and 4 months of age in the neocortex and by 6 months of age in the CA1 pyramidal neurons. Extracellular deposits of Aβ first appear in 6-month-old mice in the frontal cortex and in 12 month-old in the hippocampus. Tau pathology only appears after 12-month of age, particularly in hippocampal neurons (Oddo et al., 2003).

1.9. Objectives

It has been recognized that Aβ can be internalized following the interaction with several membrane receptors (Bi et al., 2002) and accumulate intracellulary where it interacts with several cellular components, namely the mitochondria (Pagani and Eckert, 2011) and the nucleus. There is also recent evidence that Aβ can act as a transcription modulator of AD-related genes (e.g. Maloney and Lahiri, 2011). On the other hand, oxidative stress and mitochondrial biogenesis are important features in AD pathogenesis and are present in several models of AD, including the 3xTg-AD mice (Yao et al., 2009).

Thus, the overall objective of this work was to define AD-related changes in transcription factors and target genes linked to mitochondrial biogenesis, antioxidant defenses and NMDAR-linked cAMP signaling, namely PGC-1α and Tfam, Nrf2 and CREB in both animal and cell model of AD. More specifically, we aimed to: (1) determine whether these transcription factors and their target genes are altered in young versus old 3xTg-AD brain cortex, respectively, mainly bearing intracellular and extracellular Aβ; (2) understand the effects of exogenous applied $A\beta_{1-42}$ enriched oligomers preparation on transcription factors in cultured cortical cells; and (3) elucidate the involvement of NMDARs on Aβ-mediated effects also in cultured cortical cells

These specific objectives were delineated as described next:

1) Evaluation of changes in transcription factors and target genes related to mitochondrial biogenesis, antioxidant defense and NMDAR-linked cAMP signaling.

Taking into account that Aβ interacts with several membrane receptors and might modulate the transcription of AD-related genes, we hypothesized that Aβ could modulate the transcription factor related to processes that are dysfunctional in AD. Thus, we aimed to determine the nuclear levels of PGC-1α, Tfam, Nrf2 and CREB in nuclear fractions derived from 3 (males and females) and 15 month-old 3xTg-AD male mice brain cortex. The phosphorylation of Nrf2 at Ser40 promotes Nrf2 translocation to the nucleus, whereas

phosphorylation of CREB at Ser133 promotes its activation; thus, we further evaluated whether these post-translational modifications were altered in our models.

2. Role of $A\beta_{1-42}$ enriched-oligomer preparation on transcription factor modifications in cultured cortical cells

To evaluate the role of $A\beta_{1-42}$ we aimed to determine the nuclear levels of PGC-1α, Nrf2 and CREB, as well as protein levels of their targets genes in cortical cells exposed to $A\beta_{1-42}$ for short periods, 5 minutes and 2 hours, possibly related with modified signaling pathways following the interaction and/or activation of selective membrane receptors in the presence of Aβ, and for a longer period of time, 24 hours, sufficient for Aβ internalization (Ohyagi et al., 2005). Moreover, we aimed to correlate the changes in Nrf2 evoked by $A\beta_{1-42}$ with cellular ROS production

3. Elucidate the involvement of NMDARs on Aβ-mediated effects in cultured cortical cells

Taking into account that $A\beta_{1-42}$ may interact with NMDARs and mediate intracellular calcium rise (Ferreira et al., 2012), we aimed to elucidate the role of this glutamate receptor subtype by testing the effect of selective antagonists of NMDARs on the Aβ-mediated effects in transcription factors.

CHAPTER 2 – METHODS

2.1. Primary cortical cultures

Primary cultures of rat cortical neurons were prepared as described previously (Agostinho and Oliveira, 2003) with minor modifications (Ferreira et al., 2012). Female Wistar rats with 16 days of gestation were sacrificed after anesthesia with 2-bromo-2-chloro-1,1,1-trifluoroethane followed by cervical dislocation. Frontal cerebral cortices, free of meninges, were dissected out from fetal rats and collected in Ca^{2+}, Mg^{2+}- free Krebs medium (containing 120.9 mM NaCl, 4.83 mM KCl, 1.22 mM KH_2PO_4, 25.5 mM $NaHCO_3$, 13 mM glucose, 10 mM Hepes, pH 7.4), containing 0.3% fatty acid-free BSA (Sigma Aldrich). After treatment with 0,035% trypsin (Sigma Aldrich) in BSA-Krebs medium for 5 min at 37ºC, 0.038% trypsin inhibitor (Sigma Aldrich) was added in order to block enzymatic digestion and cells were then centrifuged at 180 x g for 5 min, and further resuspended in Neurobasal Medium (Gibco, Invitrogen) supplemented with 1% B27 (Gibco, Invitrogen), 0.1% gentamycin (Gibco Invitrogen) and 0.5 mM glutamine (Lonza).

The cells were plated in poly-D-lysine coated 6-well plates, for the nuclear fractions and total extracts preparation or in 96-well plates for the ROS production evaluation assay at a density of about 0.16 $x10^6$ cells/cm^2 and 0.35 x 10^6 cell/ml, respectively. Alternatively, cells were plated in 16 mm poly-D-lysine coated coverslips (0,09 cell x 10^6/cm^2) for immunocytochemistry studies. The cells were maintained in a humidified incubator at 37°C containing 95% air and 5% CO_2 for 15 days. Half of medium was changed at 7 and 11 days in culture with fresh B27 supplemented Neurobasal Medium.

2.2. 3xTg-AD and WT mice cortices isolation

The 3xTg-AD mice express three mutations, the APP_{swe}, the PS1M146V and tauP301L (Oddo et al., 2003). Both 3xTg-AD and control wild-type (WT) mice are from the same 129/C57BL/6 hybrid background strain and were provided by Dr. Frank LaFerla (University of California,

Irvine, Irvine, CA). The mice were kept on a 12 h dark/light schedule and were given ad libitum food and water access. All animal experiments were carried out following the Guide for laboratory animal practice of the Center for Neuroscience and Cell Biology, University of Coimbra, with care to minimize the number of animals and their suffering.

Three and fifteen months-old transgenic and non-transgenic mice were sacrificed by cervical dislocation, brains were dissected out and cortices were separated from other brain regions. Tissues were maintained in liquid nitrogen containers until use.

2.3. $A\beta_{1-42}$ oligomers preparation

Briefly, synthetic Aβ peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to a final concentration of 1 mM. HFIP was then removed in a Speed Vac (Ilshin Lab. Co. Ltd., Ede, The Netherlands), and dried HFIP film was stored at −20 ◦C. The peptide film was resuspended to make a 5 mM solution in anhydrous dimethyl sulfoxide. Aβ peptides were further prepared by diluting the solution in phenol red-free Ham's F-12 medium without glutamine to a final concentration of 100 µM and incubated overnight at 4°C. The preparation was centrifuged at 15,000 × g for 10 min at 4°C to remove insoluble aggregates, and the supernatant containing soluble oligomers and monomers was transferred to pre-lubrificated clean tubes (Costar) and stored at -20°C. Protein content was determined by by using the BioRad protein assay and quantified by using a microplate reader Spectra Max Plus 384 (Molecular Devices, USA). Samples containing 10 µg of protein were diluted (1:2) with sample buffer (containing: 40% glycerol, 2% SDS, 0.2 M Tris–HCl, pH 6.8 and 0.005% Coomassie G-250). The presence of different assembly peptide forms (monomers, oligomers and/or fibrils) in the preparation was evaluated by 4–16% Tris–Tricine SDS-PAGE gel electrophoresis and further staining with Coomassie blue. Aβ 1-_{1-42} preparation contained low-n oligomers (about 50%) and monomers (about 50%) (Figure 2.1).

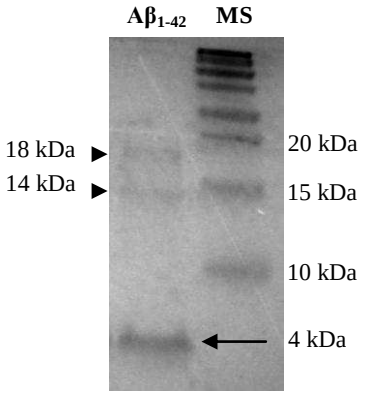


Figure 2.1. Representative gel of electrophoretic separation of $A\beta_{1-42}$ peptide forms prepared from synthetic $A\beta_{1-42}$. Oligomeric forms of $A\beta_{1-42}$ are represented by the arrow heads and monomeric form is represented by the arrow. MS – molecular weight standard.

2.4. Subcellular Fractionation

Ten female mice with 3 month of age, fourteen male with 3 month of age and eight male mice with 15 month of age were sacrificed and their cortices were submitted to subcellular fractionation to obtain nuclear and mitochondrial fractions. Also, primary rat cortical cell cultures were submitted to subcellular fractionations to obtain nuclear fractions.

2.4.1. Nuclear fractions from rat cortical cells and mice cortical tissue

Nuclear fractions from primary rat cortical cell cultures and from cortices isolated from 3xTg-AD and WT mice were obtained using the Nuclear/Cytosolic fractionation kit (Biovision, CA, USA). Cultured cortical cells with 15 DIV were washed three times with PBS (containing 137mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM $Na_2HPO_4.2H_2O$, pH 7.4) at room temperature and then scrapped by using a cytosolic extraction buffer provided by the manufacturer. Rat cortical cell suspension was submitted to several cycles of centrifugation and vortexing in the presence of cytosolic and nuclear extraction buffers provided by the manufacturer.

Mice cortical tissues were homogenized (20 strokes) by using a tissue homogenizer at 280 rpm in ice-cold PBS and then centrifuged to pellet the cells at 500 x g, 2 or 3 minutes, which were

then ressuspended in the cytosolic extraction buffer provided by the manufacturer followed by several cycles of centrifugation and vortex as mentioned for rat cortical cells. Protein content was determined by using the BioRad protein assay as described in section 2.3, and the samples were stored at -20°C until use.

2.4.2. Mitochondrial fractions from mice cortical tissue

Mice cortices were homogenized (20 strokes) in a tissue homogenizer at 280 rpm, in sacarose medium (containing 250 mM sacarose, 20 mM Hepes, 10 mM KCl, 1,5 mM $MgCl_2$, 1mM EGTA, 1 mM EDTA and 1% NP40) supplemented with 1mM DTT, 1µg/ml protease inhibitors (chymostatin, pepstatin A, leupeptin and antipain) and 100 µM PMSF. The cell suspension was then centrifuged at 560 x g for 12 min at 4°C. The resulting pellet was discarded and the supernatant was centrifuged at 11900 x g for 20 min at 4°C. The mitochondrial pellet was ressuspended in supplemented sacarose medium. Protein content was determined as described above and samples were stored at -20°C until use.

2.5. Total extract preparation

Cultured rat cortical cells were washed 3 times in ice-cold PBS and then scrapped in RIPA extraction buffer (containing 150 mM NaCl, 50 mM Tris HCl, 5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, pH 7.5) supplemented with 100 µM okadaic acid, 1 mM PMSF, 25 mM NaF, 1 mM Na_3VO_4, 1 mM DTT and 1 µg/ml protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin and antipain).

Mice cortices from WT and 3xTg-AD mice brain were homogenized in 22,5 ml of ice cold supplemented RIPA buffer per gram of tissue in a tissue homogenizer at 280 rpm (20 strokes) and homogenates were then centrifuged at 14000 x g for 10 min at 4°C. Protein content was determined as described above and the samples were stored at - 80°C until use.

2.6. Western Blotting

Nuclear, mitochondrial or total extracts were denaturated with 6x concentrated loading buffer (containing 300 mM Tris-HCl pH 6.8, 12% SDS, 30% glycerol, 600 mM DTT, 0.06% bromophenol blue) at 95°C for 5 min. Equivalent amounts of protein samples (20µg-60µg) were separated by 10% SDS-PAGE gel electrophoresis and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were further blocked with 5% fat-free milk or 5% BSA (for phosphorylated protein detection) and incubated overnight at 4°C with primary antibodies directed against the desired protein. In order to normalize the amount of protein per lane anti-lamin B1, anti-Hsp60, anti-tubulin or anti-actin were used as loading controls. Membranes were further incubated with alkaline phosphatase-conjugated secondary antibodies for 2 hours, at room temperature. Immunoreactive bands were visualized by alkaline phosphatase activity after incubation with ECF reagent and visualized by using a BioRad Versa Doc 3000 imaging system and quantified using Quantity One analysis software (BioRad). All the primary and secondary antibodies used are described in Table 2.1.

Table 2.1. Primary and Secondary antibodies used for Western Blot experiments

Primary antibody	Dilution	Host	Manufacturer
Actin (5316)	1:20000	Mouse	Sigma Aldrich
CBP (sc7300)	1:200	Mouse	Santa Cruz Biotechnology
CREB (#9192)	1:1000	Rabbit	Cell signaling
pCREB (#9196)	1:500	Mouse	Cell signaling
Cytochrome c (#556433)	1:500	Mouse	BD Pharmingen
GCLc (ab17926)	1:1000	Rabbit	Abcam
Lamin B1 (ab16048)	1:1000	Rabbit	Abcam
Nrf2 (ab31163)	1:500	Rabbit	Abcam
pNrf2 (bs2013R)	1:200	Rabbit	BIOSS
PGC-1α K-15 (sc5816)	1:500	Goat	Santa Cruz Biotechnology
SOD1 (ab16831)	1:1000	Rabbit	Abcam
Tfam (sc30965)	1:200	Goat	Santa Cruz Biotechnology
Tubulin (T6199)	1:20000	Mouse	Sigma Aldrich

Secondary Antibody		
Anti-Goat IgG-AP (sc2022)	1:3000	Santa Cruz Biotechnology
Anti-Mouse (Alkaline Phosphatase	1:20000	GE Healthcare
Anti Rabbit (Alkaline Phosphatase)	1:20000	GE Healthcare

2.7. Immunocytochemistry

In order to characterize our preparation, cortical cells cultured for 15 days in glass coverslips were washed 3 times (2 minutes), in PBS and fixed in 1:1 methanol-acetone (3 minutes). Cells were then washed 3 times (5 minutes) in PBS and blocked in 3% BSA for 1 hour at room temperature and further incubated with the primary antibody, overnight, at 4°C. Then, the cells were washed again 3 times (5 minutes) in PBS, further incubated with the secondary antibody for 2 hours and then washed again 3 times in PBS (5 minutes). During the second washing step, 1µg/ml Hoechst was added to the PBS, and washed again. The coverslips were mounted in Dako Fluorescence mounting medium. Images were examined and scored using the Axioscope 2 Plus upright microscope (Zeiss, Jena, Germany) or a confocal microscope (LSM 510 Meta/Zeiss). Primary and Secondary antibodies are described in Table 2.2.

Table 2.2. Primary and Secondary antibodies used for immunocytochemistry

Primary Antibody	**Dilution**	**Host**	**Manufacturer**
Aβ1-16 (6E10) (SIG 39300)	1:200	Mouse	Covance
APP (ab2072)	1:200	Rabbit	Abcam
S100 (s2657)	1:200	Mouse	Sigma
MAP-2 (AB5622)	1:200	Rabbit	Chemicon
Secondary antibody			
Anti-Rabbit (Alexa594)	1:200		Invitrogen
Anti Mouse (Alexa488)	1:200		Invitrogen

2.8. ROS levels determination

Intracellular ROS generation was determined in rat cortical neurons, by following 2,7-dichlorofluorescein (DCF) fluorescence. Cells were briefly washed with Na^+ medium and then incubated with 20 µM Dichlorodihydrofluorescein-diacetate (DCFH2-DA), a stable non-

fluorescent cell-permeable compound, at 37°C in Na^+ medium (containing 140 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 10 Glucose, 10 Hepes, pH 7.4/NaOH). Once internalized by the cell, $DCFH_2$-DA is hydrolyzed to $DCFH_2$ by intracellular esterases and rapidly oxidized to the highly green fluorescent component DCF by endogenous hydroperoxides. Intracellular hydroperoxides were measured in cells subjected to NMDA (100 µM) or Aβ (0.5 µM) direct stimulation in Na^+ medium without added $MgCl_2$ and in the presence of 20 µM glycine (to drive the maximum activation of NMDARs), by using a microplate reader Spectrofluorometer Gemini EM (Molecular Devices, USA) (488 nm excitation, 530 nm emission). Experiments were performed in the absence or in the presence of NMDA receptor antagonists NVP-AA077 (50 nM), ifenprodil (10 µM), memantine (10 µM) or MK-801 (10 µM). When the antagonists were tested, a preincubation of 5 minutes was performed before the addition of NMDA or Aβ. The values were normalized to the percentage of basal condition.

2.9. Data and statistical analysis

Data were analyzed by using Excel (Microsoft, Seattle, WA, USA) and Prism (GraphPad Software, San Diego, CA, USA) softwares. Data were expressed as the mean ± S.E.M. Comparisons among multiple groups were performed by one-way ANOVA, followed by Tukey's post hoc test. Student's t-test was also performed for comparison between two Gaussian populations, as described in figure legends. Significance was accepted at $p < 0.05$.

CHAPTER 3 - RESULTS

Changes in transcription factors related to mitochondrial biogenesis and antioxidant defense in the 3xTg-AD mice

3.1. Characterization of the subcellular fractions

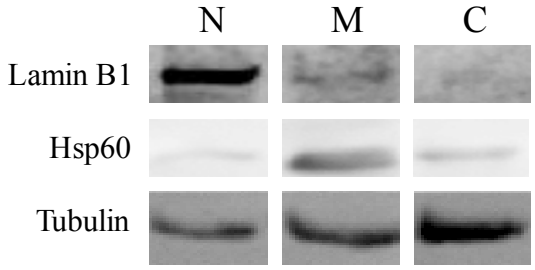


Figure 3.1. Characterization the nuclear and mitochondrial fractions. The purity of the fractions was evaluated by Western Blotting. N – Nuclear fractions, M – Mitochondrial fractions, C – Cytosolic fraction.

3.2. Modified protein levels of transcription factors in nuclear fractions from 3 and >15 month-old 3xTg-AD mice cortex

Mitochondrial dysfunction is an early event in AD pathology and impaired mitochondrial biogenesis may explain the bioenergetic deficits that occur in this disease. Since PGC-1α is a key transcription co-activator of mitochondrial biogenesis, its protein levels were analysed in nuclear fractions prepared from cortices of 3 month-old (3 mo) and 15 month-old (15 mo) of 3xTg-AD versus WT male mice (Figure 3.2).

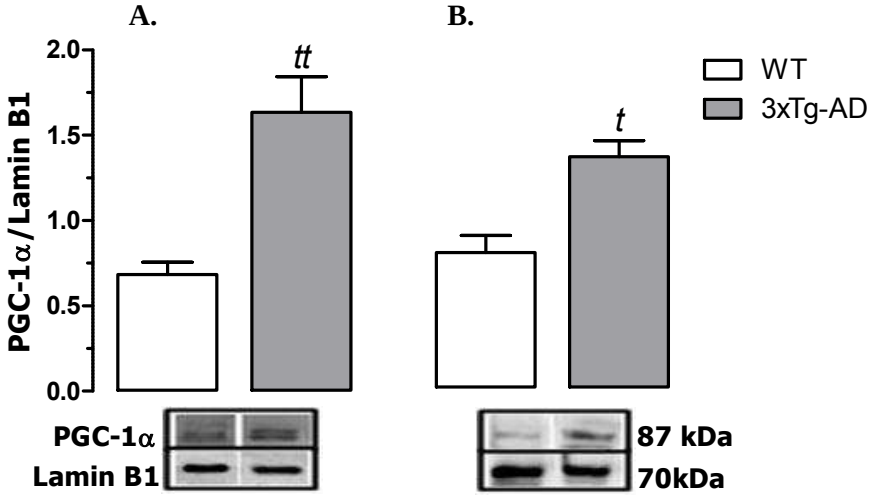


Figure 3.2. PGC-1α protein levels in 3xTg-AD vs WT male mice cortex. Protein levels of PGC-1α in nuclear fractions of **(A)** 3 mo and **(B)** >15 mo 3xTg-AD mice cortex were analyzed by Western Blotting. Data represents the mean ± SEM of 3-6 independent experiments. Statistical analysis: $^{t}p<0.05$; $^{tt}p<0.01$ vs WT mice. (Student's t-test).

Our results demonstrate a significant increase in PGC-1α protein levels in nuclear fractions of both young (Figure 3.2A) and old (>15 month-old) (Figure 3.2B) 3xTg-AD male mice cortex ($p<0.01$ and $p<0.05$, respectively), when compared to WT mice. These data may represent a compensatory response against early mitochondrial dysfunction and increased oxidative stress described in this AD animal model (Yao et al., 2009). Along with mitochondrial dysfunction, oxidative stress has been described in AD and several oxidative parameters have been found to be altered in 3xTg-AD as early as 3 months of age (Yao et al., 2009; Resende et al., 2008). Thus, we evaluated the involvement of a key transcription factor known to modulate the expression of several antioxidant defense proteins, Nrf2, by determining the nuclear levels of total Nrf2 and phosphorylated Nrf2 (pNrf2) at Ser40, a residue shown to be phosphorylated by PKC and to be important for the translocation of Nrf2 from the cytosol to the nucleus (Jaiswal, 2004). Because evidence of oxidative stress in 3xTg-AD was previously described in females at 3 mo (Resende et al., 2008), Nrf2 was analysed in nuclear fractions obtained from brain cortices of 3 month-old (3 mo) males and females and in 15 month-old (15 mo) males 3xTg-AD versus WT mice (Figure 3.3)

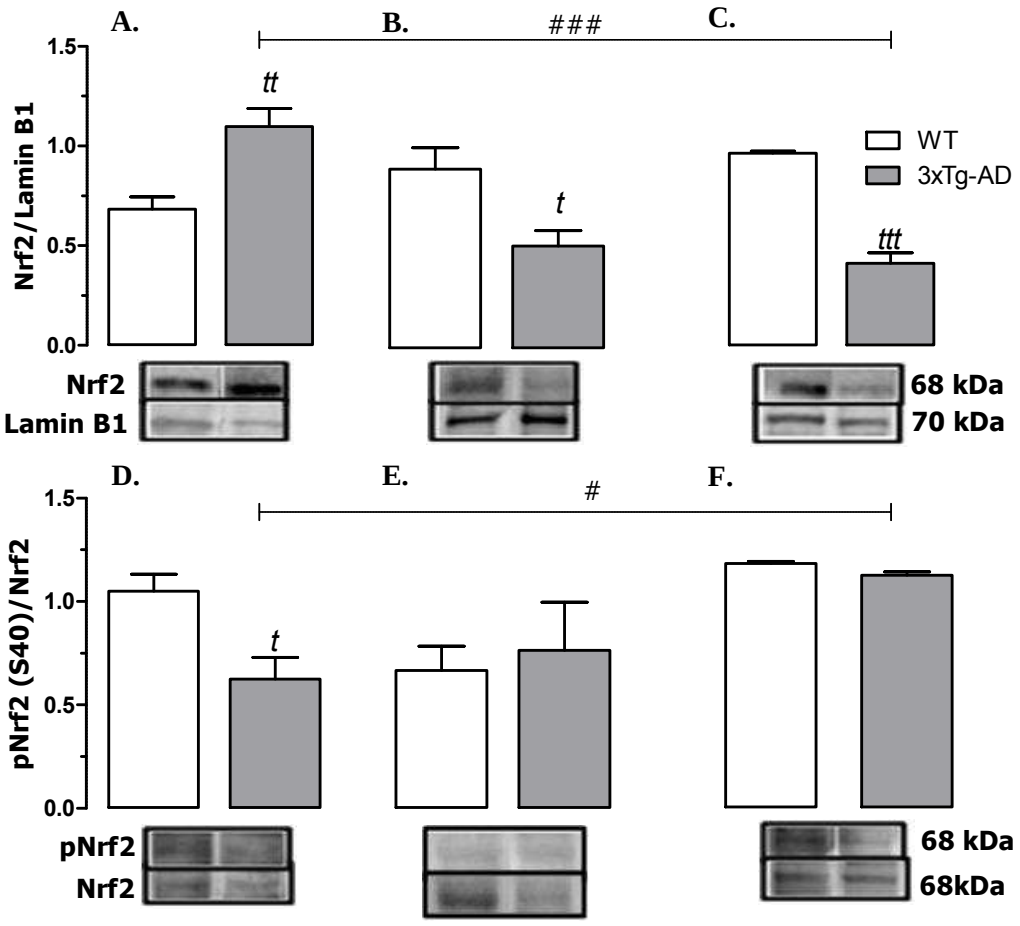


Figure 3.3. Total Nrf2 and pNrf2 protein levels in nuclear fractions of young (3mo) and old (15 mo) 3xTg-AD mice cortex. Total protein levels of Nrf2 in cortical nuclear fractions of (**A**) 3 mo male, (**B**) 3 mo female and (**C**) > 15 month-old male 3xTg-AD mice and levels of pNrf2 in cortex nuclear fractions of (**D**) 3 mo male, (**E**) 3 mo female and (**F**) >15 mo male 3xTg-AD mice were analyzed by Western Blotting. Data represent the mean ±SEM of 3-6 independent experiments. Statistical analysis: $^{t}p<0.05$; $^{tt}p<0.01$; $^{ttt}p<0.001$ vs WT mice; #p<0.05 and ###p<0.001, >15 mo male 3xTg-AD vs 3 mo 3xTg-AD mice (Student's t test).

A significant increase ($p<0.01$) in nuclear protein levels of Nrf2 (Figure 3.3A) along with a significant decrease in nuclear levels of pNrf2 (Figure 3.3D) ($p<0.05$) were detected in 3 mo 3xTg-AD males, relatively to WT mice, suggesting an increased translocation to the nucleus of total Nrf2 associated with decreased phosphorylation status in young 3xTg-AD. Interestingly, a slight decrease in total Nrf2, but unchanged pNrf2 were observed in nuclear fractions from young female 3xTg-AD mice cortex (Figure 3.3 B and E). Similarly, in old (>15 mo) 3xTg-AD males cortex, total nuclear Nrf2 levels were significantly decreased ($p<0.001$) (Figure 3.3 C), whereas no changes in pNrf2 levels were observed (Figure 3.3 F). Our results suggest that Nrf2

signaling is differentially modified in young 3xTg-AD mice males and females. Indeed, increased nuclear Nrf2 levels in 3 mo 3xTg-AD males may represent an attempt to increase the expression of antioxidant proteins, whereas in 3 mo 3xTg-AD females and 15 mo males altered nuclear Nrf2 is very similar, largely implicating this transcription factor in enhanced oxidative stress in the brain of this AD animal model.

Synaptic dysfunction is an early event in AD pathology, which may in part be caused by the Aβ-mediated internalization of the NMDARs. On the other hand, $A\beta_{1-42}$ was recently shown to mediate intracellular Ca^{2+} rise through NMDAR activation, revealing an important role of these receptors in neuronal dysfunction caused by Aβ (Ferreira et al., 2012). Significantly, CREB is a NMDAR-linked cAMP signaling factor and CBP is a CREB co-factor. Therefore, the protein levels of these two transcription factors, namely CREB and pCREB (Figure 3.4) as well as CBP levels (Figure 3.5) were analyzed in nuclear fractions obtained from3 mo and >15 mo male 3xTg-AD mice cortices.

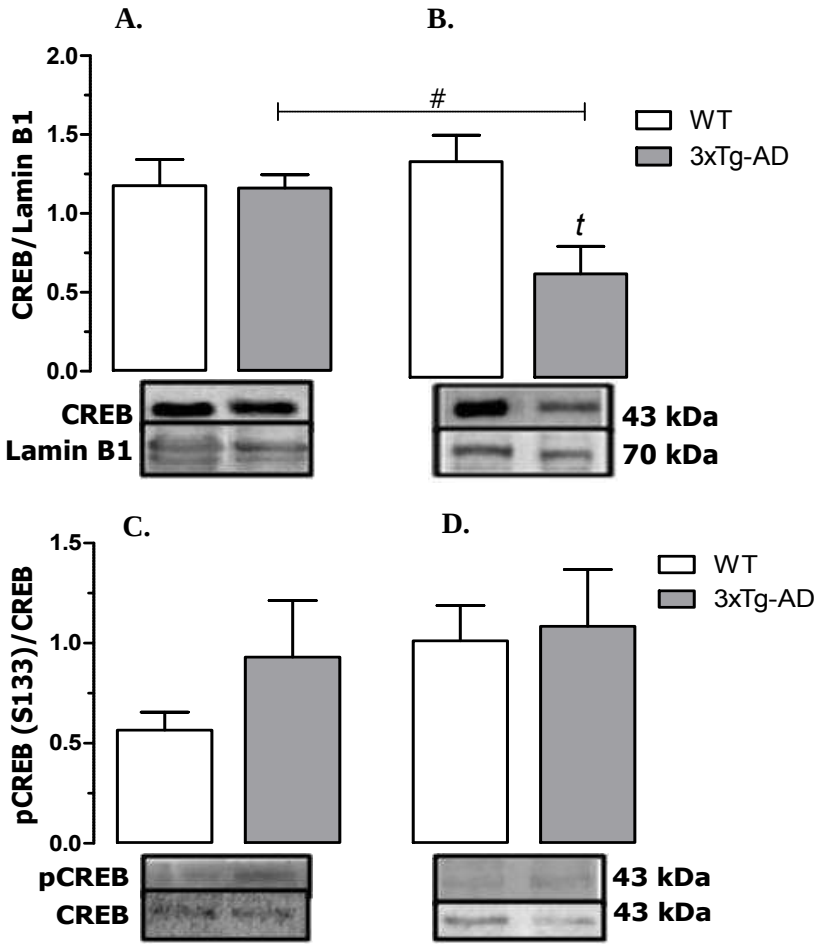


Figure 3.4. Protein levels of CREB in nuclear fractions of 3xTg-AD mice cortex (males). Total protein levels of CREB in (**A**) 3 mo (**B**) > 15 mo 3xTg-AD mice cortex nuclear fractions and levels of pCREB in (**C**) 3 mo and (**D**) >15 mo 3xTg-AD mice cortex nuclear fractions were analyzed by Western Blotting. Data represent the mean ±SEM of 3-5 independent experiments. Statistical analysis: $^{t}p<0.05$ vs WT mice; #$p<0.05$ vs 3 mo 3xTg-AD mice (Student's t test).

Our results demonstrated that nuclear CREB levels are not significantly altered in young 3xTg-AD male mice cortex (Figure 3.4A.); however, there is a tendency (although not statistically significant) for an increase in nuclear pCREB levels (Figure 3.4C). On the other hand, total CREB protein levels were significantly decreased ($p<0.05$)(Figure 3.4B), while pCREB levels were not altered in old 3xTg-AD mice male cortex (Figure 3.4D), in a similar manner as observed for Nrf2 in old 3xTg-AD mice. Decreased nuclear CREB levels may underlie reduced activation of cell survival pathways.

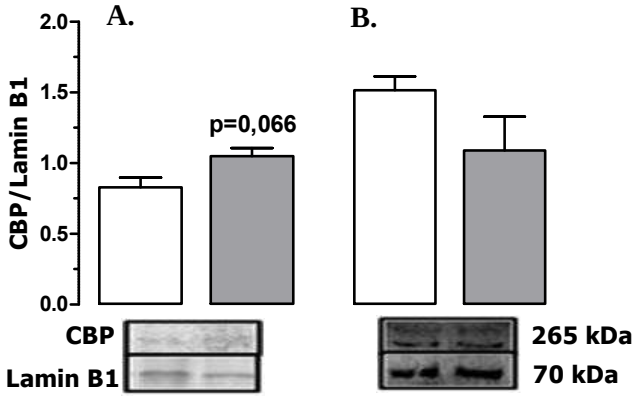


Figure 3.5 Protein levels of CBP in nuclear fractions of 3xTg-AD mice cortex (males). Total protein levels of CBP in cortex nuclear fractions of (**A**) 3 mo and (**B**) > 15 mo 3xTg-AD mice were analyzed by Western Blotting. Data represents the mean ±SEM of 3-4 independent experiments.

Nuclear protein levels of CBP (Figure 3.5 A and B) were not significantly altered in 3 mo or 15 mo 3xTg-AD, when compared to WT mice cortex; however, there was a slight tendency for an increase in CBP nuclear levels in young 3xTg-AD mice, which accompanied the tendency for an increase in pCREB nuclear levels (Figure 3.4C).

3.3. Alterations in protein targets of key transcription factors in 3 mo and >15 mo 3xTg-AD mice cortex

Taking into account the changes in nuclear levels and activity of the transcription factors PGC1α, Nrf2 and CREB in 3xTg-AD mice cortex, we further investigated if there were modification in their direct or indirect protein targets. Therefore, protein levels of Tfam (Figure 3.6), an important target of PGC-1α that regulates mitochondrial biogenesis by activating transcription and replication of the mitochondrial genome and of SOD1 and GCLc (Figure 3.7), two direct targets of Nrf2 were studied.

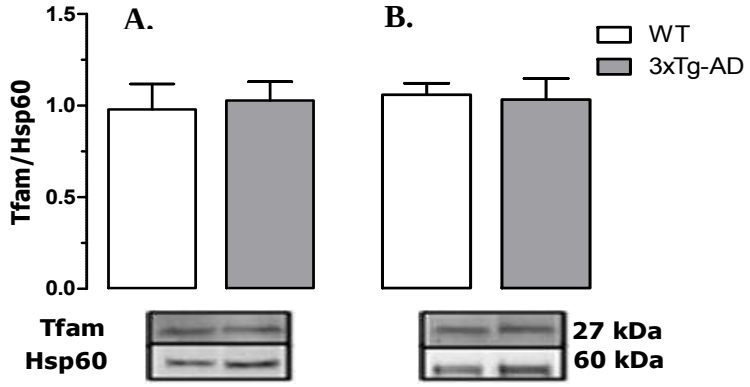


Figure 3.6. Tfam protein levels in mitochondrial fractions of young and old 3xTg-AD mice cortex (males). Protein levels of Tfam in mitochondrial fractions of (**A**) 3 mo and (**B**) > 15 mo 3xTg-AD mice cortex were analized by Western Blotting. Data represent the mean ±SEM of 4 independent experiments.

Despite the increase in PGC-1α protein levels in nuclear fractions of 3 and 15 mo 3xTg-AD mice cortex (Figure 3.2), no significant changes in Tfam were observed (Figure 3.6).

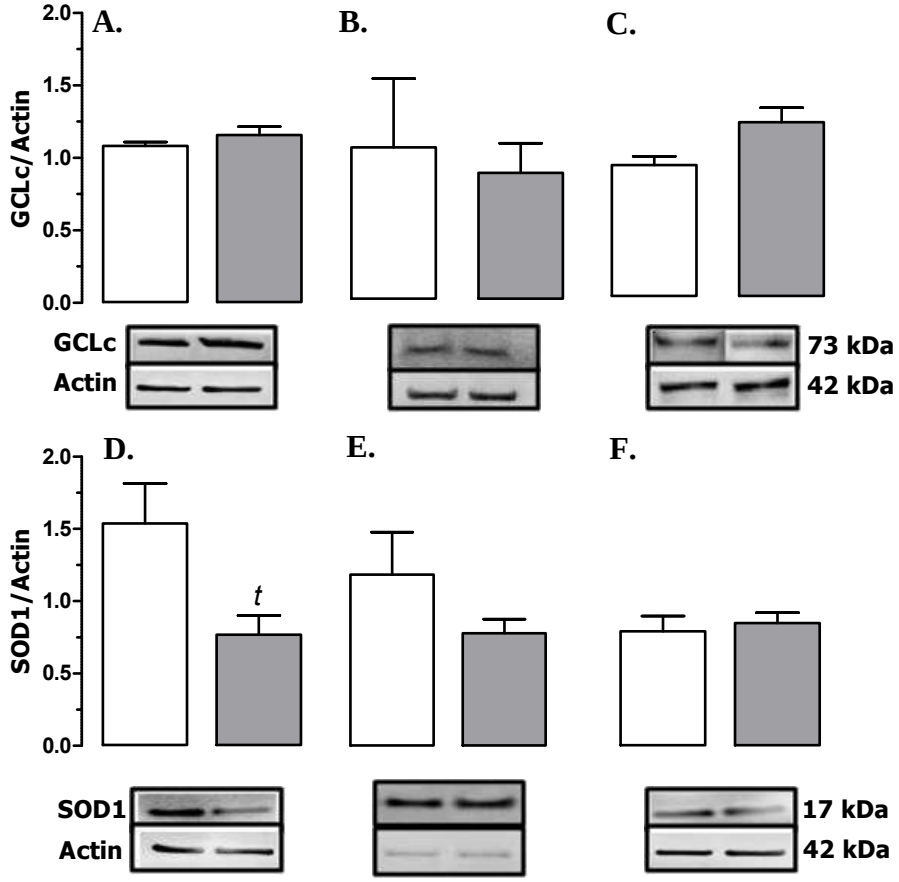


Figure 3.7. Nrf2 protein targets: GCLc (**A-C**) and SOD1 (**D-F**) total protein levels in (**A,D**) 3 mo males, (**B,E**) 3 mo females and (**C,F**) >15 mo 3xTg-AD male mice cortex; were analyzed by Western Blotting. Data represent the mean±SEM of 3-6 independent experiments. Statistical analysis: $^{t}p<0.05$ vs WT mice (Student's t test).

Concordant with the decreased nuclear pNrf2, SOD1 protein levels are decreased in young 3xTg-AD mice cortex. In contrast, SOD1 protein levels are not altered in old 3xTg-AD mice comparatively to WT mice cortex despite the significant decrease in nuclear Nrf2 protein levels, which suggests SOD1 might be regulated by other transcription factor. Concordantly with the tendency for a decrease in Nrf2 protein levels, SOD1 and GCLc levels tend to be decreased in female 3xTg-AD mice cortex relatively to WT mice. However, GCLc protein levels are not significantly altered in 3 and 15 mo male 3xTg-AD mice comparatively to WT.

CHAPTER 4 - RESULTS

Interplay between transcription factors and oxidative stress in rat cortical cells exposed to $A\beta_{1-42}$

4.1. Characterization of the cultures and subcellular fractions

Since cortical cell cultures were grown for 15 DIV in the absence of cytosine arabinose, facilitating the proliferation of glia cells we further evaluated the percentage of neurons and glial cells in our culture preparation. Immunocytochemistry analysis by using antibodies against the neuronal marker microtubule associated protein-2 (MAP-2) and the marker of astrocytic proliferation (s-100, beta subunit) demonstrated that cortical cultures with 15 days in vitro contained about 40% glial cells and 60% of neurons (Figure 4.1).

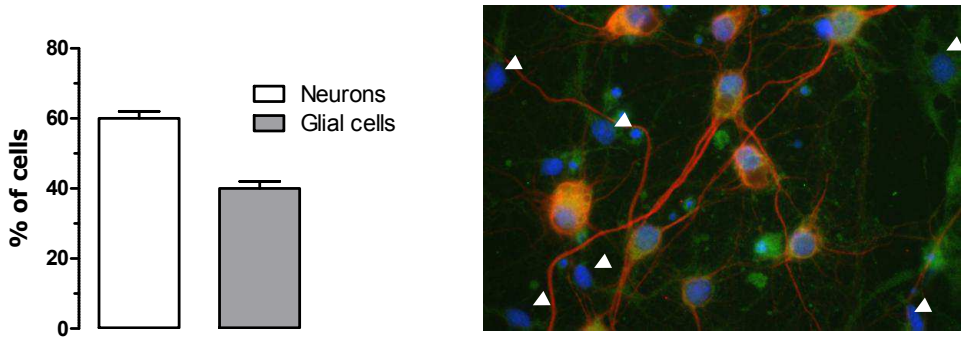


Figure 4.1. Characterization of the rat cortical cultures with 15 DIV. The percentage of glial cells vs neurons was evaluated by immunocytochemistry, using antibodies against s100 (green) and MAP 2 (red). Data represents the mean ± SEM of 3 independent experiments. The nuclei were labeled with Hoechst (blue). Arrowheads indicate non-neuronal cells.

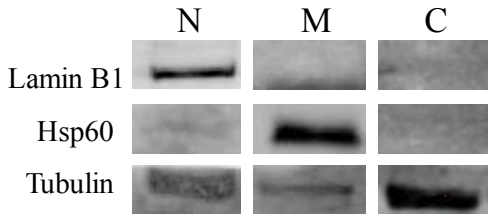


Figure 4.2. Characterization of the nuclear and mitochondrial fractions. The purity of the fractions was evaluated by Western Blotting. N – Nuclear fractions, M – Mitochondrial fractions, C – Cytosolic fraction.

4.2. Aβ-mediated ROS production

Since oxidative stress is a hallmark of AD (Zhu et al., 2007) and NMDAR activation increases intracellular Ca^{2+} levels, with consequent ROS production (Bolanos et al., 2009), we evaluated whether ROS production was altered in mature cortical cells exposed to $A\beta_{1-42}$ and further determined the effect of NMDAR selective antagonists. ROS production following exposure to 0.5 μM $A\beta_{1-42}$ preparation, containing oligomers (at higher percentage) and monomers (as described in Chapter 2), was compared to 100 μM NMDA, used as a positive control for NMDAR activation. To determine the role of NMDARs and particularly of GluN2A and GluN2B subunits we also tested the effect of memantine and MK-801, respectively the uncompetitive and non-competitive NMDAR antagonists, and NMDARs selective subunit-antagonists, namely NVPAA077 (GluN2A antagonist) and ifenprodil (GluN2B antagonist) in Aβ- and NMDA-evoked ROS production (Figure 4.3).

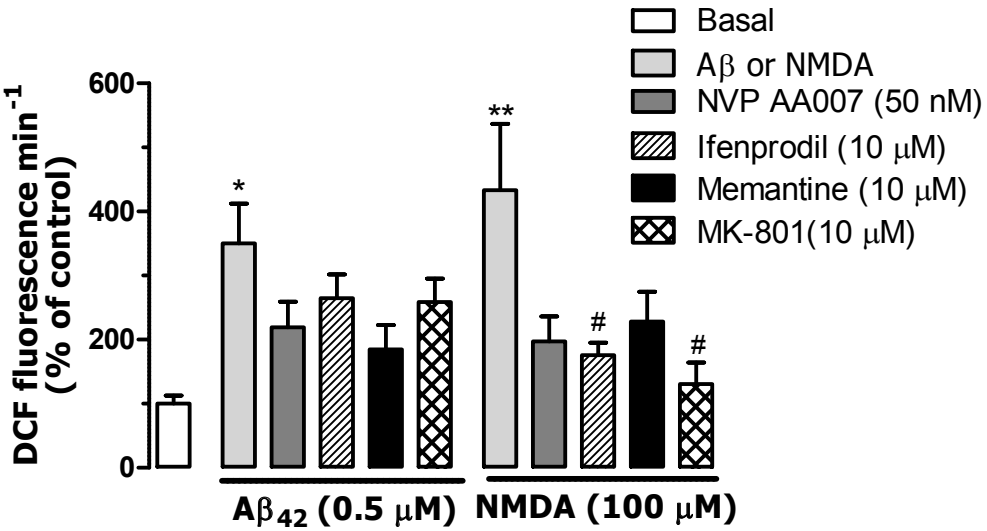
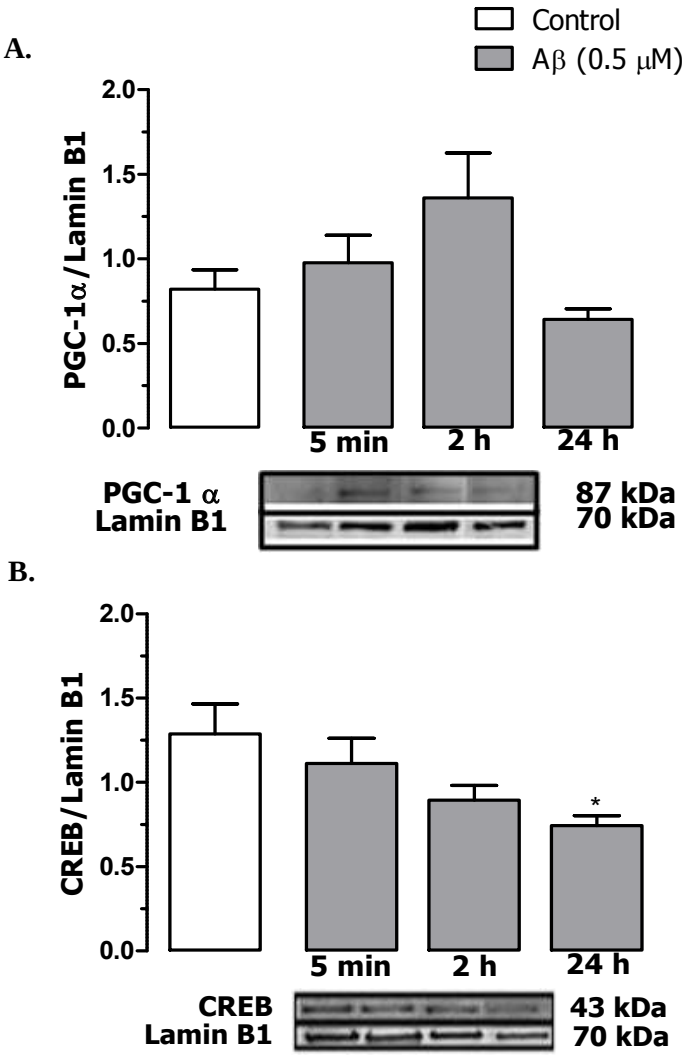


Figure 4.3. ROS levels in rat primary cortical neurons (DIV 15) exposed to $A\beta_{1-42}$ (0.5 μM) and NMDA (100 μM) in Mg^{2+} free medium. When present, NMDAR antagonists were pre-treated for 5 minutes before adding $A\beta_{1-42}$ or NMDA and remained throughout the experiment (15 minutes). ROS levels were determined by using DCFH2-DA fluorescent probe. Data are the mean±SEM of 2-6 experiments performed in quadruplicates. Statistical analysis:*$p<0.05$; **$p<0.01$ vs basal, and #$p<0.05$ vs respective control (Tukey's Multiple Comparison test).

Exposure to $A\beta_{1-42}$ enhanced ROS production ($p<0.05$) in cortical cells. However, it was not significantly prevented by NMDAR antagonists, in contrast to what was observed after exposure to NMDA. These data suggest that NMDAR activation may only partially mediate Aβ-induced ROS production.

4.3. Alterations of transcription factors in nuclear fractions of mature cortical neurons exposed to $A\beta_{1-42}$

In order to determine the effect of $A\beta_{1-42}$ on transcription factors related to mitochondrial biogenesis, antioxidant defenses and NMDAR-mediated signaling pathways (as in Chapter 3), primary cultures of rat cortical cells were exposed to $A\beta_{1-42}$ for 5 minutes, 2 hours or 24 hours and nuclear protein levels of PGC1α, Nrf2 and CREB were analyzed (Figure 4.4).



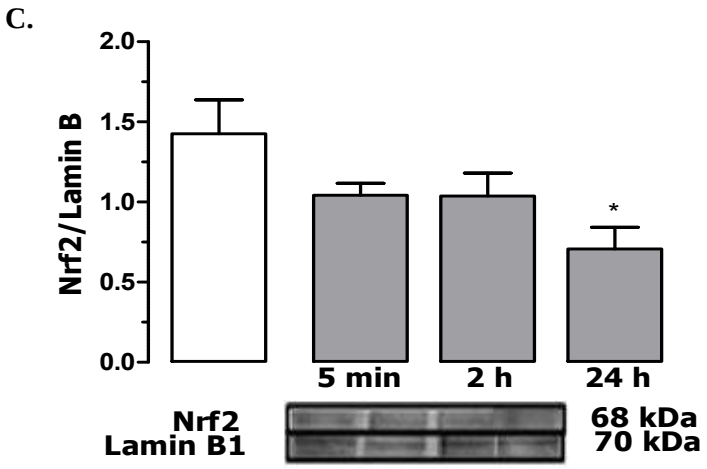


Figure 4.4. Time-dependent changes in transcription factors protein levels in nuclear fractions of rat primary cortical neurons (DIV 15) exposed to 0.5 μM Aβ1-42. Protein levels of (**A**) PGC-1alpha, (**B**) CREB and (**C**) Nrf2 were analyzed by Western Blotting. Cells were treated with Aβ1-42 in culture medium during 5 minutes, 2 or 24 hours. Data are the mean ±SEM of 5-9 independent experiments. Statistical analysis: *p<0.05 vs control, in the absence of Aβ (Tukey Multiple comparison test).

Although not significant, there was a clear tendency (p=0.0542 by Student's t test) for an increase in the PGC-1α levels in cortical cells exposed to $A\beta_{1-42}$ for 2 hours (Figure 4.4A). On the other hand, Nrf2 (Figure 4.4B) and CREB (Figure 4.4C) nuclear levels were significantly decreased in cortical cells exposed to $A\beta_{1-42}$ for 24 hours.

4.4. Involvement of NMDA receptors in $A\beta_{1-42}$-mediated changes in transcription factors

Since $A\beta_{1-42}$ slightly increased the levels of PGC-1α (Figure 4.4A) and significantly decreased both CREB (Figure 4.4B) and Nrf2 (Figure 4.4C) protein levels and taking into account the close link between Aβ and NMDAR activation, we further hypothesized that NMDAR-related signaling could be involved in the alteration of these transcription factors in the nucleus. Therefore, we evaluated the protein levels of the same transcription factors upon treatment with a GluN2B subunit antagonist, ifenprodil, and with the uncompetitive NMDAR antagonist, memantine (Figure 4.5).

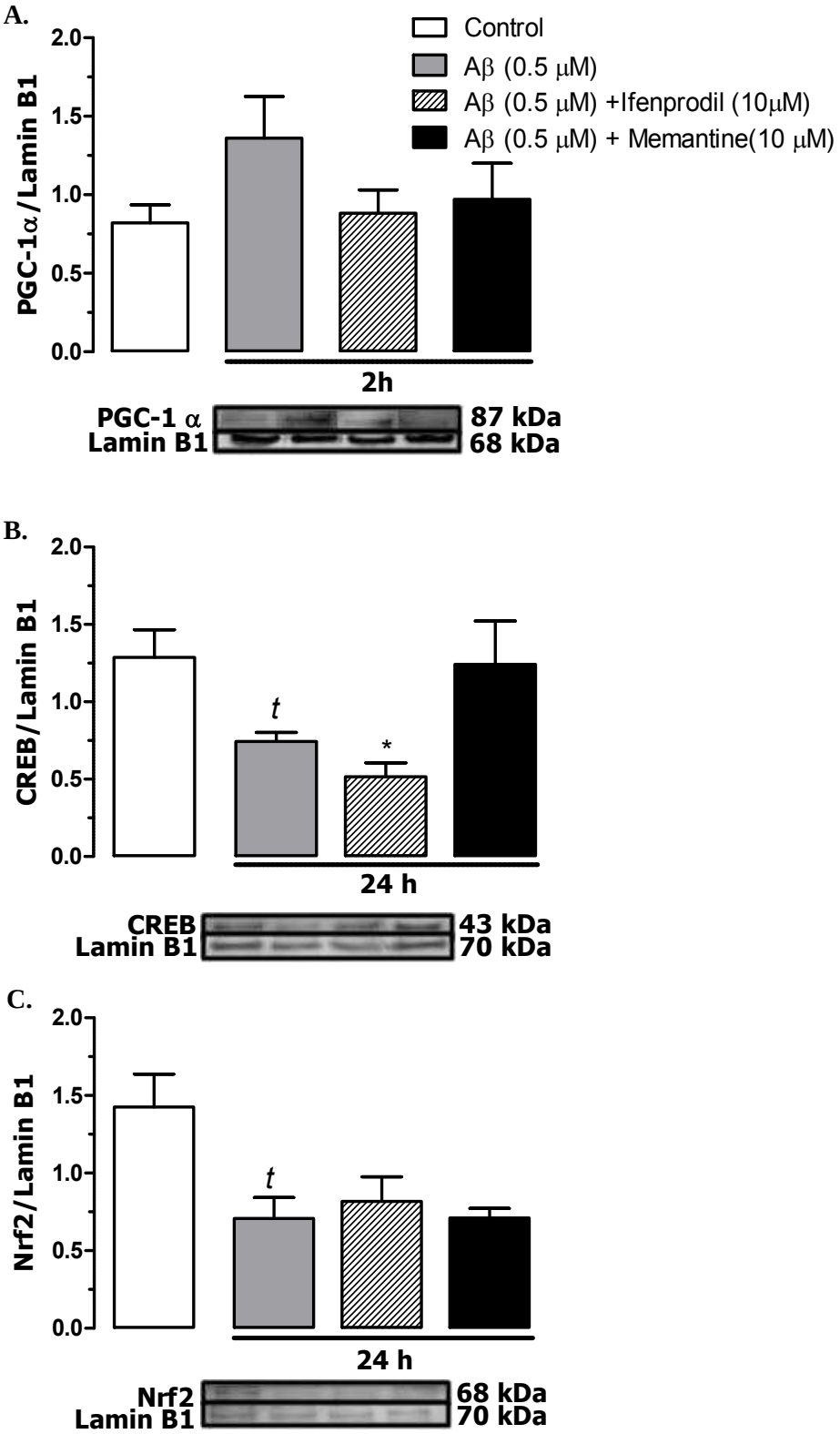
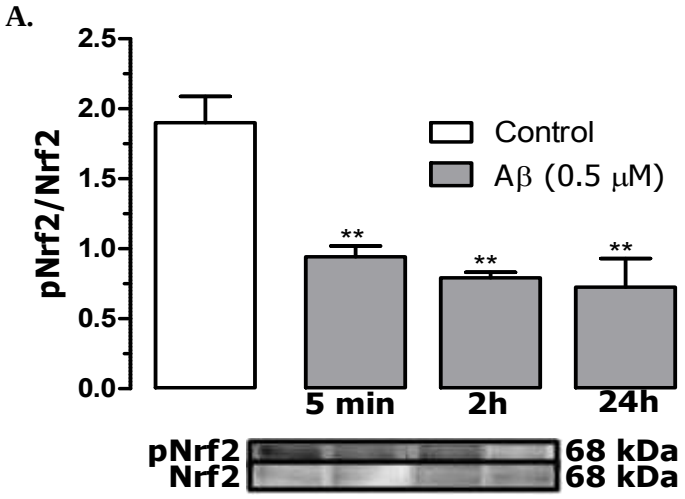


Figure 4.5. Effect of NMDAR antagonists on protein levels of transcription factors in nuclear fractions of rat primary cortical neurons (DIV 15) exposed to 0.5 μM $A\beta_{1-42}$ for 2 or 24 hours. $A\beta_{1-42}$ was added to the culture medium 5 minutes after exposure to NMDAR antagonists, and remained during $A\beta_{1-42}$ exposure. Protein levels of (**A**) PGC-1a, (**B**) CREB and (**C**) Nrf2 were analyzed by Western Blotting. Data are the mean±SEM of 3-9 independent experiments. Statistical analysis: $^{t}p<0.05$ vs control (Student's t test); *P<0.05vs control (Tukey Multiple Comparison test).

Our results demonstrate that $A\beta_{1-42}$-induced increase in PGC-1α protein levels was completely prevented by ifenprodil or memantine (Figure 4.5A), suggesting an involvement of GluN2B subunit in the observed effects; however, the decrease in CREB nuclear protein levels induced by $A\beta_{1-42}$ were prevented by memantine, but not by ifenprodil (Figure 4.5B), suggesting that other subunits than GluN2B may be involved in $A\beta_{1-42}$-induced CREB decrease. Moreover, the decrease in nuclear Nrf2 protein levels induced by $A\beta_{1-42}$ was not prevented by ifenprodil nor memantine (Figure 4.5.C); although other antagonists should still be tested, these data seems to exclude the involvement of NMDARs in this effect; other receptors or factors may be involved in Aβ-mediated changes in Nrf2 protein levels, such as the RAGE or α7AChRs.

As described in the previous Chapter 3, phosphorylation of Ser 40 of Nrf2 leads to the translocation of this transcription factor to the nucleus, leading to the transcription of genes encoding antioxidant defense proteins, whereas the phosphorylation of Ser 133 of CREB activates it, allowing the transcription of genes involved in several cell processes, namely anti-apoptotic or neurotrophic factors (Figure 4.6).



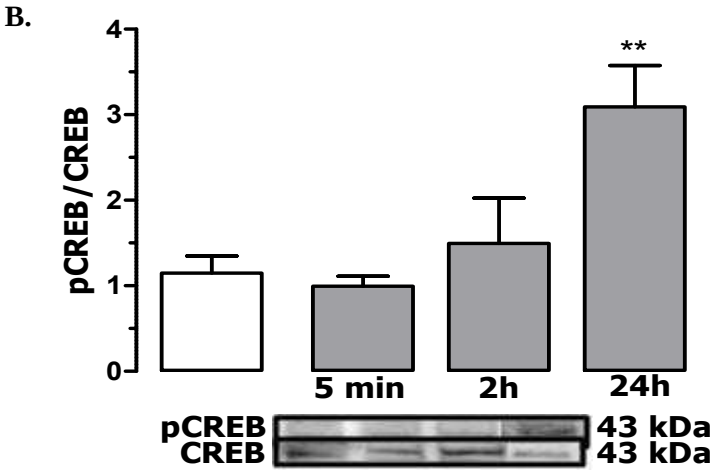
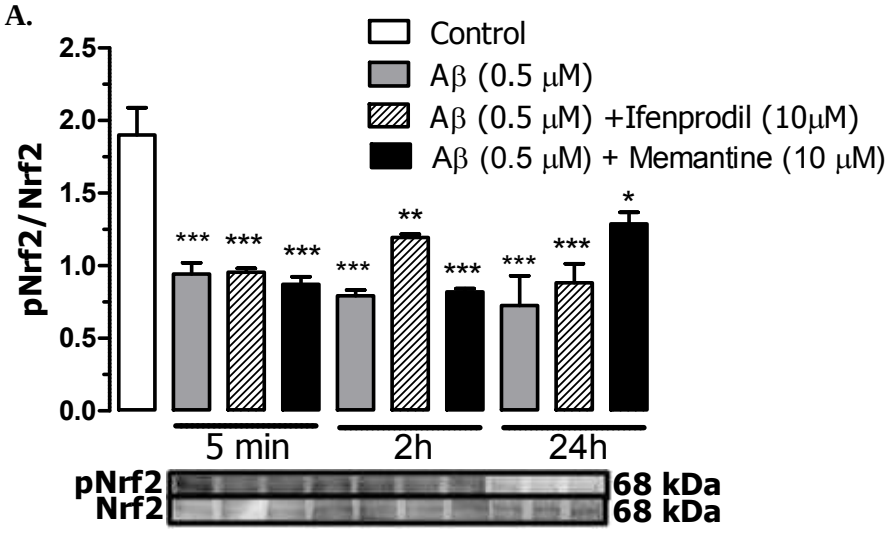


Figure 4.6. Activation of transcription factors in nuclear fractions of rat cortical neurons (DIV 15) exposed to 0.5 µM $A\beta_{1-42}$ for 5 minutes, 2 or 24 hours. (**A**) pNrf/Nrf2 and (**B**) pCREB/CREB were determined by western blotting. Data are the mean±SEM of 2-6 independent experiments. Statistical analysis: **p<0.01 vs control (Tukey Multiple Comparison test).

Interestingly, nuclear pNrf2 levels (Figure 4.6A) were significantly decreased after $A\beta_{1-42}$ exposure for 5 min and no additional effect was observed with advanced time exposure for 2 and 24 hours (p<0.01), indicating that $A\beta_{1-42}$ impairs Nrf2 phosphorylation. In contrast, and surprisingly, pCREB/CREB nuclear levels (Figure 4.6B) were significantly increased in cells exposed to $A\beta_{1-42}$ for 24 hours (p<0.01), suggesting a late event when compared to nuclear Nrf2 or PGC-1alpha.

To evaluate the involvement of the NMDARs activation induced by $A\beta_{1-42}$ in Nrf2 and CREB phosphorylation, the effects of ifenprodil or memantine were also studied (Figure 4.7).



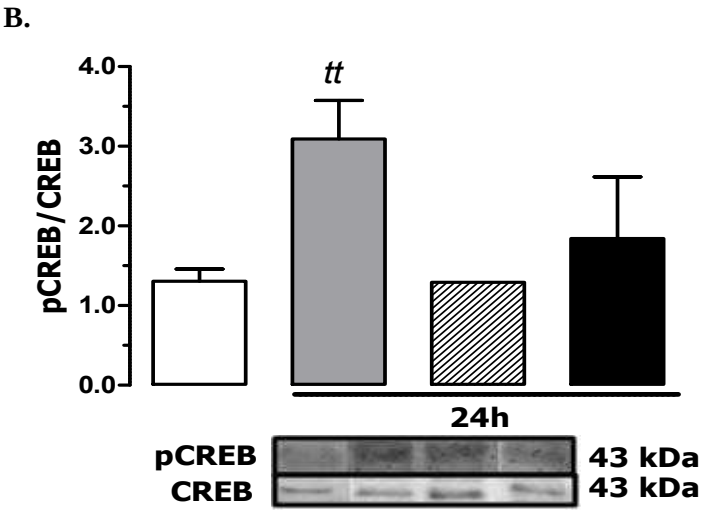


Figure 4.7. NMDAR antagonists involvement in transcription factor post-translational modifications in nuclear fractions of rat cortical neurons (DIV15) exposed to 0.5 μM $A\beta_{1-42}$ for 5 minutes, 2 or 24 hours. $A\beta_{1-42}$ (was added to the culture medium 5 minutes after NMDARs antagonists treatment, which remained during $A\beta_{1-42}$ exposure. Phosphorylation levels of (**A**) Nrf2 and (**B**) CREB were analyzed by Western Blotting. Data are the mean±SEM of 1-4 independent experiments. Statistical analysis: *p<0.05; **p<0.01; ***p<0.001 vs control (Tukey Multiple comparison test); ^{tt}p<0.01 vs respective control (Student's t test)

Results showed that the decrease in the pNrf2 levels caused by $A\beta_{1-42}$ exposure were not rescued by ifenprodil or memantine, suggesting that Aβ-mediated effects on Nrf2 phosphorylation, as for Nrf2 protein levels, do not depend on NMDAR activation; however for longer periods of exposure (2 and 24 h) there is a tendency for a recovery in pNrf2 levels.

In contrast, the effects in pCREB levels (Figure 4.7B) seem to be prevented by ifenprodil (very preliminary data) and partially prevented by memantine, suggesting a possible relation between the GluN2B subunit and Aβ-mediated increase in pCREB levels.

4.5. $A\beta_{1-42}$-induced changes in Nrf2 target proteins –role of NMDA receptors

$A\beta_{1-42}$ largely decreased both Nrf2 and pNrf2/Nrf2 levels, suggesting that antioxidant defense proteins, which are targets of Nrf2, might also be altered. Therefore, we evaluated the protein levels of SOD1 and GCLc, which are important for the maintenance of the celllular redox status (Figure 4.8).

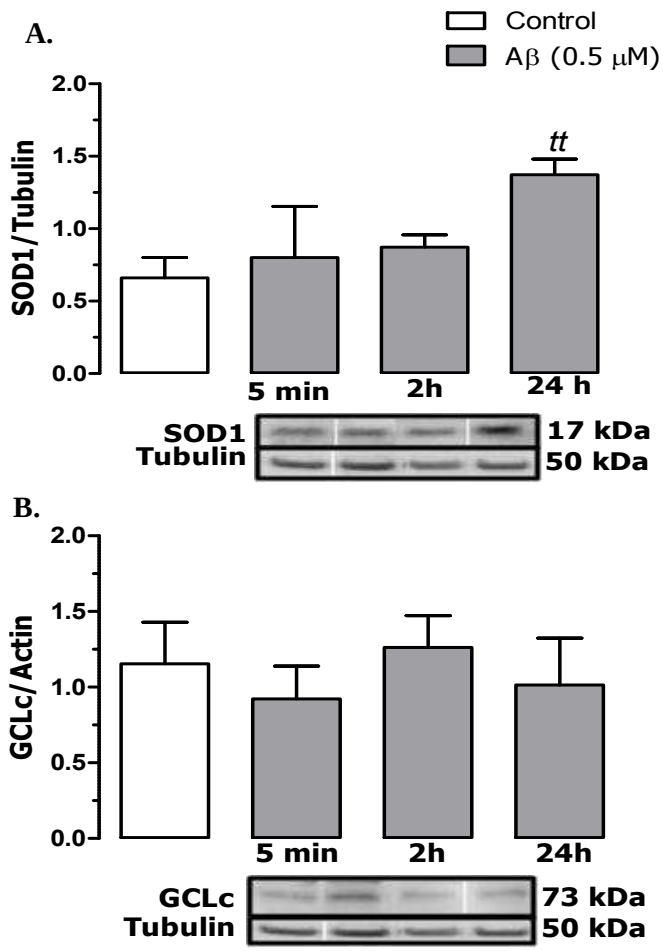


Figure 4.8. Nrf2 protein targets. Protein levels of (**A**) SOD1 and (**B**) GCLc in rat cortical neurons exposed to $A\beta_{1-42}$ were analyzed by Western Blot. Data represent the mean ±SEM of 3 independent experiments. Statistical analysis: $^{t}p<0.05$ vs control. (Student's t test).

In contrast to what would be expected (based on decreased nuclear levels of total and pNrf2 observed in Figure 4.4C and 4.6A), our results demonstrate that SOD1 protein levels are increased upon 24 hours $A\beta_{1-42}$ exposure ($p<0.05$) (Figure 4.8A); suggesting that SOD1 might be regulated by other transcription factor(s). Accordingly, GCLc protein levels (Figure 4.8B) are not influenced by treatment with $A\beta_{1-42}$ for 5 min, 2 and 24 hours.

The involvement of NMDARs activation on increased SOD1 levels was further analyzed by using ifenprodil and memantine (Figure 4.9).

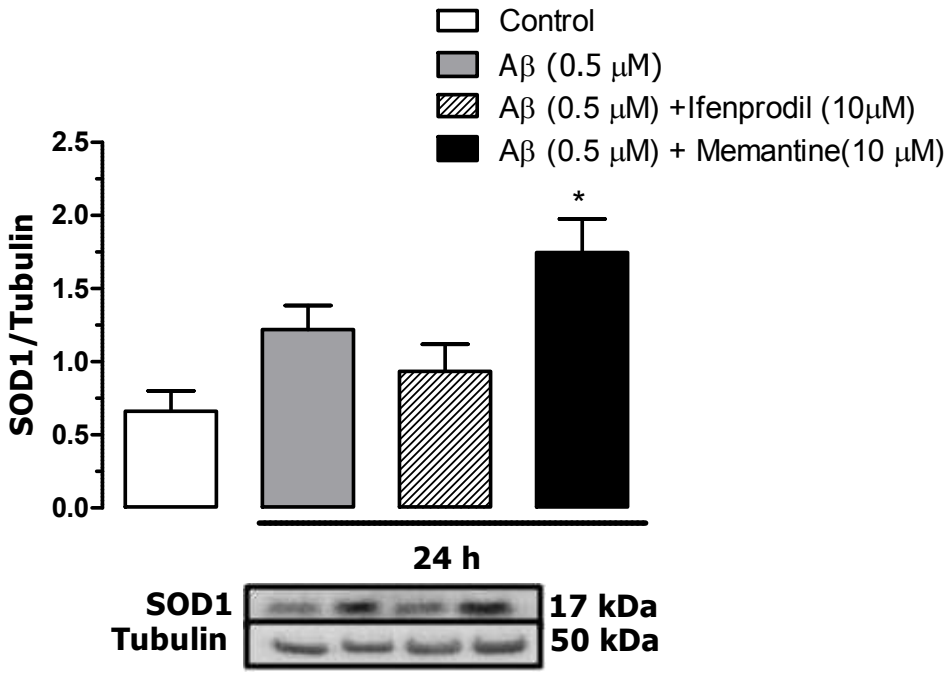


Figure 4.9. SOD1 protein levels in the presence of NMDAR antagonists. $A\beta_{1-42}$ (0.5 μM) was added to the culture medium 5 minutes after NMDARs antagonists treatment, and remained during $A\beta_{1-42}$ exposure. SOD1 protein levels were analyzed by Western Blotting. Data are the mean ±SEM of 3 independent experiments. Statistical analysis: *$p<0.05$ vs control. (Tukey Multiple Comparison test).

Our results showed that SOD1 protein levels were not prevented to values equivalent to the control by none of the antagonists (Figure 4.9), indicating that SOD1 levels are not related to NMDAR activation. Indeed, memantine treatment for 24h further enhanced SOD1 levels in Aβ-treated neurons.

4.6. p65 protein levels in nuclear fractions of cortical cells exposed to $A\beta_{1-42}$

RAGE has been linked to intracellular Aβ levels and its activation leads to stimulated NF-kB signaling cascades. NF-kB acts as a transcription factor and is also implicated in SOD1 regulation; therefore we evaluated the nuclear levels of p65 in rat cortical cells exposed to $A\beta_{1-42}$ (Figure 4.10.)

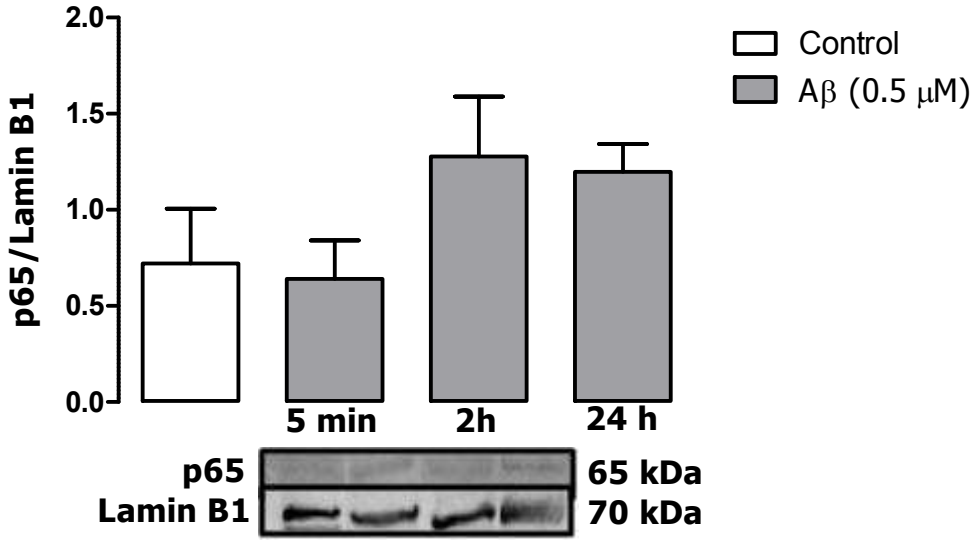


Figure 4.10. p65 nuclear protein levels in nuclear fractions of rat cortical cells exposed to 0.5 μM $A\beta_{1-42}$ for 5 minutes, 2 or 24 hours. p65 protein levels analyzed by Western Blotting. Data represents the mean ±SEM of 3-4 independent experiments.

Nuclear levels of p65 tend to increase in cortical cells exposed to $A\beta_{1-42}$ for 2 and 24 hours, suggesting that NF-kB is activated by $A\beta_{1-42}$ exposure, possibly through the interaction with RAGE. Furthermore, the increase in p65 nuclear levels may explain the increase in SOD1 levels at 24 hours, despite the decrease in Nrf2 and pNrf2 levels.

4.7. $A\beta_{1-42}$ cellular localization under oxidative stress

Since we hypothesized that Aβ could directly or indirectly interact with transcription factorswe performed immunocytochemistry analysis in rat primary cortical cells aiming to determine if $A\beta_{1-42}$ could be found intracellularly or even in the nucleus under oxidative stress induction, mimicking the oxidative environment present in AD (Figure 4.11).

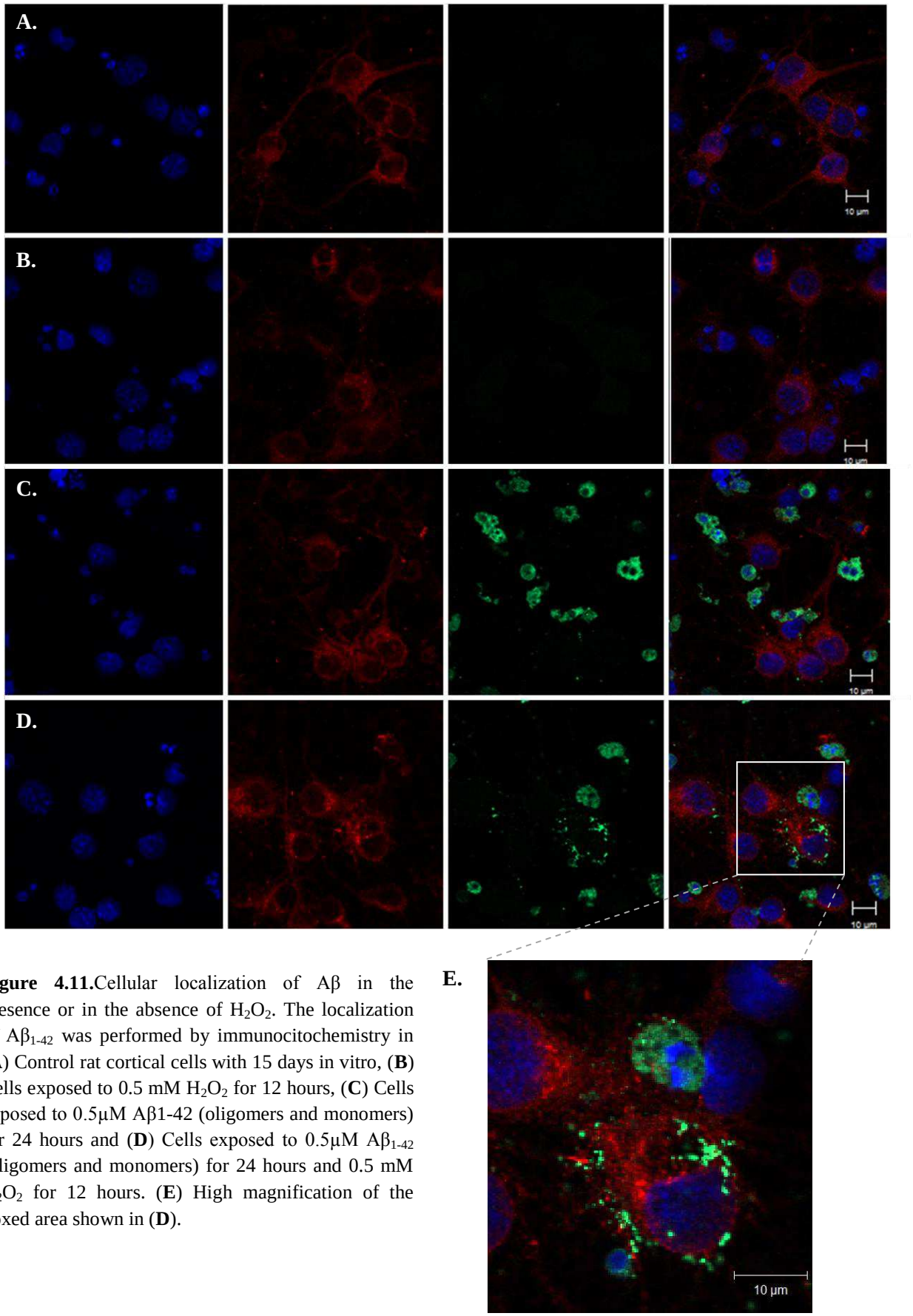


Figure 4.11. Cellular localization of Aβ in the presence or in the absence of H_2O_2. The localization of $A\beta_{1-42}$ was performed by immunocitochemistry in (**A**) Control rat cortical cells with 15 days in vitro, (**B**) Cells exposed to 0.5 mM H_2O_2 for 12 hours, (**C**) Cells exposed to 0.5µM Aβ1-42 (oligomers and monomers) for 24 hours and (**D**) Cells exposed to 0.5µM $A\beta_{1-42}$ (oligomers and monomers) for 24 hours and 0.5 mM H_2O_2 for 12 hours. (**E**) High magnification of the boxed area shown in (**D**).

Sinthetic $A\beta_{1-42}$ preferentially colocalizes with apoptotic cells (Figure 4.11 C and D) but we were also able to visualize $A\beta_{1-42}$ in the nucleus of cells treated with $A\beta_{1-42}$ and H_2O_2 (Figure 4.11E) but not in cells only exposed to $A\beta_{1-42}$ (Figure 4.11 C), suggesting that Aβ is translocated to the nucleus under high oxidative stress.

CHAPTER 5 – DISCUSSION

Increasing evidence implicates reduced mitochondrial function and oxidative stress in AD pathology. Also, transcription deregulation has been recently associated with this neurodegenerative disease. Indeed, a direct relation of Aβ peptide in gene transcription regulation has been proposed. Also, growing evidence link Aβ internalization to the activation of NMDAR and other receptors and, consequently, to the activation of signaling cascades with different outcomes. Thus, in this work we hypothesized that Aβ could regulate the activity of transcription factors related to mitochondrial biogenesis, antioxidant defenses and NMDAR activation. Intraneuronal Aβ immunoreactivity is one of the earliest neuropathological manifestations in 3xTg-AD mice occurring between 3 and 4 months of age in the neocortex, coinciding with early cognitive impairment (Oddo et al., 2003; Billings et al., 2007); however, by 12 months of age, extracellular Aβ deposits are evident in other cortex regions and in the hippocampus (Oddo et al., 2003). Evidence shows that mitochondrial dysfunction starts early in this model. By 3 months of age, 3xTg-AD mice show decreased mitochondrial respiration as well as decreased protein levels and activity of pyruvate dehydrogenase (PDH) in the hippocampus (Yao et al., 2009). Accordingly, Aβ may interact with several components of mitochondria, leading to mitochondrial dysfunction and also to the deregulation of the expression of several mitochondrial proteins (Manczak et al., 2006; Wang et al., 2008).

Oxidative stress is also a hallmark of the disease. Increased protein nitration and oxidation, DNA oxidation and lipid peroxidation have been described in AD brain and also in MCI patients (Butterfield et al., 2007). Also, 3xTg-AD mice show increased levels of H_2O_2, and age-related increase in lipid peroxidation, which appears to be more evident in animals at 12 months of age (Yao et al., 2009); increased activity of SOD, glutathione peroxidase (GPx) and glutathione redutase (GRd) and decreased GSH/GSSG ratio and vitamin E levels were also observed in 3 to 5 month-old 3xTg-AD female mice cortex (Resende et al., 2008). Concordantly, we demonstrate here, increased production of ROS in rat cortical cells exposed to $A\beta_{1-42}$.

In the present work we observed increased nuclear levels of PGC-1α both in 3 month-old and 15 month-old 3xTg-AD mice and in rat cortical cells exposed to 0.5μM $A\beta_{1-42}$ for 2 hours. Accordingly, an analysis of mitochondrial proteome of cerebral cortices of 6 month-old male 3xTg-AD and non-transgenic mice revealed that ATP synthase subunit beta (complex V), which is a target of PGC-1α (Puigserver et al., 1999) was upregulated in 3xTg-AD mice (Chou et al., 2011). In fact, there is evidence that PGC-1α promoter activity is enhanced under oxidative stress (St-Pierre et al., 2006). Thus, there is a possibility that the observed increase in nuclear PGC-1α might be a consequence of an increased expression of PGC1α, that is then transported to the nucleus. Alternatively, the increase in nuclear PGC-1α may constitute a cellular compensatory mechanism in order to counteract mitochondrial dysfunction. Interestingly, Aβ-mediated effects in PGC-1α nuclear levels were completely prevented by NMDAR antagonists, ifenprodil and memantine, implicating the activation of GluN2B-composed NMDAR in Aβ-mediated effects. In fact, mice cortical neurons subjected to oxygen deprivation (OGD) express more PGC-1α than control mice, and blockade of NMDAR by MK801 reduced PGC-1α mRNA expression in OGD neurons; accordingly, NMDA directly induced the expression of PGC-1α in neuronal cells (Luo et al., 2009). Thus, the evaluation of PGC-1α mRNA levels under Aβ exposure as well as the study of post-translation modifications that would favor PGC-1α translocation to the nucleus could give important information about the mechanism prevailing in Aβ-mediated effects in the nuclear levels of PGC-1α. In contrast, PGC-1α was decreased in both AD hippocampal tissue and APP_{swe} M17, correlating with decreased mitochondrial DNA/nuclear DNA ratio, ATP content and cytochrome c oxidase activity (Sheng et al., 2012). The differences observed suggests PGC-1α expression varies along the different stages of the disease and may be influenced by variations in Aβ amount around and inside the cells.

Nuclear Nrf2 and pNrf2 levels vary with gender and age. In fact, sex variance in 3xTg-AD mice has been previously described, since female 3xTg-AD mice exhibit enhanced cognitive deficits compared with age-matched males (Clinton et al., 2007). Also, there is evidence that mitochondrial dysfunction is exacerbated by reproductive senescence in 3xTg-AD female mice

(Yao et al., 2009). Our results evidenced that in 3 month-old 3xTg-AD male mice a significant increase in nuclear Nrf2 levels was observed, while pNrf2 levels were significantly decreased when compared to WT mice, suggesting that, due to oxidative stress, Nrf2 is translocated to the nucleus, and rapidly dephosphorylated and thus inactivated, which is in accordance with the decreased protein levels of one of its target, SOD1. In 15-month-old male mice nuclear levels of Nrf2 were shown to be significantly decreased which might exacerbate oxidative stress by reducing the transcription of antioxidant defense protein such as peroxiredoxin and glutathione peroxidase; nevertheless, GCLc and SOD1 remained unaltered in 3xTg-AD comparatively to WT mice, but a decrease in SOD1 could be detected in old WT mice; these data suggest that other regulators of these proteins may be involved in old AD mice brain. Indeed, SOD1 is regulated by several proteins, including CCAAT/enhancer binding proteins (C/EBP), activating protein 1 (AP1) and NFκB (Milani et al., 2011). The later is a transcription factor that is activated upon RAGE activation, a receptor previously reported to be related with Aβ internalization. In 3 month-old female 3xTg-AD mice, decreased nuclear levels of Nrf2 suggests that its translocation to the nucleus may already be compromised at this age in 3xTg-AD female mice (similarly to old 3xTg-AD males); moreover, this appears to be correlated with a slight reduction in SOD1 protein levels.

In cultured cortical cells a significant decrease in Nrf2 nuclear levels was observed after $A\beta_{1-42}$ exposure for 24 hours, along with a decrease in pNrf2 in cells exposed to $A\beta_{1-42}$ for 5 minutes, 2 or 24 hours, suggests that exogenous applied $A\beta_{1-42}$ initially impairs Nrf2 phosphorylation at Ser 40, the residue responsible for Nrf2 transport to the nucleus; for longer periods of $A\beta_{1-42}$ exposure phosphorylation-dependent transport of Nrf2 to the nucleus is also affected. In contrast, SOD1 protein levels increase upon 24 hours of $A\beta_{1-42}$ exposure; this result is not in accordance with Nrf2 levels, but might be explained by the observed increase in p65 nuclear levels in cells exposed to $A\beta_{1-42}$ for 2 and 24 hours. We did not observed any alteration in GCLc protein levels in cortical cells exposed to $A\beta_{1-42}$; however, as for SOD1, GCLc expression is regulated by several transcription factors, namely AP1, AP2, NF-kB and CREB. GCLc activity

is also regulated by a negative feedback by GSH (Franklin et al., 2009) which levels are balanced by Nrf2 expressed by glial cells (Shih et al., 2003). Studies that aim at understand the mechanisms underlying the pathogenesis of AD rely on the evaluation of alterations in several types of cells and tissues, such as lymphocytes (Bartolome et al., 2010; Saresella et al., 2011), lymphoblasts (Bartolome et al., 2007; Munoz et al., 2008), CSF (cerebrospinal fluid) (Halim et al., 2011), (Leinonen et al., 2011); postmortem samples (Ansari and Scheff, 2010), blood (Baldeiras et al., 2010) and AD cybrids (Cardoso et al., 2004; Onyango et al., 2005)

Interestingly, we recently showed a significant decrease in Nrf2 and SOD1 protein levels in MCI patient lymphocytes and an increased production of ROS at this stage while no changes in the other transcription factors were observed (Table 5.1.), suggesting that Nrf2 signaling impairment may occur early in the pathogenesis of AD. Results obtained in cortical cells also evidenced that $A\beta_{1-42}$ caused an immediate increased in ROS production, which seems to be concordant with decreased nuclear levels of Nrf2.

Table 5.1 Transcription factor and target protein levels in MCI, AD patients and non-demented controls. Data show the variation of protein levels relatively to controls.Symbols: ↓ significant decrease ($p<0,05$ by Student's t test); ↓↓↓ significant decrease ($p<0,001$ by Student's t test); ↗ tendency to increase; ↙ tendency to decrease; - no variation.

Transcription factor	**Control**	**MCI**	**Mild**	**Moderate/Severe**
Nrf2	-	↓	-	-
PGC-1α	-	-	-	-
CREB	-	↗	↗	↗
CBP	-	-	-	-
Target proteins				
SOD1	-	↓↓↓	↗	↙
GCLc	-	-	-	-

NMDAR antagonists, namely NVP-AAM077, ifenprodil, MK801 and memantine only partially prevented $A\beta_{1-42}$-mediated ROS production. These data suggest that elevated ROS production may not fully depend on NMDAR activation, and may also be a consequence of the activation of other glutamatergic receptors, namely AMPARs(Carriedo et al., 2000) and mGluR5 (Li et al., 2011). Furthermore, ABAD was shown to be up-regulated in AD hippocampus (He et al., 2005) and an ABAD inhibitor prevented Aβ-induced apoptosis and ROS production in neurons

(Lustbader et al., 2004), suggesting Aβ-mediated ROS production may be originated by the interaction of intracellular Aβ with mitochondrial components. In addition, the effect of $A\beta_{1-42}$ on Nrf2, pNrf2 and SOD1 in cortical cells were not prevented by the NMDAR antagonists, excluding a significant contribution of NMDARs in these results. Thus, activation of alternative signaling pathways and/or other receptors, such as RAGE or α7AChRs may be associated with impaired Nrf2 phosphorylation under these conditions. On the other hand, intracellular Aβ could directly impair Nrf2 phosphorylation through the inhibition of kinases. In fact, Aβ directly interacts and inhibits PKC (Lee et al., 2004), a kinase protein responsible for the phosphorylation of Nrf2 at Ser 40 (Bloom and Jaiswal, 2003). Concordantly with the observed decrease in nuclear levels of Nrf2, are findings showing that after binding to ARE elements, Nrf2 undergoes Ser 40 dephosphorylation by an unknown phosphatase, being then exported from the nucleus by Fyn kinase-mediated phosphorylation of Tyr 568 (Jain and Jaiswal, 2006). Fyn kinase was also shown to be altered in AD and this kinase was further responsible for tau phosphorylation (Yang et al., 2011); thus, it is possible that $A\beta_{1-42}$ may interfere with Fyn activity, enhancing Nrf2 export from the nucleus. Analysis of PKC activity and other kinases that may phosphorylate Nrf2, such as PERK, under $A\beta_{1-42}$ exposure, and a screening of phosphatases and kinases that interact with Nrf2 and which activity is altered by Aβ would be helpful in elucidating the signaling pathways involved in the impairment of Nrf2 phosphorylation.

CREB nuclear protein levels were shown to be decreased in 15 month-old 3xTg-AD mice, whereas pCREB levels were not significantly altered in old 3xTg-AD, comparatively to WT mice. Decreased nuclear levels of CREB (despite unchanged phosphorylation of this pool of proteins) may suggest reduced NMDARs signaling due to internalization of NMDARs. Striatal-enriched phosphatase (STEP) was previously shown to be involved in NMDARs endocytosis, being elevated in the pre-frontal cortex of human AD patients and also in Tg2576 transgenic mice. Accordingly, a genetic-manipulated decreased activity of STEP reversed cognitive and cellular deficits in 3xTg-AD mice (Zhang et al., 2010).Moreover, CREB is involved in neuronal

survival since it triggers signaling pathways that culminate in the transcription of several neuroprotective factors, namely IGF-1, BDNF and estrogens, and regulates the expression of anti-apoptotic factors, such as Bcl2 (Walton and Dragunow, 2000). Thus, the reduction in CREB protein levels in old 3xTg-AD mice is in accordance with previously observed neuronal death in 12 month-old 3xTg-AD (Janelsins et al., 2008). Interestingly, whereas total nuclear CREB was decreased in cortical cells exposed to $A\beta_{1-42}$ for 24 hours, pCREB levels were enhanced in these nuclear fractions. Concordantly, it has been previously reported that long-term treatment with a low concentration of H2O2 led to an increase in pCREB accompanied by a decrease in CREB protein abundance in cardiomyocytes, suggesting, once again, a role for oxidative stress in the regulation of transcription factors (Ozgen et al., 2009). Prevention of $A\beta_{1-42}$-mediated effects on CREB by the NMDARs antagonist memantine but not by ifenprodil implicate the GluN2A subunits in the observed effects. The involvement of NMDARs in these results led to suggest that $A\beta_{1-42}$-mediated desensitization of the NMDARs in the first minutes and posterior activation of NMDARs (by increased glutamate in the synaptic cleft and/or remaining $A\beta_{1-42}$ oligomeric/momeric forms in the medium) could explain the late increase in CREB phosphorylation. Indeed, Aβ was shown to inhibit neuronal glutamate uptake in the synapse, further leading to NMDAR desensitization and consequently to synapse depression (Li et al., 2009). Analysis of CREB and pCREB levels in GluN2A knockdown cells would give us more precise information about the role of selective NMDAR subunits

In summary, despite the fact that Aβ can be found in the nucleus, our results strongly suggest that the observed alteration in PGC-1α and CREB both in mice and rat cortical cells exposed to $A\beta_{1-42}$ is probably a consequence of Aβ-mediated oxidative stress along with NMDAR signaling cascades activation by direct interaction of the receptor with Aβ. The fact that CREB alteration in 3xTg-AD mice was only noticed in later phases, when Aβ is mainly localized extracellularly, corroborates the hypothesis of Aβ-mediated extrasynaptic NMDAR activation, altering the signaling cascade and favoring the consequent decrease in CREB activation (Hardingham and Bading, 2010).

Regarding Nrf2 and pNrf2 protein levels, the results obtained in old 3xTg-AD mice are consistent with the results obtained in rat cortical cells exposed $A\beta_{1-42}$, specially for a longer period (24h), when Aβ is localized both intracellularly and extracellularly, as evaluated by immunocytochemistry in the present study, suggesting that the effects in Nrf2, pNrf2 and in their protein target are due not only to the activation of membrane receptors but also by the direct interaction of Aβ with intracellular kinases and possibly phosphatases implicated in the translocation and activity of Nrf2 in the cell. Understanding the mechanisms responsible for the alterations in the transcription factors that are involved in the extremely important mechanisms of defense against Aβ-mediated insults will be very important for the development of strategies aiming to restore cell homeostasis and consequently avoid or revert the known deficits inherent to AD

CONCLUSION

This work is a major contribution to the characterization of changes in transcription factors occurring in 3xTg-AD mice, a model that presents several hallmarks of AD, including intracellular and extracellular deposition of Aβ, hyperphosphorylation of tau, mitochondrial dysfunction and oxidative stress. PGC-1α, Nrf2 and CREB are transcription factors related to important cellular processes shown to be altered in AD; because we were able to observe changes in pre-symptomatic 3xTg-AD animals at 3 months of age, knowledge about the regulation of these transcription factors will be helpful in understanding AD pathogenesis. Although the mechanisms by which $A\beta_{1-42}$ mediates the changes in each transcription factor is still not clear, this work provide useful information regarding the involvement of particular receptors and therefore specific signaling pathways in the Aβ-mediated alteration in transcription factor nuclear levels and post translational modifications. The promotion of mitochondrial biogenesis as well as an improvement in antioxidant defense strength would be extremely beneficial to the cells, and could in fact lead to the reversion of AD-related symptoms.

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