NMDA receptors-associated events and oxidative stress in models of Alzheimer's disease

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Aos meus pais

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Abbreviations

3xTg	Triple transgenic
α-CTF	α carboxyl terminal fragment
Aβ	Amyloid beta
AD	Alzheimer's disease
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
Apl-1	Amyloid precursor-like protein 1
ApoE	Apolipoprotein E
ApoER	Apolipoprotein E receptor
APP	Amyloid peptide precursor
APPL	Amyloid precursor protein-like protein
Arp	Actin-related protein
ASK1	Apoptosis signal regulating kinase 1
ATP	Adenosine triphosphate
BACE	β-site APP cleaving enzyme
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma 2
Ca^{2+}	Calcium
$Ca^{2+}{}_i$	Intracelular calcium
CBP	CREB-binding protein
CHOP	C/EBP homologous protein
CK2	Casein kinase 2
CREB	*cAMP response element-binding*
CSF	Cerebrospinal fluid
Dab1	Disabled-1 protein
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
DRG	Donepezil, Rivastigmine, Galantamine
ER	Endoplasmic reticulum
FDG	Fluorodeoxyglucose ^{18}F
GADD153	growth arrest and DNA damage-inducible gene 153
GCLc	Glutamate-cysteine ligase catalytic subunit
GFAP	Glyceraldehyde 3-phosphate dehydrogenase
GRP78	Glucose regulated protein 78
GSH	Glutathione
H_2O_2	Hydrogen peroxide
IP_3R	Inositol 1,4,5-trisphosphate receptors
JNK	c-Jun N-terminal kinase
LTD	Long term depression
LTP	Long term potentiation
MAP	Microtubule associated protein
MAPT	Microtubule associated protein tau
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	*Nicotinamide adenine dinucleotide*
NFTs	Neurofibrillary tangles

NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
Nrf2	Nuclear factor erythroid derived 2-related
N-WASP	Wikott-Aldrich Syndrome protein
PBMCs	Peripheric blood mononuclear cells
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-α
PET	Positron emission tomography
PERK	*Protein* kinase RNA-like endoplasmic reticulum
PIB	^{11}C-Pittsburgh Compound B
PIP_2	Phosphatidylinositol bisphosphate
PS	Presenilin
PSD	Post synaptic density
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SAP102	Synaptic-associated protein 102
sAPPα	Soluble ectodomain of APP
SOD	Superoxide dismutase
STEP	Tyrosine phosphatase striatal enriched protein
TGN	Trans-Golgi-network
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPR	Unfolded protein response
VLDLR	Very-low-density-lipoprotein receptor
WT	Wild Type
XBP-1	X box binding protein

Resumo

A doença de Alzheimer (DA) é a doença neurodegenerativa mais comum a nível mundial, apresentando-se associada a uma severa perda de memória. Alterações na ativação dos recetores do tipo *N*-metil-D-aspartato (NMDA) têm sido implicadas na disfunção sináptica que ocorre na DA, em condições em que a acumulação do peptídeo beta amilóide (Aβ) assume uma maior importância. No entanto, a alteração na composição e ativação dos receptores NMDA que ocorre na DA ainda é pouco conhecida. Neste trabalho, propusemo-nos avaliar a importância dos recetores NMDA na progressão da doença, usando culturas neuronais tratadas com $A\beta_{1-42}$ e amostras de hipocampo e córtex de murganhos 3xTg-AD, duas regiões cerebrais envolvidas nos processos de memória. Propusemo-nos também investigar possíveis biomarcadores relacionados com o stresse oxidativo associados à progressão da doença, usando células mononucleares de sangue periférico (CMSPs) derivadas de doentes de Alzheimer. Para tal, o nosso trabalho dividiu-se em três partes, descritas seguidamente. Numa primeira parte avaliámos o papel dos receptores NMDA na disfunção e morte neuronal induzidas por Aβ e as alterações da polimerização dos microtúbulos em culturas maduras de hipocampo (Capítulo 3). Analisámos de seguida a dependência do sexo e da idade nos níveis de expressão proteica e ativação das subunidades GluN1, GluN2A e GluN2B dos recetores NMDA em homogeneizados de córtex e hipocampo de murganhos transgénicos 3xTg-AD *versus* murganhos "wild type" (WT) (Capítulo 4). Para além disso, tendo em conta o envolvimento do stresse oxidativo e da desregulação da homeostasia do cálcio intracelular (Ca^{2+}_i) em mecanismos que precedem a morte neuronal associada à DA, na terceira parte do nosso trabalho avaliámos marcadores de lesão celular relacionados com o stresse oxidativo usando CMSPs isoladas de doentes com DA ligeira, moderada e severa e indivíduos com défice cognitivo ligeiro (DCL) *versus* indivíduos controlo com idades comparáveis e que não apresentavam disfunção cognitiva (Capítulo 5).

Assim, demonstrámos que a exposição de células de hipocampo a uma preparação de $A\beta_{1-42}$ enriquecida em oligómeros causa a diminuição dos níveis de beta-III tubulina total e polimerizada, e dos níveis de alfa-tubulina polimerizada, sugerindo uma desorganização dos microtúbulos. Para além disso, os efeitos do Aβ na polimerização da tubulina neuronal mostraram estar correlacionados com uma redução do comprimento das neurites e com a fragmentação do ADN, demonstrando a ocorrência

de morte celular por apoptose causada por Aβ. Curiosamente, a proteção promovida pelo MK-801 e pela memantina sugere o envolvimento de receptores NMDA extrasináticos, em particular os recetores contendo a subunidade GluN2B, na toxicidade induzida por Aβ.

A análise dos níveis totais das subunidades dos recetores NMDA no córtex e no hipocampo de murganhos 3xTg-AD machos e fêmeas demonstrou que os níveis das subunidades GluN1, GluN2A e GluN2B dos recetores do NMDA não alteram com a idade ou com o sexo. Um resultado similar foi obtido para a proteína PSD-95 (do inglês "post-synaptic density-95"), responsável pela ancoragem dos recetores NMDA à membrana e ao citosqueleto. Da mesma forma, no córtex, não observámos alterações dos níveis de fosforilação das subunidades GluN2A ou GluN2B nos resíduos Ser1232 e Tyr1472, respetivamente. Além disso, no hipocampo dos murganhos 3xTg-AD machos e fêmeas, não encontrámos alterações na fosforilação da Ser1232 da subunidade GluN2A. No entanto, a fosforilação no resíduo Tyr1472 da subunidade GluN2B diminui no hipocampo de3xTg-AD machos jovens, com 3 meses de idade, e aumenta no hipocampo de fêmeas 3xTg-AD com 15 meses de idade, revelando diferenças dependentes do sexo na ativação da subunidade GluN2B na DA. As alterações precoces na fosforilação da subunidade GluN2B no hipocampo correlacionam-se com a atividade da Src. Curiosamente, o decréscimo da atividade da Src no hipocampo e no córtex aos 3 meses dos 3xTg-AD machos também se correlaciona com um decréscimo dos níveis totais e fosforilados da proteína Dab1, um alvo da Src implicado na estabilização do citosqueleto. Além disso, observámos uma diminuição dos níveis totais da cortactina, outro alvo da Src, também envolvida na estabilização do citosqueleto. Assim, os resultados obtidos no hipocampo sugerem uma destabilização do citosqueleto (que deverá ser testada em estudos futuros) em jovens machos 3xTg-AD que mostram uma marcação para Aβ intracelular, acompanhado de uma diminuição da ativação dos receptores NMDA contendo a subunidade GluN2B.

Na terceira parte desta tese observámos uma desregulação da homeostasia do Ca^{2+}_{i} nas CMSPs de doentes com DCL e DA ligeira. Estas alterações ocorrem de forma concomitante com um aumento dos níveis de espécies reactivas de oxigénio em CMSPs de doentes com DCL mas não estão relacionadas com alterações dos níveis de proteínas pró- ou anti-apoptóticas. De forma concordante, demonstrámos uma diminuição dos níveis de Nrf2 (do inglês "nuclear factor erythroid derived 2-related"), um fator de

transcrição que regula a expressão de antioxidantes importantes. De facto, observámos uma diminuição dos níveis de superóxido dismutase 1 (SOD1), um dos genes alvo do Nrf2, em CMSPs de doentes com DCL. Em conclusão, os nossos dados sugerem que a disfunção neuronal induzida por Aβ em células maduras de hipocampo deve-se, em parte, à desregulação da rede de microtúbulos associada a uma retração das neurites e à fragmentação do ADN por um mecanismo dependente dos recetores NMDARs. Além disso, utilizando murganhos 3xTg-AD, os nossos resultados evidenciaram alterações da ativação da subunidade GluN2B dependentes do sexo e da idade, e um decréscimo da ativação da subunidade GluN2B associada a um decréscimo dos níveis e atividade de proteínas envolvidas na regulação da estabilidade do citosqueleto em machos 3xTg-AD jovens, correspondente a fases iniciais da DA. Assim, demonstrámos um envolvimento precoce dos recetores do NMDA na progressão da doença e mais particularmente da subunidade GluN2B. Os resultados obtidos em CMSPs de doentes com DCL e AD ligeira evidenciaram a ocorrência de modificações na homeostasia do Ca^{2+}_{i} e da capacidade redox, que se correlacionam com uma diminuição do factor de transcrição Nrf2. Estes dados sugerem que as alterações observadas em células humanas de sangue periférico durante a fase pre-clínica e as fases iniciais da DA podem refletir as alterações observadas no cérebro dos doentes e serem utilizadas para investigar os mecanismos básicos de disfunção celular na DA. De facto, mostramos neste trabalho que as alterações moleculares relacionadas com o stresse oxidativo podem ser detetadas muito antes do aparecimento dos primeiros sintomas, nos indivíduos com DCL.

Summary

Alzheimer's disease (AD) is the most common neurodegenerative disorder worldwide associated with severe memory loss. *N*-methyl-D-aspartate (NMDA) receptors (NMDAR) activation has been recently implicated in AD-related synaptic dysfunction, when oligomeric amyloid beta (Aβ) peptide accumulation assumes a greater importance. However, little is known about the complex changes in NMDAR subunit composition and activation in AD. In this thesis we propose to evaluate the importance of NMDARs in AD progression using $A\beta_{1-42}$ treated neuronal cultures, hippocampal and cortical brain samples from the 3xTg-AD mice, two brain areas involved in memory processes, and to investigate possible biomarkers of pathological progression linked to oxidative stress using peripheral blood mononuclear cells (PBMCs) derived from AD patients. To accomplish these objectives, our work has been divided in three parts, described below. We first evaluated the role of NMDARs on Aβ-evoked neuronal dysfunction and cell death through changes in microtubule polymerization in mature hippocampal cultures (Chapter 3). Then, we analyzed age-and gender-dependent changes in NMDAR subunit (GluN1, GluN2A, GluN2B) protein levels and activation using cortical and hippocampal homogenates from the 3xTg-AD *versus* age-matched wild-type (WT) mice (Chapter 4). Moreover, because oxidative stress and deregulation of intracellular calcium (Ca^{2+}_i) homeostasis are believed to be implicated in mechanisms upstream of AD-associated neuronal loss, in the third part of this work we studied markers of peripheral cell injury related with oxidative stress using PBMCs isolated from mild, moderate and severe AD patients and individuals with mild cognitive impairment (MCI) *versus* non-demented age-matched control subjects (Chapter 5).

In this thesis, we showed that exposure of mature hippocampal cells to $A\beta_{1-42}$-enriched oligomer preparation caused a decrease in total and polymerized levels of beta-III tubulin, and polymerized alpha-tubulin, suggesting microtubule disassembly. Furthermore, effects of Aβ on neuronal tubulin polymerization were highly correlated with reduced neurite length and neuronal DNA fragmentation, a feature of cell death by apoptosis. Importantly, the protection provided by MK-801 and memantine suggested the involvement of extrasynaptic NMDARs, in particular GluN2B subunit-containing NMDARs, on Aβ toxicity.

Analysis of total levels of NMDAR subunits in the cortex and the hippocampus of 3xTg-AD males and females evidenced unchanged levels of NMDAR subunits, namely

GluN1, GluN2A and GluN2B. A similar result was obtained regarding the levels of post-synaptic density (PSD)-95, a protein responsible for the attachment of NMDARs to the membrane and the cytoskeleton. Similarly, no major changes regarding GluN2A Ser1232 phosphorylation or GluN2B Tyr1472 were observed in the cortex. Moreover, in the hippocampus of 3xTg-AD mice, females or males, no age-dependent changes were detected regarding GluN2A Ser1232 phosphorylation. Nevertheless, GluN2B Tyr1472 phosphorylation decreased in 3xTg-AD males at 3 months of age, and increased in 3xTg-AD females at 15 months of age, revealing gender differences in the activation of the GluN2B subunit during AD. Moreover, early alterations of GluN2B phosphorylation status in hippocampus correlated with the activity of the Src Tyr kinase. Interestingly, early decrease Src activation in both hippocampus and cortex of 3xTg-AD males also correlated with decrease in total and activated levels of Dab1, a target of Src implicated in cytoskeleton stabilization. Furthermore, we observed decreased levels of total cortactin, another target of Src and a protein involved in cytoskeleton stability. Thus, our data suggest weakened actin cytoskeleton stabilization (which should be tested in future studies) in young 3xTg-AD mice males exhibiting intracellular Aβ labeling, which was accompanied by a decrease in activated GluN2B-composed NMDARs in the hippocampus.

In the third part of this work, we observed deregulation of Ca^{2+}_i homeostasis in PBMCs from MCI subjects and mild AD patients. These alterations occurred concomitantly with increased reactive oxygen species (ROS) production levels in MCI PBMCs but were unrelated with changes in pro- or anti-apoptotic proteins. Concordantly, we demonstrated the downregulation of nuclear factor erythroid derived 2-related (Nrf2), a ROS-related transcription factor, which regulates the expression of several important antioxidants. Indeed, we observed decreased levels of superoxide dismutase (SOD)1, one of the Nrf2 target genes, in MCI's PBMCs.

In conclusion, our data suggest that Aβ-induced hippocampal neuronal dysfunction occurs through NMDAR-dependent microtubule disassembly associated to neurite retraction and DNA fragmentation in mature hippocampal cells. Moreover, results in 3xTg-AD evidenced age- and gender-dependent changes in GluN2B subunit activation and early decreased GluN2B activation in males, which was accompanied by decreased levels and activation of protein involved in cytoskeleton stabilization in early stages of AD. Indeed, we demonstrate an early involvement of NMDARs in disease progression and more particularly of the GluN2B subunit. Results obtained in PBMCs from pre-

clinical and mild AD evidenced changes in Ca^{2+}_i and redox homeostasis that correlate with decreased Nrf2. These data suggest that alterations observed in human peripheral blood cells during pre-clinical and initial stages of the disease might reflect brain changes and can thus be used to elucidate about the early molecular basis of cell dysfunction in AD. Indeed, we show that molecular changes related with oxidative stress may be detected at MCI, long before the first symptoms of the disease.

Chapter 1

Introduction

1.1. Main features of Alzheimer's disease

1.1.1 Clinical features

Alzheimer's disease (AD) is a progressive, neurodegenerative disease with seriously impairment in memory, thinking and behavior. It was first identified in 1906 by Alois Alzheimer who reported the autopsy results of a 55 year-old woman named Auguste Deter. Alzheimer observed the presence of two distinctive pathologies in her patient's brain: intracellular neurofibrillary tangles and extracellular plaques (Alzheimer et al., 1995).

Diagnostic criteria for AD were recently updated by the National Institute of Neurological and Communicative Disorder and Stroke and the Alzheimer's Association. Currently, AD diagnosis focuses on signs of problems in thinking, learning and memory. However, research indicates that alterations due to AD begin long before symptoms appear. Therefore, new criteria describe three phases of AD, a preclinical AD wherein changes in biomarkers are measurable and indicate very earliest signs of the disease (Sperling et al., 2011), mild cognitive impairment (MCI) due to AD (Albert et al., 2011) and dementia due to AD (McKhann et al., 2011). Individuals diagnosed for MCI can be differentiated from healthy control subjects and those with mild AD, indicating that MCI is an intermediate stage with an increased risk of progressing to AD (Petersen et al., 1999). Interestingly, many individuals with MCI revert to normal or do not progress to AD (Petersen et al., 2009;Matthews et al., 2008). Individuals diagnosed with AD can be separated in three phases of the disease: mild, moderate and severe. In mild AD, patients start suffering from a significant impairment of learning and memory and from modest vocabulary. However, patients may still be able to live independently in the most cases, needing support for a variety of organizational matters. In moderate AD stage, logical reasoning, planning and organizing are significantly deteriorated until patients cannot survive in community without close supervision. Severe AD patients show gravely impairment in almost cognitive functions: early biographical memories can be lost, language is dramatically reduced and misinterpret of nursing intervention may lead to aggressive reactions (Forstl and Kurz, 1999). The mean survival of AD patients is 5.7 years after clinical diagnosis (Molsa et al., 1986) but it can be influenced by factors such as race/ethnicity, comorbid diabetes and hypertension (Helzner et al., 2008).

1.1.2 Histopathological features: Amyloid beta peptide and tau protein

Neuropathologically, AD is characterized by two hallmark proteinaceous aggregates, extracellular senile plaques composed by deposits of the amyloid beta peptide, or Aβ, and intracellular neurofibrillary tangles formed by hyperphosphorylated tau (Fig. 1.1).

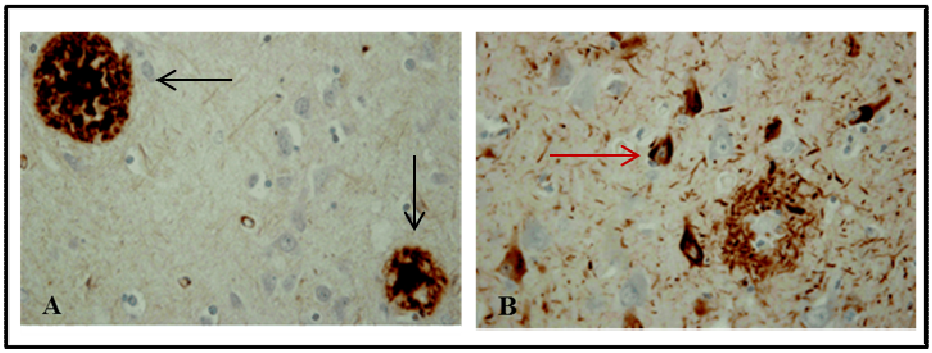

Figure 1.1: Classic pathological hallmarks for Alzheimer's disease. (A) Neuritic plaques (black arrow), stained with anti-$A\beta_{1-42}$ antibodies; (B) neurofibrillary tangles (red arrow), stained with paired helical filament tau antibody. (Vandenberghe and Tournoy, 2005)

The first report of isolation and characterization of the peptide composing extracellular plaques in the brain was made by Glenner and Wong (1984) who showed that it was a 4.2 kDa peptide with primarily 40 or 42 amino acids length, known today as Aβ (Glenner and Wong, 1984). These authors speculated that Aβ was generated from a unique precursor, which was verified very few years later when complementary DNA coding for the Aβ was cloned. Results suggested that the Aβ peptide accumulation was caused by an aberrant catabolism of a cell-surface protein, the amyloid precursor protein, or APP (Kang et al., 1987). The APP gene maps to human chromosome 21 bands q21.105-q21.05 (Korenberg et al., 1989) and encodes for three major isoforms derived from alternative splicing, with 695, 751 and 770 amino acids, which mRNA levels were shown to be significantly elevated in AD human brains (Tanaka et al., 1988). The physiological function of APP remains unknown, but numerous studies suggest a positive effect on cell growth. Neuroprotective effects of APP have been demonstrated recently in *Drosophila* models of progressive neurodegeneration (Wentzell et al., 2012) and in different transgenic mice expressing different human APP isoforms (Masliah et al., 1997). In PC12 cells, tunicamycin-induced apoptosis is prevented following overexpression of human APP, suggesting that one of the protective pathways of APP is related with impairment of endoplasmic reticulum (ER)-induced apoptosis (Kogel et al., 2003).

There are multiple alternate pathways for APP proteolysis, not all leading to generation of Aβ (Fig.1.2). Full-length APP is a transmembrane protein (Selkoe et al., 1988), synthesized in the ER and then transported through the Golgi apparatus to the trans-Golgi-network (TGN). From the TGN, APP can be transported to the cell surface or re-internalized via an endosomal compartment. On the cell surface, APP can be proteolytically cleaved by an α-secretase, ADAM10 (Kuhn et al., 2010), resulting in the production of α carboxyl terminal fragment (α-CTF) and a secreted APP α (sAPPα). The α-CTF fragment is further cleaved by γ-secretase, generating the P3 fragment and the intracellular domain of APP (AICD) (Zhang et al., 2011b). This succession of cleavages is considered as the non-amyloidogenic pathway (Sisodia, 1992). In the amyloidogenic pathway, APP is processed by BACE1 (a β-secretase also known as β-site APP cleaving enzyme 1), generating the β-CTF fragment, further cleaved by a γ-secretase, generating Aβ and AICD (Xu et al., 1997;Kuentzel et al., 1993) (Fig.1.2).

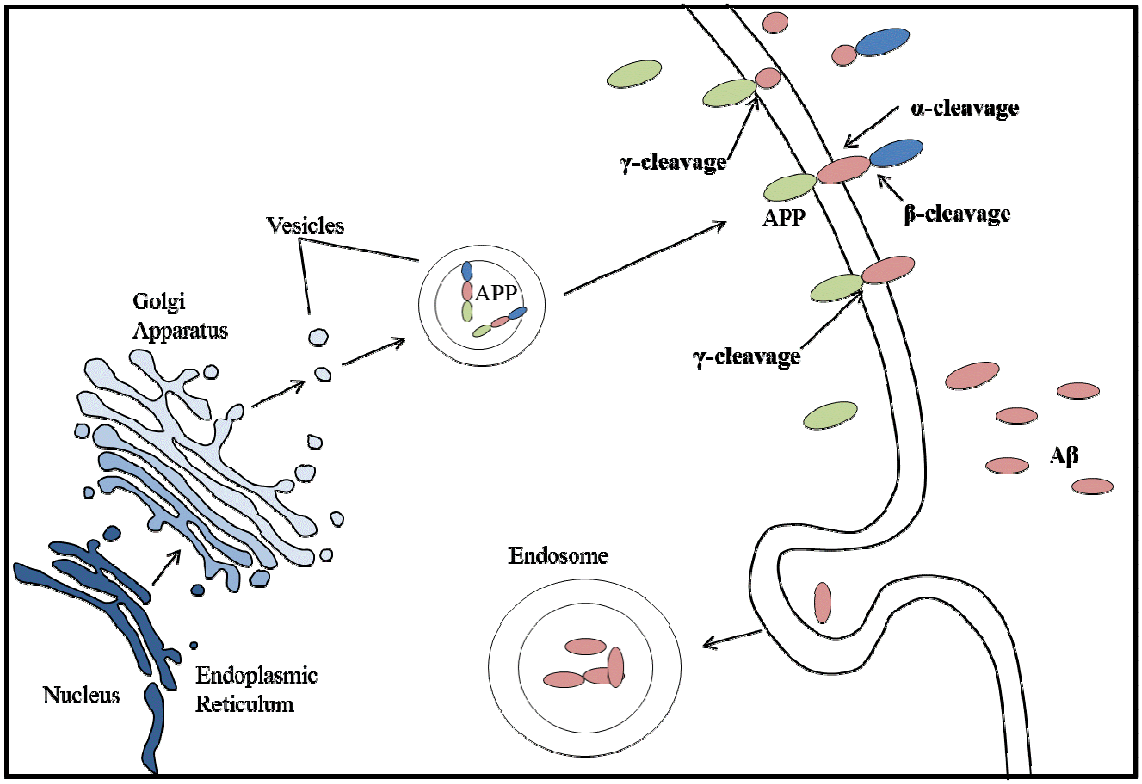

Figure 1.2: Schematic cleavage of APP. APP is transported into vesicles, to the cell surface where it can be cleaved or re-internalized. Non-amyloidogenic pathway implicates the cleavage of APP by α- and γ-secretases. Cleavage by β-secretase and γ-secretase leads to the formation of Aβ through the amyloidogenic pathway. After cleavage, the Aβ peptide can be internalized by endocytosis.

The γ-secretase-mediated cleavage takes place within the transmembrane domain of APP, though the exact site can vary generating, among others, $A\beta_{1-40}$, the majority

specie, and $A\beta_{1-42}$, the highly amyloidogenic specie (Cook et al., 1997;Hartmann et al., 1997). γ-Secretase is a multi-subunit protease complex composed by the assembly of four integral membrane proteins, presenilin (PS), nicastrin, presenilin enhancer 2 and anterior pharynx-defective 1 (Kaether et al., 2006).

Only about 10% of AD cases are hereditary forms, linked to mutations in genes encoding for APP, PS1 or PS2, of the late two being part of the γ-secretase complex (Bertram and Tanzi, 2012). Remaining 90% are considered as sporadic forms. The apolipoprotein E ε4 gene polymorphism is considered as a major risk factor for sporadic AD (Verghese et al., 2011) since ApoE regulates both intracellular and extracellular clearance of Aβ and the ε4 variant leads to a less efficient clearance than the other variants (Castellano et al., 2011) (Fig. 1.3).

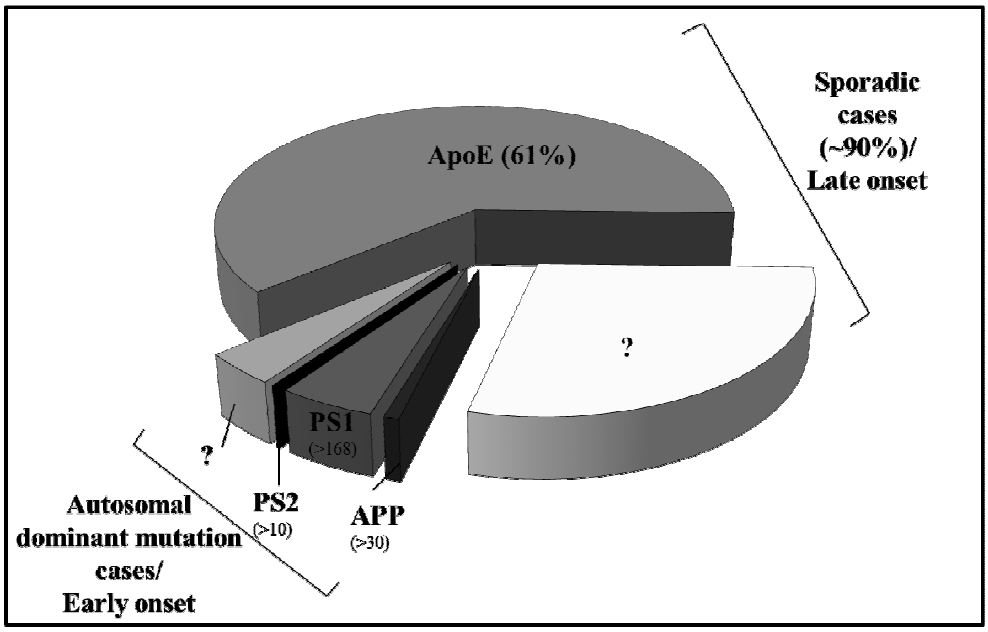

Figure 1.3: Distribution of AD forms and their related principal genetic susceptibility factors. Sporadic cases may be explained in 61% by the ApoE polymorphism, about 29% remain with unknown origin. 4% of genetic cases of AD are unrelated to APP, PS1 or PS2 mutations. Moreover, the number of mutations known for APP is above 30, 168 for PS1 and 10 for PS2, as indicated in the figure.

The process by which Aβ aggregates to form plaques has been largely studied. Several studies evidenced that the peptide oligomerizes before forming protofibrils or large aggregates, which may be considered the intermediate species to fibers or plaques formation (Walsh et al., 1997;Tjernberg et al., 1999). In a preparation of $A\beta_{1-40}$ peptide free of aggregates, the peptide rapidly assembles to form monomers, dimers, trimers and tetramers in a rapid equilibrium, whereas the same preparation of $A\beta_{1-42}$ peptide

preferentially forms pentamer- or hexamer-composed small oligomers that further assemble to form large oligomers and protofibrils (Bitan et al., 2003). In AD brains and Tg2576 mouse which express the human gene for APP with a double mutation, $A\beta_{1-42}$ aggregation into oligomers occurs within endosomal vesicles and along microtubules, being associated with pathological alterations of synaptic function and compartments (Takahashi et al., 2004). Interestingly, Aβ accumulation within the brain can be used to diagnose AD, as described in the next section.

Tau proteins are mainly expressed in neurons and are the product of an alternative splicing from a single gene microtubule associated protein (MAP) tau (MAPT) (Goedert et al., 1989;Goedert et al., 1988). Tau promotes microtubule assembly and stability and seems to be involved in the maintenance of neuronal polarity (Mandell and Banker, 1996). Tau C-terminus binds axonal microtubules whereas the N-terminus binds neural plasma membranes components, suggesting that tau functions as a linker protein between both (Pooler and Hanger, 2010). The first evidences of the presence of abnormal (hyper)phosphorylated tau protein in neurofibrillary tangles appeared in the 80's when a study demonstrated that dephosphorylation of brain tissue sections increased dramatically the immunolabeling of neurofibrillary tangles obtained with an antibody against tau (Grundke-Iqbal et al., 1986). Hyperphosphorylated tau is not found only in tangles but also in the cytoplasm of neurons lacking neurofibrillary tangles suggesting that accumulation of abnormally hyperphosphorylated tau is one of the earliest cytoskeletal changes in the process of tangle formation (e.g. Bancher et al., 1989). Moreover, the presence of ubiquitin epitopes in pathological fibers suggests that neurofibrillary tangles may reflect an unsuccessful attempt of proteolytic degradation (Bancher et al., 1989). Importantly, hyperphosphorylation of tau seems to be a condition necessary to its accumulation (Fig 1.4). Indeed, Herrmann and colleagues (1999) demonstrated significant elevations of phosphorylated tau levels in the frontal and parietal cortex and in hippocampus of AD patients, which significantly correlated with the presence of neurofibrillary tangles (Herrmann et al., 1999).

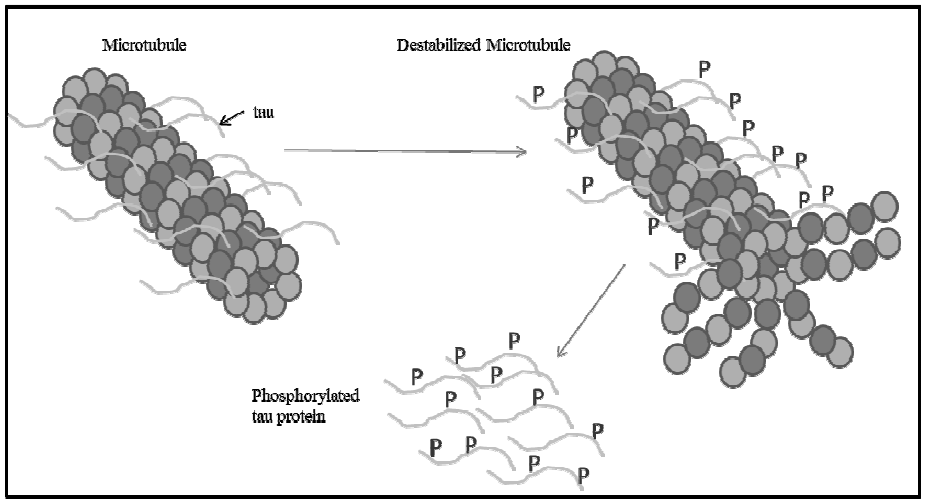

Figure 1.4: Schematic hyperphosphorylation of tau protein. Once phosphorylated, tau loses its ability to link microtubules and aggregates.

Interestingly, tau was shown to interact with APP both *in vitro* and *in vivo* (Barbato et al., 2005) and $A\beta_{1\text{-}42}$ aggregates promote *in vitro* tau aggregation in a dose-dependent manner (Rank et al., 2002), suggesting a direct link between senile plaques and neurofibrillary tangles in AD. On the other hand, other studies suggest that hyperphosphorylated tau may be benefic as demonstrated in adult flies which expand their lifespan following expression of hyperphosphorylated tau (Yeh et al., 2010). This last observation appears in contradiction with the toxic properties granted to hyperphosphorylated tau, closely associated to AD progression.

1.1.3 Biomarkers of Alzheimer's disease

Biomarkers are defined as "parameters (physiological, biochemical and anatomical) that can be measured *in vivo* and that reflect specific features of disease related pathophysiological processes" (Jack, Jr. et al., 2011). According to the new criteria of AD diagnosis, biomarkers of the disease can be divided in two major categories: biomarkers of Aβ accumulation and biomarkers of neuronal degeneration or injury (Jack, Jr. et al., 2011). Cerebral Aβ accumulation can be evaluated by the retention of Aβ identifying compounds on positron emission tomography (PET) imaging and by decreased levels of $A\beta_{1\text{-}42}$ in cerebrospinal fluid (CSF). On the other hand, neuronal degeneration or injury can be studied by the evaluation of tau CSF levels, ^{18}F fluorodeoxyglucose (FDG) uptake in brain by PET imaging in the temporo-parietal cortex and the atrophy of specific brain regions by structural magnetic resonance imaging (MRI).

In AD brain, levels of soluble Aβ are 6-fold higher than in control brains (Kuo et al., 1996). Moreover, a correlation between levels of soluble oligomers of Aβ, cognitive decline and neuropathological hallmarks in AD patients has been demonstrated (Tomic et al., 2009). Aβ accumulation in the brain is commonly evaluated by PET through the ^{11}C-Pittsburgh Compound B (PIB). Retention of PIB is significantly higher in areas of associated cortex of AD patients than in age-matched controls (Klunk et al., 2004); however, PIB retention can be also observed in non-demented individuals, but in these case PIB labeling correlates with memory deficit, indicating a possible preclinical stage of AD (Mintun et al., 2006;Pike et al., 2007). Recent studies evidenced that PET-PIB enables detection of early changes and the prediction of further conversion from MCI to AD (Jia et al., 2011;Zhang et al., 2012a). Moreover, PET-PIB can be combined with the evaluation of glucose uptake by PET-FDG, an indicator of neurodegeneration, to accurate AD and MCI diagnosis (Li et al., 2008). In fact, temporo-parietal cortex of AD patients shows relatively less glucose uptake than in controls (Friedland et al., 1983;Foster et al., 1984). It is also possible to associate different imaging methods as the PET-PIB with volumetric magnetic resonance. Indeed, there is a positive correlation between cerebral amyloid plaque load observed by PET-PIB and rates of cerebral atrophy evaluated by volumetric MRI (Archer et al., 2006). As described above in this section, CSF can also be used as a peripheral indicator of AD progression. Thereby, senile plaques and hyperphosphorylated tau in cortical brain biopsies correlated with low $A\beta_{1-42}$ and high total and phosphorylated tau CSF levels (Seppala et al., 2012). Nevertheless, a recent study points out that a correct classification of AD patients relatively to other dementias, like frontotemporal lobar degeneration or vascular dementia, needs the evaluation of more than one parameter in CSF (Schoonenboom et al., 2012). In fact, tau appears to be non-specific since it is also increased in CSF from patients with vascular dementia (Andreasen et al., 1998). Interestingly, phosphorylated tau protein levels in CSF were demonstrated to be a more specific diagnostic marker for AD than total tau (Ishiguro et al., 1999). Very recently, it was demonstrated that levels of $A\beta_{1-42}$ are fully decreased in CSF at least 5 to 10 years before conversion to AD dementia, whereas total and phosphorylated tau levels seem to be a later marker (Buchhave et al., 2012), suggesting detection of $A\beta_{1-42}$ CSF levels as an early proof of AD.

Interestingly, other parameters not yet accepted as biomarkers have been evaluated to be used in AD diagnosis and correlate with disease progression or known markers. In this

line, angiotensin-converting activity enzyme in CSF may be used as an index of neuronal dysfunction in AD (Zubenko et al., 1985) and elevated levels of ubiquitin (Kudo et al., 1994) and pyruvate were also shown to be remarkably higher in AD patient's CSF and positively correlated with the severity of the disease (Parnetti et al., 1995). Furthermore, antibodies against microglia proteins from CSF can also be used to evaluate neurodegenerative processes in AD before the onset of dementia (McRae et al., 1993). Importantly, biochemical analysis in CSF should not be dissociated from imaging studies. Moreover, the necessity to obtain a non-invasive and earlier diagnosis of AD led to the development of studies looking at changes occurring in the blood, which is easier to obtain. Thus, levels of antioxidant defenses in MCI, AD and control individuals have been evaluated in peripheral blood samples, suggesting that progression to AD might be accompanied by antioxidant depletion in a peripheral model (Baldeiras et al., 2008). Considering the importance of having an accurate diagnosis of AD, research for new biomarkers is a constant concern for researchers.

1.2. Transgenic mice models of Alzheimer's disease

Mice offer several advantages in research. Indeed, they are small which make them manageable, their progeny are abundant and gestation is very short and their whole genome has been mapped. Furthermore, mice respond well in learning and memory tasks enabling the study of these two capacities typically compromised in AD. Nevertheless, as described next, a single model does not recapitulate all features of the disease seen in humans.

1.2.1 Tau transgenic mice

The presence of intracellular neurofibrillary tangles composed by the abnormally hyperphosphorylated protein tau is one of the brain histopathological hallmarks of AD. Indeed, overexpression of human tau in mice appears one of the ways to study some of the alterations observed in AD.

The ALZ7 tau transgenic mouse, constructed before the identification of pathogenic tau mutations, expresses the longest human isoform of tau under the control of a brain and neuron specific promotor element, Thy-1 (Gotz et al., 1995). In these mice, tau labeling was detected not only in the axon but also in cellular body of neurons indicating pre-tangle changes similar to those preceding neurofibrillary tangles formation in AD-affected neurons; however, detection of neurofibrillary tangles failed (Gotz et al., 1995). Insertion of the shortest human tau isoform in mice led to maintained expression of transgenic tau along age in dendrites and cell body of neurons; in this model, dendrites were shown to be reactive for phosphorylated tau in axons and dendrites, but neurofibrillary tangles were not detected (Brion et al., 1999). In the same way, overexpression of human tau using an artificial chromosome derived from the DNA of P1 bacteriophage did not evidence hyperphosphorylation of tau or neurofibrillary tangles (Duff et al., 2000). These evidences suggest that, in these models of transgenic tau mice, overexpression of human wild type tau is sufficient to induce pre-tangle changes similar to those observed in AD, but not enough to induce neurofibrillary tangles.

Since wild-type human tau *per se* is not sufficient to trigger neurofibrillary tangles accumulation, research focused further on the effect of mutant tau proteins. Expression of mutant (P301L) tau protein in mice resulted in age- and gene-dose-dependent

development of neurofibrillary tangles, accompanied by motor and behavioural deficits (Lewis et al., 2000). When the mutant (P301L) tau was expressed under the control of promotor Thy1.2, high expression of tau was observed in the cortex and hippocampus along with hyperphosphorylated tau accumulation, accompanied by apoptotic cell death (Gotz et al., 2001). More recently, a transgenic mouse line overexpressing the human tau with a double mutation (V337M/R406W) showed increased levels of total and phosphorylated soluble tau and spatial learning impairment since 5 months of age (Flunkert et al., 2012).

These few models of tau associated pathology demonstrated their utility to study tauopathies but are clearly not sufficient to be used as a model of AD. Moreover, expression of mutant form of human tau appears to be a necessary condition to observe neurofibrillary tangles characterizing advanced tauopathies.

1.2.2 Mice overexpressing Aβ

The mouse overexpressing the mutant form of human APP (hAPP717V3F) under the control of platelet-derived growth factor promoter (the PDAPP mouse) was one of the first transgenic mice models of AD. Overexpression of hAPP717V3F in transgenic mice induced first intracellular and extracellular amyloid deposition in the form of fibrils and plaques, respectively (Masliah et al., 1996), and then accumulation of phosphorylated tau (Masliah et al., 2001). In this mouse model, the levels of APP remained constant at all ages; however, the levels of Aβ, and particularly $A\beta_{1\text{-}42}$, dramatically increased in specific brain regions, and notably in the cortex and hippocampus from 4 month-old animals (Johnson-Wood et al., 1997). Interestingly, these mice showed synaptic dysfunction before plaque formation (Larson et al., 1999), pointing out the importance of early modifications in AD, deficits in visuospatial learning, thermoregulation, motor activity and circadian cycle (Huitron-Resendiz et al., 2002) and evidences of oxidative stress in the brain (Butterfield et al., 2010), but not presenting overt neuronal loss (Irizarry et al., 1997b).

The double mutant APP (K670N,M671L) transgenic line, or Tg2576, harbors the Swedish mutation in the gene coding for human APP (Lamb et al., 1997). This model showed elevated Aβ levels at an early age and developed extracellular Aβ deposits in the cortex and hippocampus at 9-12 months of age (Irizarry et al., 1997a). Importantly, Tg2576 mice do not exhibit neither widespread or profound cognitive impairment (King and Arendash, 2002), nor neuronal loss (Irizarry et al., 1997a), clearly revealing the

limitations of this model; however, the model presented evidences of inflammatory processes (Benzing et al., 1999) and impairment of synaptic transmission (Fitzjohn et al., 2001). One attempt to improve Tg2576 model was achieved by crossing these mice with a mutant PS1M146L transgenic line expressing the mutant form of PS1 found in some familial cases of AD, generating the APP/PS1 mice. The new model showed a selective 41% increase in $A\beta_{1-42}$ in the brain and developed large number of fibrillar Aβ deposits in cerebral cortex and hippocampus in early age, compared to Tg2576 (Holcomb et al., 1998), indicating that the development of AD-like pathology is significantly enhanced when PS1 mutation is introduced into Tg2576-derived mice.

TgCRND8 mice expressing a double mutant form of APP (K670N,M671L+V717F) under the control of the prion protein gene promoter evidenced extracellular Aβ deposits at 3 months of age and dense-cored plaques and neuritic pathology at 5 months of age (Chishti et al., 2001). The presence of hyperphosphorylated tau at different sites and insoluble aggregates in neocortex and hippocampus of these mice was also observed after the onset of Aβ deposition (Bellucci et al., 2007). Moreover, accumulation of $A\beta_{1-42}$ correlated with early impairment in learning from 3 month-old TgCRND8 mice (Chishti et al., 2001), which may be counteracted by immunization (Janus et al., 2000). In older (9 month-old) animals, at advanced stages of Aβ pathology, significant deficits in spatial and non-spatial learning and memory, as well as increased locomotion could be observed (Walker et al., 2011). This mouse model showed an early activation of inflammatory processes (Dudal et al., 2004) and metabolic perturbations (Salek et al., 2010), as well as Aβ-mediated changes in calcium homeostasis (Supnet et al., 2006). The data gathered from these AD mice models show that overexpression of human APP harboring mutations, found in hereditary forms of AD offers an interesting model to study the disease. In fact, APP transgenic mice present many pathological aspects found in AD human brain, such as the presence of Aβ plaques, inflammatory processes and synaptic dysfunction, often accompanied by cognitive decline. Interestingly, Aβ deposition was enhanced following the expression of human mutant forms of PS1 associated with AD. Moreover, in mice overexpressing APP, evidences of tau pathology can also be found, implicating a link between Aβ deposition and tau phosphorylation.

APP_{SL}/PS1 transgenic mice express human APP with the Swedish and London (V717I) mutations together with the human PS1 with the M146L mutation. In this model, intraneuronal Aβ accumulation precedes plaques formation which starts at 3 months of age (Wirths et al., 2001). Importantly, APP_{SL}/PS1 mice present substantial neuronal loss

with significant decrease in pyramidal neurons in the hippocampus at 17month´s of age, as well as synaptic loss. Interestingly, neuronal and synaptic death are observed not only at sites of Aβ aggregation and surrounding astrocytes but also in areas distant from Aβ plaques (Wirths et al., 2002).

Another model showing extensive neuronal loss is the APP/PS1KI mice which express the human APP with both London and Swedish mutations and the human PS1 with two knock in mutations (M233T/L235P). It is the model with the most aggressive pathology. Indeed, extracellular Aβ deposits are visible at 2.5 months of age and preceded by a strong intraneuronal Aβ accumulation in the hippocampus. At 6 month-old, Aβ deposits are found in hippocampus, cortex and thalamus and $A\beta_{1-42}$ oligomers are highly abundant (Casas et al., 2004) and concomitant with axonal degeneration and impaired working memory and motor tasks (Wirths et al., 2007;Wirths et al., 2006). Moreover, at 6 months, a loss of 33% of hippocampal CA1 pyramidal neurons together with an atrophy of the entire hippocampus was observed (Breyhan et al., 2009), as well as cortical neurons loss correlated with intraneuronal Aβ accumulation (Christensen et al., 2008). This model shows also a significant astrogliosis in the areas of strong intraneuronal Aβ accumulation and neuronal loss (Casas et al., 2004).

One of the latest AD mouse model is the APP transgenic mouse harboring the mutation E693Delta which induces the formation of Aβ oligomers without fibrils (Tomiyama et al., 2010). Interestingly, this model presents intraneuronal Aβ oligomers at 8 month-old but no extracellular plaques even at older ages (24 month-old) (Tomiyama et al., 2010). This model presents, concomitantly with intracellular Aβ, impaired hippocampal synaptic plasticity and memory as well as hyperphosphorylated tau (Tomiyama et al., 2010). At 12 months of age, microglial activation is detected and astrocyte activation at 18 months of age; finally, whereas neuronal loss is detected at 24 months of age (Tomiyama et al., 2010). This model is particularly interesting because of its ability to develop neuronal loss, allowing the study of Aβ oligomers involvement in AD in the absence of plaques formation.

1.2.3 The triple transgenic (3xTg-AD) mice

As suggested by models presented above, overexpression of a single AD-related mutated human protein is not enough to reproduce all features observed along the disease. An attempt to better mimic AD was the production of triple transgenic mice (3xTg-AD).

3xTg-AD harbors three transgenes APP (K670N,M671L), PS1 (M146V) and tau (tauP301L), enabling the development of Aβ and tau pathologies in a temporal and regional profile that closely mimic their development in human AD (Oddo et al., 2003b). Interestingly, in the 3xTg-AD mice Aβ deposits initiated in the cortex and further progressed to the hippocampus (Fig. 1.5). Aβ was detectable in the cortex at 3 months of age whereas extracellular Aβ deposits were observed at 6 months of age; however, only mice older than 12 month-old present Aβ deposits in the hippocampus. Conversely, tau pathology was first apparent in the hippocampus and then progressed to the cortex (Oddo et al., 2003b) (Fig. 1.5). Furthermore, synaptic dysfunction correlated with Aβ intracellular accumulation (Oddo et al., 2003b), before tangle pathology (Oddo et al., 2003a). This model corroborates the relationship between Aβ and tau pathologies, believed to exist in AD. In fact, immunization against Aβ led to the clearance of both Aβ deposits and early tau pathology before the appearance of aggregate of hyperphosphorylated tau (Oddo et al., 2004).

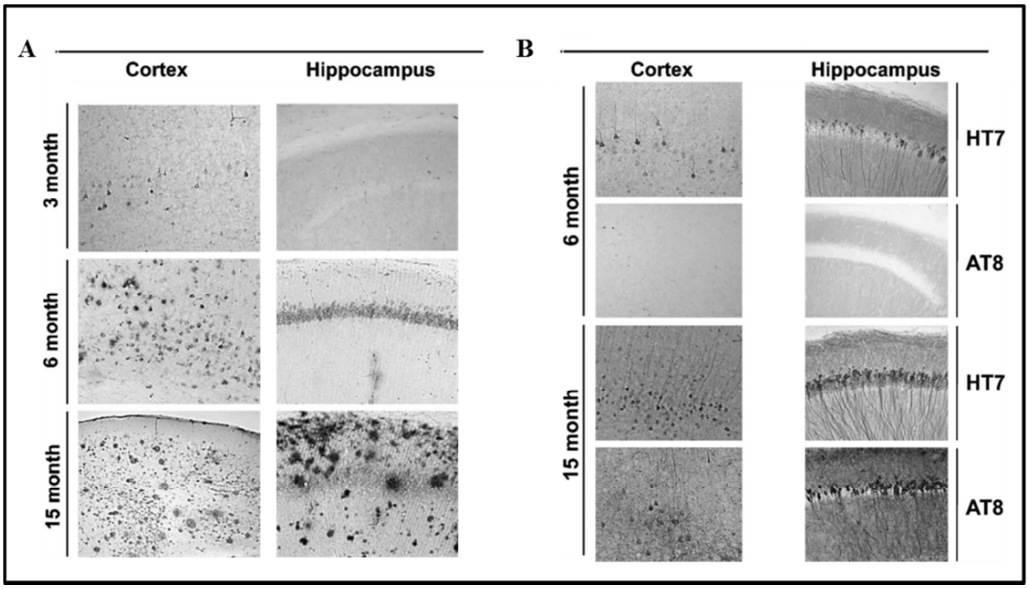

Figure 1.5: Aβ and tau deposition in 3xTg-AD cortex and hippocampus. A Coronal sections were evaluated with an anti $A\beta_{42}$ specific antibody. Aβ deposition initiates in the cortex and progresses to the hippocampus. Extracellular Aβ deposits are evident by 6 months of age in the cortex but do not appear in the hippocampus until the mice are older. **B** Coronal sections were evaluated with a human-specific tau antibody (HT7) and the antibody AT8, which detects serine 202 and threonine 205 phosphorylated residues. Tau pathology initiates in the hippocampus and progresses to the cortex. Although human tau immunoreactivity is apparent in 6 month-old mice, it does not appear to be hyperphosphorylated until the mice are older. Adapted from (Oddo et al., 2003a).

In 3xTg-AD, the presence of soluble intracellular Aβ alone or combined with hyperphosphorylated tau and extracellular Aβ plaques alone can be correlated with dendritic spine loss (Bittner et al., 2010). Importantly, intracellular accumulation of Aβ is not accompanied by an overt neuronal loss (Oddo et al., 2003b). Moreover, 3xTg-AD mice exhibited significant region-specific alterations in myelination patterns prior to appearance of Aβ and tau accumulation (Desai et al., 2009), suggesting the importance of alterations of supporting cells in AD progression. Importantly, this model enables the study of numerous mechanisms believed to be involved in AD progression. Indeed, intracellular levels of calcium (Ca^{2+}_i) were shown to be significantly higher in 3xTg-AD than in wild type mice due to an aberrant Ca^{2+} entry (Lopez et al., 2008). Recently, it has been demonstrated that 3xTg-AD present impaired mitochondrial respiratory chain and oxidative phosphorylation system and shown a high percentage of damaged mitochondria (Carvalho et al., 2012). Moreover, the levels of antioxidants, such as reduced glutathione and vitamin E, and the activity of antioxidant enzymes, namely glutathione peroxidase and superoxide dismutase, were decreased, whereas lipid peroxidation was increased in 3xTg-AD mice, when compared to wild type mice; these changes occurred in early stages of the disease (3-5 month-old), before plaque formation (Resende et al., 2008b).

1.2.4 The five familial AD mutations (5xFAD) mice

Transgenic 5xFAD mice express human APP with Swedish, Florida (I716V9) and London mutations together with mutant PS1 (M146L/L286V) regulated by the Thy1 promoter. Intraneuronal accumulation of $A\beta_{1-42}$ begins at 1.5 month-old, just before the appearance of $A\beta_{1-42}$ deposits, which start at 2 months of age in deep cortical layers and progress further to the whole cortex and hippocampus. Importantly, 5xFAD presents synaptic loss since 4 months of age, becoming significant at 9 month-old, at the same time that significant reduced numbers of pyramidal neurons in cortex are observed. Interestingly, synaptic loss is accompanied by deficits in spatial memory tasks and impairments in trace and contextual fear conditioning tests (Oakley et al., 2006).

Altogether, these different models of AD illustrate the limitations of animal models in mimicking the human disease; indeed, none of the transgenic lines models reproduces exactly the AD pathology. However, they provide tools for the study of disease mechanisms making them good models to test therapeutic agents.

1.3. Pathological role of amyloid beta peptide in Alzheimer's disease

Aβ is one of the major histopathological hallmarks of AD. Numerous animal models and observation of AD human brains showed that soluble and oligomeric forms of Aβ are intracellular and appears early during the disease, whereas Aβ-composed plaques are extracellular and represent a late stage AD progression. Oligomeric forms of Aβ have been shown to be more toxic than monomers or insoluble forms (Townsend et al., 2006;Resende et al., 2008a), being $A\beta_{1-42}$ the most toxic form of the peptide (Selkoe, 1996). However, it was recently demonstrated that crude $A\beta_{1-42}$ preparations, consisting of monomeric and oligomeric Aβ forms, are more toxic than purified monomeric, protofibrillar fractions or fibrils in different cell lines and primary neurons (Jan et al., 2011), suggesting the importance of all soluble forms of the peptide to trigger toxicity. Concordantly, $A\beta_{1-42}$ monomers spontaneously assemble to form globular structures or oligomers, ranging from trimers to 24mers (Chromy et al., 2003). Moreover, a recent study showed that aggregation in oligomers is followed by the formation of protofibrils, which then form fibrils and further increase in size to self-organize into tangles (Serem et al., 2011).

In the next sub-sections we will discuss the role of Aβ on mechanisms involved in AD pathogenesis, namely synaptic dysfunction, excitotoxicity, ER stress and mitochondrial dysregulation, cytoskeleton disruption and cell death.

1.3.1 Modified neurotransmission

Synaptic transmission or neurotransmission comprehends the release of neurotransmitters that in turn may bind and activate selective postsynaptic or presynaptic receptors. Neurotransmission also implicates the synthesis, storage, release, interaction and deactivation of a neurotransmitter, being all of these steps altered in AD. Mice models overexpressing human APP and presenting Aβ accumulation enabled the establishment of a link between deficits in synaptic transmission and Aβ. Indeed, synaptic dysfunction was observed in Tg2576 mice (Calkins et al., 2011) showing early increased Aβ levels, as previously described in this thesis. Moreover, these synaptic alterations in Tg2576 mice seem to be early changes detectable at 3 months of age (Tamagnini et al., 2012). Alterations of cholinergic and glutamatergic neurotransmission have been associated to AD pathogenesis.

Changes in cholinergic system in AD have been largely documented by assessing the acetylcholine synthesizing (choline acetyltransferase) and degrading (acetylcholinesterase) enzymes, acetylcholine transport into vesicles, as well as cholinergic receptors. Numerous studies report that levels of acetylcholine are significantly decreased in AD brain (Sims et al., 1980;Bowen et al., 1983). Fresh cortical biopsy samples of AD patients evidenced that decreased acetylcholine levels are due not only to decreased choline acetyltransferase activity (Sims et al., 1980), but also to a severe and specific degeneration of cholinergic neurons from the basal forebrain (Whitehouse et al., 1982). Moreover, vesicular acetylcholine transporter, responsible for loading acetylcholine into vesicles, is decreased in AD brains as shown by decreased mRNA and protein levels (Chen et al., 2011), contributing to cholinergic system impairment. Decreased acetylcholinesterase activity was also found in the hippocampus and temporal cortex of AD patients (Perry et al., 1978)fffect of Aβ on the cholinergic system has been demonstrated in different AD models. Indeed, treatment with Aβ of SN56 cells derived from mouse basal forebrain cholinergic neurons led to a decrease in acetylcholine levels and choline acetyltransferase activity (Pedersen et al., 1996); moreover, intracerebrovascular administration of Aβ peptide in mice triggered amnesia, which was prevented by the use of a cholinergic agent, suggesting a relationship between Aβ and cholinergic dysfunction in AD (Maurice et al., 1996). Moreover, transgenic mice APP/PS1, overexpressing Aβ, exhibit decreased acetylcholine levels and choline acetyltransferase activity concomitant with spatial memory deficits in early stages of AD, before plaque formation (Zhang et al., 2012b). Importantly, there are evidences for decreased acetylcholinesterase activity in human AD brain (Davies, 1979;Perry et al., 1978) and in old APP/PS1 mice (Harrison et al., 2009). Cholinergic receptors are ion channel and G protein-coupled receptors, respectively, nicotinic and muscarinic receptors. Muscarinic receptors are downregulated by Aβ (Pavia et al., 2000). Aβ effects on nicotinic acetylcholine receptors have been also observed, although there are conflicting reports of upregulation or inhibition of various receptor subtypes (reviewed by (Yakel, 2013)). An important role of the α7 subtype of nicotinic receptors has been suggested in different models, such as in rat hippocampal neurons in which Aβ decreased receptor activity (He et al., 2013); furthermore, the interaction between $A\beta_{1\text{-}42}$ -and α7 nicotinic acetylcholine receptor was previously described (Wang et al., 2000).

Glutamate is the major excitatory neurotransmitter in the brain. Glutamatergic neurotransmission impairment has been described as an important process in AD; deficiencies in numerous stages of glutamate cycle have been observed leading to increased glutamate levels, as described below. Vesicular transporters of glutamate have been shown to be significantly decreased in prefrontal cortex of AD patients (Kashani et al., 2008;Chen et al., 2011). On the other hand, expression and levels of excitatory amino acid transporter, responsible for astrocyte glutamate reuptake , were reduced in the cortex and hippocampus of AD brains (Jacob et al., 2007), suggesting impaired clearance of glutamate from the synapse. Concomitantly, there is a decrease in glutamine synthase (Robinson, 2001), which transforms glutamate into glutamine in astrocytes, enabling its transport back to presynaptic neurons. Thus, both alterations in glutamate transport and conversion into glutamine contribute for increased glutamate concentrations at the synapse and subsequently to excitotoxicity (addressed in the next section). Furthermore, reports suggest that levels of metabotropic glutamate receptors are modified in AD. Indeed, mGluR1 levels are decreased, whereas mGluR2 are increased in the hippocampus of AD patients, correlating with hyperphosphorylated tau levels (reviewed by (Revett et al., 2013)). Alterations in the levels of ionotropic glutamate receptors remains inconclusive since reports are contradictory. However, the studies seem to indicate that alterations in receptor levels may be region-specific (reviewed by (Revett et al., 2013)). Effects of Aβ on glutamatergic synaptic dysregulation have been largely discussed (reviewed by (Parameshwaran et al., 2008)). Among other effects, Aβ decreases glutamate uptake in rat brain astrocytes (Matos et al., 2008) and has been associated to a significant loss of group II metabotropic glutamate receptors in a transgenic mice model of AD (Richards et al., 2010). Interestingly, stimulation of group II metabotropic glutamate receptors in intact nerve terminals of AD transgenic mice brain triggers production and release of $A\beta_{1-42}$ (Kim et al., 2010).

Altogether, these reports suggest that alterations in neurotransmission in AD are linked to the presence of Aβ. Furthermore, the studies indicate that impairment in synaptic plasticity is likely an early event in AD pathogenesis, occurring before plaque formation and cell death.

1.3.2 Excitotoxicity

Excitotoxicity is the pathological process by which neurons are damaged and die after excessive stimulation of excitatory glutamate receptors, namely the NMDAR (section 1.3.2.1), allowing the entry of high levels of Ca^{2+} (reviewed by (Mehta et al., 2013)). Indeed, Ca^{2+} is a key mediator of excitotoxicity damage. Under physiological conditions Ca^{2+} activates a number of Ca^{2+}-dependent enzymes, which influence a wide variety of cellular components governing numerous cellular processes. Consequently, maintenance of Ca^{2+}_i concentration, spatially and temporally, is fundamental for cell integrity. Interestingly, in APP/PS1 transgenic mice Ca^{2+} overload is found preferentially in the proximity of Aβ plaques (Kuchibhotla et al., 2008), suggesting an effect of Aβ in Ca^{2+}_i homeostasis dysregulation observed in AD.

1.3.2.1 NMDA receptors

NMDARs have critical roles in excitatory synaptic transmission, plasticity and excitotoxicity in the central nervous system. Activation of NMDARs leads to Ca^{2+}_i increase (MacDermott et al., 1986) and is required for long-term potentiation and long-term depression (Muller et al., 2009;Fetterolf and Foster, 2011), and more generally for synaptic plasticity regulation (MacDonald et al., 2006;Lau et al., 2009).

1.3.2.1.1 NMDA receptors subunits expression and localization

NMDAR subunits are encoded by three families of genes coding for GluN1, GluN2 and GluN3 subunits (Cull-Candy et al., 2001). Functional NMDARs are heterotetramers composed of two glycine-binding GluN1 subunits and two glutamate-binding GluN2 (GluN2A-D) subunits (Fig. 1.6), or in some cases glycine-binding GluN3 (GluN3A/B) subunits (Kohr, 2006).

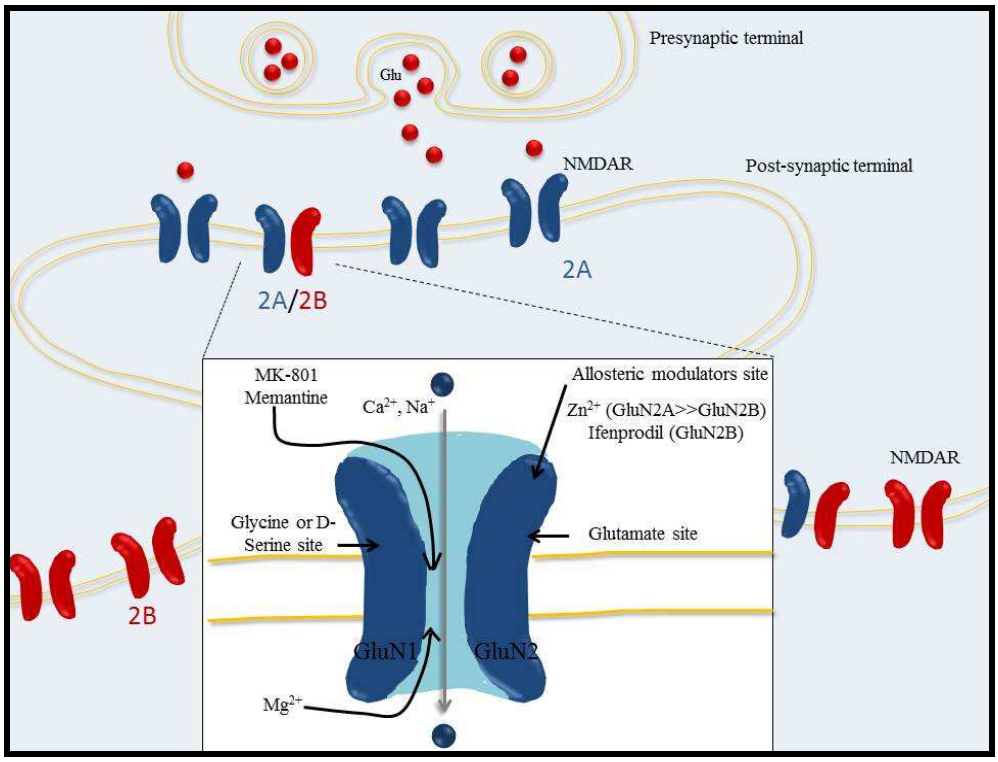

Figure 1.6: NMDARs assembly. Most NMDARs are believed to assemble as tetramers, associating two NR1 and two NR2 subunits in a 'dimer of dimers' quaternary architecture. The GluN2 subunit binds glutamate, whereas the GluN1 binds the co-agonists glycine or D-serine. Sites for competitive agonists and antagonists and allosteric modulators such as endogenous zinc or ifenprodil-like compounds, both acting as non-competitive antagonists are indicated by arrows. The ion-channel domain also forms binding sites for pore blockers such as endogenous Mg^{2+}, MK-801, memantine or ketamine, acting as antagonists.

Expression of different NMDARs subunits differs in localization and development stage. GluN1 mRNA expression represents 67-88% of total subunit gene expression in the brain (Goebel and Poosch, 1999). In the rat fetal brain, GluN1 as well as GluN2A and GluN2C subunits are mildly expressed in restricted areas such as the temporal region of the cerebral cortex and the hippocampus and become widely expressed throughout the whole brain in neonates (Takai et al., 2003). On the other hand, GluN2B subunit, which is mildly expressed in hippocampus and temporal cortex in fetal brain, becomes hardly expressed in the neonatal brain after 7 days of life, being absent from the brain stem (Takai et al., 2003). In human, GluN1 levels are low in embryonic prefrontal cortex and increases after birth (Henson et al., 2008) remaining constant with aging in whole brain (Law et al., 2003). Moreover, expression of GluN2B mRNA is higher in the neonate than in older brains, whereas GluN2A mRNA remains constant after birth, leading to an age-related increase in GluN2A/2B transcript ratio (Law et al.,

2003). Furthermore, in early postnatal stages protein level of GluN3A is high in many brain regions, whereas GluN3B is almost undetectable. Along postnatal development there is a switch in GluN3 subunit expression and, while GluN3B protein levels increase, there is an obvious decrease in GluN3A protein levels (Low and Wee, 2010). It is believed that the pre- and postnatal progressive changes in subunits expression could contribute to the variation in NMDARs-mediated synaptic plasticity during development. Interestingly, GluN2B subunit levels are reduced in old mouse frontal cortex, suggesting alterations in memory processes during aging (Kuehl-Kovarik et al., 2000).

NMDARs subunits differ not only in temporal expression but also in cellular localization. Synaptic NMDARs composition changes quickly after synapse formation. Synapses containing predominantly GluN1/GluN2B represent immature sites, whereas mature sites are more predominantly composed by NMDARs composed of GluN1/GluN2A subunits (Tovar and Westbrook, 1999;Lopez de and Sah, 2003). Recently, it was demonstrated that extrasynaptic NMDARs are usually concentrated at points of contact containing adhesion factors with adjacent processes such as axons, axon terminals or glia (Petralia et al., 2010) and are largely composed by GluN1/GluN2B heteromers (Tovar and Westbrook, 1999;Petralia, 2012).

1.3.2.1.2 NMDA receptors subunits regulation and trafficking

Regulation of NMDARs function and localization is a complex process involving numerous proteins in the cell, particularly a variety of protein kinases. Phosphorylation of GluN2B Tyr1472 enhances NMDARs activity increasing its number at the synaptic membrane (Goebel et al., 2005;Goebel-Goody et al., 2009), whereas phosphorylation of Tyr1336 is associated with enrichment of extrasynaptic NMDARs (Goebel-Goody et al., 2009). Tyrosine kinase Src, as well as Fyn kinase, are involved in upregulation of GluN2B-containing NMDARs at plasma membrane surface (Sinai et al., 2010);(Xu et al., 2006). On the contrary, the tyrosine phosphatase striatal enriched protein ($STEP_{61}$) leads to decrease of GluN1/GluN2B receptor complexes from the neuronal surface (Kurup et al., 2010). In the same way, phosphorylation of Ser1480 by casein kinase 2 (CK2) disrupts the interaction of GluN2B with the scaffolding protein post-synaptic density 95 (PSD-95) and synaptic-associated protein 102 (SAP102) and decreases its surface expression in neurons (Chung et al., 2004). On the other hand, decreased synaptic GluN2B leads to increase in synaptic GluN2A expression (Sanz-Clemente et

al., 2010). Moreover, NMDAR activity can be downregulated by S-nitrosylation (Lipton et al., 1998;Kim et al., 1999).

Interestingly, there are other types of NMDARs regulation not involving post-translational modifications. For example, the calcium-dependent protease calpain downregulates NMDARs function through GluN2 subunit degradation (Wu et al., 2005). Thus, overactivation of NMDARs and subsequent Ca^{2+}_i increase may activate calpains, providing a negative feedback on NMDAR activation (Wu et al., 2005). The Wnt pathway can also be involved in the regulation of NMDARs function in later stages of development; in fact, Wnt ligands are necessary to maintain basal levels of NMDARs synaptic transmission and Wnt5 specifically up-regulates synaptic NMDAR currents in rat hippocampal slices (Cerpa et al., 2011). More recently, it has been demonstrated that the glutamate metabotropic receptor mGluR7 reduces the association of NMDARs with PSD-95, and the consequent surface level of NMDARs, in an actin-dependent manner (Gu et al., 2012). There are many evidences for cytoskeletal and plasma membrane involvement in NMDARs regulation. Membrane composition also affects NMDAR localization; indeed, the membrane phospholipid phosphatidylinositol bisphosphate (PIP_2) is important for the maintenance of NMDARs at the cell surface (Mandal and Yan, 2009). Blocking PIP_2 reduces NMDAR-mediated currents, whereas application of PIP_2 enhances these currents (Mandal and Yan, 2009). Moreover, cofilin, an actin depolymerizing factor which link actin and PIP_2 is required for NMDARs regulation suggesting that PIP_2 decrease leads to cofilin release and actin depolymerization which in turn promotes NMDARs internalization (Mandal and Yan, 2009). Active myosin light chain kinase enhances NMDARs-mediated whole-cell and synaptic currents increasing actin-myosin contractility, which leads to increased membrane tension on NMDARs or to altered physical relationships between NMDAR anchored, such as PSD-95 (Kornau et al., 1995), and cytoskeleton (Lei et al., 2001). PSD-95 is linked indirectly to cortactin, a protein that promotes actin polymerization, by a succession of linker proteins. Thus, NMDARs are indirectly linked to actin cytoskeleton indicating that cytoskeleton alterations may affect NMDARs surface availability (Fig. 1.7). Indeed, binding of reelin, a secreted glycoprotein involved in synaptic plasticity, to its receptors ApoER2 (apolipoprotein E receptor 2) and VLDLR (very low density lipoprotein receptor) triggers Dab1 activation, which further leads to actin polymerization (Suetsugu et al., 2004). Moreover, Dab1 activation by phosphorylation increases NMDARs activity (Chen et al., 2005), and induces Src phosphorylation (Ballif et al.,

2003;Bock and Herz, 2003). Therefore, reelin inhibition decreases GluN2B subunit availability at synapse (Groc et al., 2007).

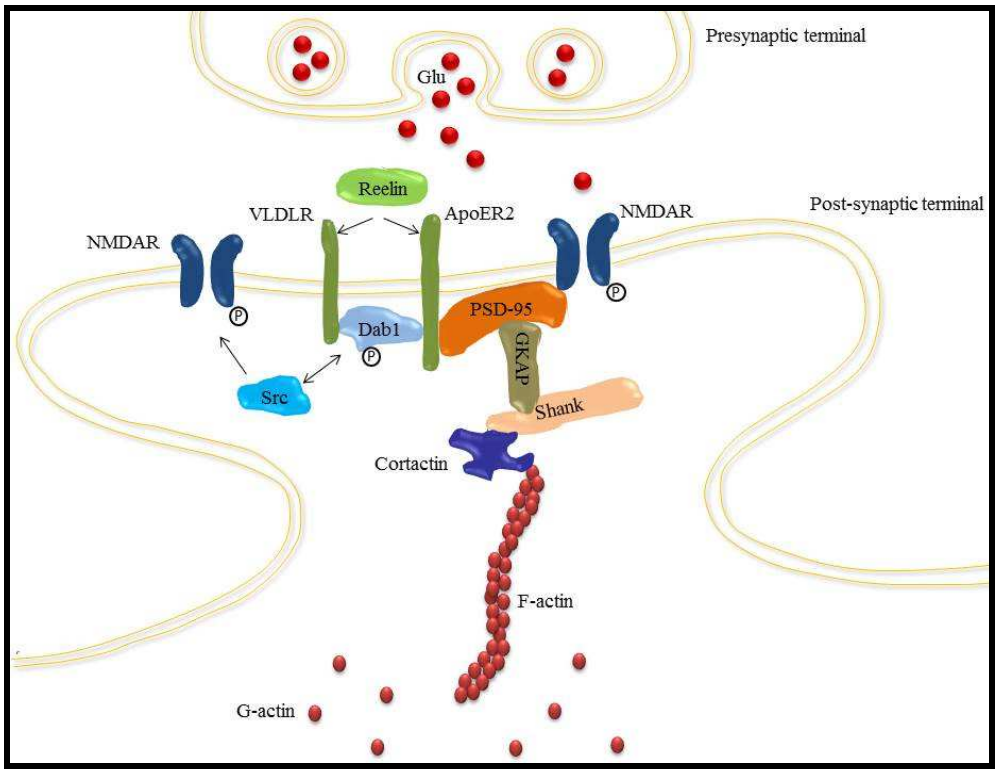

Figure 1.7: NMDARs anchorage to the synapse membrane. NMDARs are indirectly linked to actin cytoskeleton through PSD-95, GKAP, Shank and cortactin proteins. Reelin, involved in synaptic plasticity, links to its receptors ApoER2 and VLDLR and activates Dab1. ApoER2 associates with PSD-95, thus coupling the reelin signaling complex to the NMDAR. Activation of Dab1 leads not only to Src activation, but also to glomerular-actin (G-actin) polymerization into filamentous-actin (F-actin).

1.3.2.1.3 NMDA receptor dysfunction in Alzheimer's disease

Activation of NMDARs was initially hypothesized to occur at late-stage AD; concordantly memantine, an uncompetitive open channel blocker of NMDARs, has been prescribed as a memory-preserving drug for moderate- to late-stage AD patient (Reisberg et al., 2003). However, more recent reports suggest that activation of NMDARs by Aβ occurs at early stages of the disease (Parameshwaran et al., 2008). The levels of NMDAR subunits mRNA and protein have been largely evaluated in different models of AD and in AD brains. A first study in transgenic mice expressing the C-terminal of APP demonstrated that protein levels of NMDARs are unchanged compared to control mice (Sandhu et al., 1993). Conversely, recent studies demonstrated that knock-out for PS leads to early increase in GluN2A subunit expression at post-synaptic densities with a concomitant reduction at non-synaptic sites before synaptic loss (Aoki

et al., 2009). In humans, Jacob and colleagues reported a downregulation of GluN1 subunit in brains of AD patients in various stages of the disease (Jacob et al., 2007). GluN1 mRNA levels were significantly lower in AD compared with control brains (Hynd et al., 2001) and the GluN1 isoform containing an N-terminal splice cassette appeared drastically decreased, suggesting that this isoform may increase vulnerability of cells in AD (Hynd et al., 2004b). Moreover, levels of GluN2B mRNA and protein, as well as GluN2A levels were decreased in susceptible regions of postmortem human AD brain, such as the hippocampus and cortex (Hynd et al., 2004a;Bi and Sze, 2002;Mishizen-Eberz et al., 2004). In comparison to AD susceptible brain areas, there were no alterations in NMDARs subunits expression in cerebellum of AD patients (Bi and Sze, 2002). Conversely, mRNA levels of GluN1 (Bi and Sze, 2002) and GluN2A (Mishizen-Eberz et al., 2004) were unchanged in AD patient brains.

A decrease in GluN2B and GluN2A subunits could be due, in part, to a decrease in reelin levels, a protein which mediates NMDAR activity, and which is depleted in AD brains (Herring et al., 2012). On the other hand, NMDAR subunits decrease may be also due to an increase of tyrosine phosphatase $STEP_{61}$ protein levels, which contributes to the endocytosis of GluN1/GluN2B and GluN1/GluN2A receptors (Snyder et al., 2005;Kurup et al., 2010;Zhang et al., 2011a). Furthermore, reduced $STEP_{61}$ activity by genetic manipulations in different AD mice models triggers an inversion in cognitive and cellular impairment (Zhang et al., 2010), supporting a fundamental role for NMDARs activation in AD. APP and its derivate Aβ peptide have been implicated in many neurotoxic pathways linked to NMDARs dysfunction. Indeed, co-expression of mutated APP and NMDAR subunits in embryonic kidney cells increased surface levels of GluN1/GluN2B and GluN1/GluN2A, linked to enhanced NMDARs currents, through decreased receptor internalization (Cousins et al., 2009). Interestingly, APP seems to be able to bind NMDARs since anti-APP antibodies could immunoprecipitate GluN1, GluN2A and GluN2B (Cousins et al., 2009). NMDARs, more particularly those containing GluN2B subunit appear in numerous studies as mediators of Aβ-induced neurotoxicity. Indeed, Aβ oligomers induce Ca^{2+}_i dysregulation and neuronal death through activation of NMDARs (Alberdi et al., 2010), inhibit long-term potentiation (Li et al., 2011) and induce ER stress (Costa et al., 2012b) in a GluN2B-dependent manner. Moreover, we previously demonstrated that GluN2B subunit activation is involved in Aβ-induced Ca^{2+}_i homeostasis deregulation (Ferreira et al., 2012). Interestingly, in primary neuronal cultures, overexpression of human tau leads to cell death, which can

be prevented by treatment with ifenprodil, a GluN2B specific antagonist, suggesting that GluN2B subunit also mediates tau-induced neurotoxicity (Amadoro et al., 2006). On the other hand, NMDARs activation (Lesne et al., 2005), and more particularly extrasynaptic activation (Bordji et al., 2010), triggers increased production and secretion of Aβ due to amyloidogenic cleavage of APP, largely suggesting a circuit in which Aβ facilitates NMDARs activation, which in turn promotes Aβ production.

It has been suggested that enhancement of GluN2A activity and/or the reduction of GluN2B activity are key events at an early stage of the disease, (Liu et al., 2010). Since GluN2A subunits have been implicated in protective pathways, whereas GluN2B subunits appear to increase neuronal vulnerability (Liu et al., 2007), the early increase in GluN2A and decrease in GluN2B subunit-composed NMDARs activity may be an attempt to reduce Aβ-induced neuronal dysfunction.

1.3.3 Ca^{2+}_i dysregulation induced by Aβ pores

Permeabilization of the plasma membrane by Aβ seems to be one of the processes of Aβ toxicity and one of the pathways by which Aβ disturbs membrane conductance and Ca^{2+}_i homeostasis (Sepulveda et al., 2010). High resolution transmission electron microscopy allowed the visualization of Aβ pores in neuronal plasma membranes of AD brains (Inoue, 2008). Aβ-formed channels have strong selective affinity to Ca^{2+} ions (Jang et al., 2007). Moreover, there are evidences that Aβ pores are dynamic structures, which may break spontaneously after formation into ordered subunits (Jang et al., 2007). A recent study demonstrated that Aβ-induced membrane disruption occurs in two steps with the initial formation of ion-selective pores, followed by non-specific fragmentation of the lipid membrane during Aβ plaque formation (Sciacca et al., 2012). Interestingly, Aβ channel formation depends on membrane charge and fluidity (Wong et al., 2009) and seems to consist in tetrameric and hexameric β-sheet subunits (Strodel et al., 2010).

1.3.4 Endoplasmic reticulum and mitochondrial dysregulation

1.3.4.1 Endoplasmic reticulum stress

Proteins implicated in ER stress are altered in AD brains suggesting the role of ER stress in AD progression. Glucose regulated protein 78 (GRP78 or Bip) and RNA-like endoplasmic reticulum *protein* kinase (PERK), a chaperone protein and a kinase,

respectively, are activated under unfolded protein response (UPR). Moreover, these proteins were shown to be increased in cortex and hippocampus of AD patients (Hoozemans et al., 2005), suggesting the activation of ER stress response during AD. Interestingly, familial AD with mutation in PS1 have been related to deficiency in UPR pathway through decreased levels of GRP78/Bip (Katayama et al., 1999) and increased levels of growth arrest and DNA damage-inducible gene 153 (GADD153) also known as C/EBP homologous protein (CHOP), a pro-apoptotic transcription factor induced during ER stress (Milhavet et al., 2002), thus increasing vulnerability of neurons to ER stress.

Exogenous application of oligomeric Aβ was previously reported to induce its internalization and trigger ER stress (Chafekar et al., 2007). Similar results were observed following intracellularly produced Aβ (Chafekar et al., 2008). ER stress triggered in the presence of $A\beta_{1-42}$ oligomers is, at least in part, due to the release of Ca^{2+} from the ER through RyR (ryanodine receptor) and IP(3)R (inositol triphosphate receptor), leading to increased cytosolic free Ca^{2+} (Ferreiro et al., 2006). Moreover, in different mice models of AD, enhanced Ca^{2+} through RyR has been associated to increased RyR expression (Stutzmann et al., 2006). Therefore, reduction of Ca^{2+} released from ER may provide a therapeutic strategy to counteract toxic effects of Aβ (Suen et al., 2003). Importantly, Aβ-induced ER stress may result in apoptosis. Indeed, in transgenic mice expressing mutant APP and presenting intraneuronal Aβ oligomers, the levels of GRP78 are increased and ER stress activation leads to cell death (Umeda et al., 2011). Moreover, Aβ induces activation of caspase 12, which causes apoptosis under ER stress (Nakagawa et al., 2000), as well as activation of caspases 2, -3, -6, -8 and -9 (Ferreiro et al., 2006) in cortical neurons. Activation of apoptosis under ER stress could be also associated with the nuclear translocation of GADD153 and NF-kappaB, two transcription factors that regulate expression of genes involved in apoptosis, as well as decreased levels of the anti-apoptotic protein Bcl-2 (B cell lymphoma 2) (Ghribi et al., 2001).

Interestingly, Kudo and colleagues demonstrated that ER stress response facilitates the sequestration of APP by Grp78 in early compartments of ER, distant from β and γ secretases, thus regulating Aβ generation (Kudo et al., 2006), suggesting that early ER stress induction may be benefic. On the other hand, another study suggested that ER Ca^{2+} disruption may induce the expression of Aβ deposit-promoting factors (Koyama et al., 2008), and a recent study reported that ER stress induces PS1 expression resulting in

increased Aβ secretion (Ohta et al., 2011). Moreover, Aβ-induced ER stress seems to be related to mitochondrial dysfunction since mitochondrial dysfunction observed in AD patients potentiates cell susceptibility to Aβ-induced ER stress (Costa et al., 2012a). Interestingly, the increase in caspase 12 activity observed in 3xTg-AD mice is responsible for further mitochondrial failure (Quiroz-Baez et al., 2011).

1.4.4.2 Mitochondrial dysfunction and oxidative stress

Mitochondrial dysfunction has been verified in many models of AD. In the APP/PS1 transgenic mice, the degree of cognitive impairment can be linked to the extent of mitochondrial dysfunction and the levels of mitochondrial Aβ (Dragicevic et al., 2010). Mitochondrial dysfunction observed in AD may be due not only to a direct effect of Aβ on mitochondria, but also by its effect on $Ca^{2+}{}_i$ homeostasis. In fact, the massive entry of Ca^{2+} in neurons after activation of ionotropic glutamate receptors by Aβ is accompanied by mitochondrial Ca^{2+} overload, mitochondrial membrane depolarization and oxidative stress (Alberdi et al., 2010). On the other hand, classical Aβ-induced alterations of mitochondrial function in AD are related with decreased cytochrome c oxidase activity (or mitochondrial respiratory chain complex IV) in brain (Kish et al., 1992) and consequent reduction in ATP production (Casley et al., 2002).

Previous studies evidenced the involvement of oxidative stress in the pathogenesis of AD human brains (Pappolla et al., 1992). Mice models of AD overexpressing human APP displayed increased oxidative stress (Mohmmad et al., 2004), and Aβ peptide has been proposed as an inducer of free-radical-based oxidative damage (Harris et al., 1995;Pike et al., 1997). Involvement of mitochondrial oxidative stress in Aβ-induced toxicity is corroborated by numerous studies demonstrating that the use of mitochondrial-targeted antioxidants prevent synaptic loss, caspase activation and cognitive decline in 3xTg-AD mice (McManus et al., 2011); mitochondrial-targeted antioxidants also restored synaptic and mitochondrial viability and mitochondrial transport in Tg2576 mice (Calkins et al., 2011). Interestingly, Leuner and colleagues (2012) demonstrated that reactive oxygen species (ROS) derived from mitochondria are able to enhance APP processing and consequently Aβ production (Leuner et al., 2012). Moreover, Aβ-induced oxidative stress seems to be an important point to Aβ-induced impairment of energy metabolism (Pereira et al., 1999) and neuronal cell death (Abramov et al., 2004). Thus, mRNA of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a critical regulator of mitochondrial energy metabolism and

biogenesis (Wareski et al., 2009;Anderson and Prolla, 2009), was found to decrease with AD progression, being negatively correlated with Aβ and neurofibrillary tangles content (Qin et al., 2009). Moreover, PGC-1α upregulation protects against $A\beta_{1-42}$ neurotoxicity (Zhu et al., 2012) by reducing amyloidogenesis (Katsouri et al., 2011) and restoring mitochondrial biogenesis and mitochondrial deficits (Sheng et al., 2012). Importantly, induction of AD-like symptomatology also seems to require a decrease in brain's antioxidant defense system (Lecanu et al., 2006). In fact, increased lipid peroxidation observed in AD patients brain is accompanied by a decrease in the activity of Cu/Zn superoxide dismutase (SOD) and catalase (Marcus et al., 1998). Moreover, a decrease in the activity of methionine sulfoxide reductase, which allows the recovery of methionine residues after their oxidation, has been found in human AD brain (Gabbita et al., 1999). The nuclear factor (erythroid- derived 2)-like 2 (Nrf2) is a transcription factor normally activated under oxidative stress conditions and which regulates the cellular redox state through induction of cytoprotective protein coding genes transcription (Nguyen et al., 2004). Under basal conditions, Nrf2 interacts with the actin-anchored Keap1 protein and is "sequestered" in the cytoplasm, maintaining low basal levels of Nrf2-regulated genes (reviewed by (Kensler et al., 2007)). Keap1 is a cysteine rich protein, which under oxidative stress undergoes oxidation forming disulfides bridges (Wakabayashi et al., 2004). This triggers the release of Nrf2 and its migration to the nucleus following phosphorylation of the residue Ser 40 by protein kinase C (Bloom and Jaiswal, 2003). Once in the nucleus, Nrf2 regulates the expression of SOD1 (Park and Rho, 2002), GCLc, the catalytic subunit of glutamate cysteine ligase, the rate limiting enzyme for the synthesis of glutathione (Sekhar et al., 2003), heme oxygenase 1 (Alam et al., 1999) and glutathione peroxidases (Banning et al., 2005), among others. Importantly, nuclear Nrf2 is decreased in AD patient's brain, suggesting that Nrf2-mediated transcription is not induced in AD despite the presence of oxidative stress (Ramsey et al., 2007). Thus, retention of Nrf2 in the cytoplasm in AD brains (Ramsey et al., 2007) leads to decreased gene transcription of antioxidant enzymes, which may in part explain increased oxidative stress observed in AD brains. Interestingly, activation of Nrf2 in NT2N cells after induction of Aβ formation protects neurons from cell death and decreases Aβ formation (Eftekharzadeh et al., 2010); moreover Nrf2 expression in APP/PS1 mice induces expression of the Nrf2 target genes, protecting against Aβ toxicity (Kanninen et al., 2008) and triggering an improvement in spatial learning (Kanninen et al., 2009).

Interestingly, oxidative stress promotes Aβ formation (Eftekharzadeh et al., 2010) and, as observed in other toxic effects of Aβ peptide, Aβ-induced oxidative stress fosters intracellular Aβ accumulation (Paola et al., 2000) which increases the source of toxicity as in a cycle.

1.3.5 Cytoskeletal disruption

The influence of Aβ on cytoskeleton dysfunction in AD has been largely studied. Reduced synaptic density and number of dendritic spines have been observed in the cortex and the hippocampus of AD brains (reviewed by (Yu and Lu, 2012)). Studies evidenced that accumulation of soluble Aβ is the starting point to dendritic spine loss observed in AD (Selkoe, 2008;Spires-Jones and Knafo, 2012). Exposure of mature primary rat culture to Aβ oligomers causes missorting of neurofilaments and loss of dendritic microtubules (Zempel and Mandelkow, 2012), as well as abnormal spine morphology and ultimately a decrease in spine density (Lacor et al., 2007). However, the molecular mechanisms by which Aβ triggers spine loss remain uncertain. Spine loss mediated by Aβ requires NMDAR activity (Shankar et al., 2007;Roselli et al., 2009) and occurs through a pathway involving calcineurin activation (Wu et al., 2010). Recently, the involvement of GSK3β activation in Aβ-mediated spine loss has been demonstrated (DaRocha-Souto et al., 2012). Moreover, numerous studies suggest that Aβ triggers alterations in postsynaptic density proteins. Thus, PSD-95 protein appears to be decreased in human and mice AD brains, as well as Shank1 and Shank3 (Pham et al., 2010) and Homer1b (Roselli et al., 2009). Debrin, a postsynaptic protein involved in F-actin stabilization, is also decreased by Aβ, concomitantly with the dephosphorylation of cofilin, the later also involved in actin cytoskeleton polymerization when phosphorylated (Kojima and Shirao, 2007).

Fast axonal transport is achieved by microtubules and defective axonal transport or axonopathy is commonly a consequence of cytoskeleton disruption (Goellner and Aberle, 2012). Importantly, Aβ is sufficient to induce AD-like tau phosphorylation and the consequent cytoskeletal disruption and neuritic dystrophy (Jin et al., 2011), which implicate that the effect of the peptide on the cytoskeleton can be due to alterations of tau phosphorylation. Moreover, Aβ exposure leads to increased Ca^{2+} levels and breakdown of microtubules, concomitant with activation of kinases and tau phosphorylation (Zempel et al., 2010). In APP/PS1 transgenic mice elevated intracellular levels of Aβ led to axonopathy, characterized by the formation of axonal

spheroids and myelin ovoid structures (Wirths et al., 2006). Moreover, defects in axonal transport were observed in different AD models. Tg2576 and 3xTg-AD mice present an early significant impairment in axonal transport before Aβ deposition and formation of tau fibrils (Smith et al., 2007;Kim et al., 2011). Expression of different PS1 mutants, known to increase Aβ formation, impairs fast anterograde axonal transport along with abnormal phosphorylation of tau and neurofilaments (Lazarov et al., 2007). Impairment of fast axonal transport by Aβ leads, among other effects, to inhibition of both anterograde and retrograde transport of mitochondria (Wang et al., 2010). One of the signaling pathways by which Aβ induces toxicity is the activation of endogenous CK2 which phosphorylates the kinesin-1 light chains leading to the release of kinesin from its cargoes and thus disrupting cell transport (Pigino et al., 2009).

Cytoskeletal disruption and consequent axonal transport impairment appears as a key event in AD pathogenesis. Importantly, positive results obtained after treatment with microtubule-stabilizing drugs such as taxol and taxotere (Michaelis et al., 2002) corroborate the importance of cytoskeleton disruption in the progression of AD, suggesting the utilization of this type of drug as a part of AD therapeutics. Thus, in primary neurons in culture, treatment with microtubule stabilizing drugs (taxol and epothilone A) was shown to prevent the induction of the UPR by Aβ (Seyb et al., 2006). However, since not all agents promoting microtubule stability are able to trigger protection against Aβ toxicity (Michaelis et al., 2002), the involvement of additional mechanisms in Aβ toxicity remains an important theme of research in AD progression.

1.3.6 Cell death

Involvement of apoptosis during AD remains under discussion. In fact, there are some evidences of apoptosis activation in AD brains such as increased levels of Bim in the frontal cortex (Engidawork et al., 2001) and Bax (Bcl-2 associated protein) in hippocampus (Nagy and Esiri, 1997), known to promote apoptosis, and also increased levels of the anti-apoptotic protein Bcl-2 in hippocampus and enthorinal cortex (Satou et al., 1995), suggesting an attempt to protect against apoptosis. However, some authors believe that DNA fragmentation observed in AD brains (Stadelmann et al., 1998;Overmyer et al., 2000;Colurso et al., 2003) is most likely not related to apoptosis (Stadelmann et al., 1998) or cell loss (Colurso et al., 2003). Interestingly, DNA fragmentation found in human hippocampal neurons by TUNEL method (Jellinger and Stadelmann, 2000) or in human brain slices (Lassmann et al., 1995) correlates with

neurofibrillary tangles and amyloid deposits. Nevertheless, Aβ-induced death mechanisms involved in AD still remain unclear.

Chronic exposure to 25 μM Aβ peptide induced cell death in cultured cortical neurons as well as in hippocampal neurons through activation of an apoptotic pathway (Loo et al., 1993). Moreover, Aβ induces apoptosis by downregulation of Bcl-2, an anti-apoptotic protein, upregulation of Bax, a pro-apoptotic protein in human neurons (Paradis et al., 1996;Yao et al., 2005), and the release of cytochrome c (Kim et al., 2002). These *in vitro* observations were confirmed *in vivo.* Indeed, overexpression of Aβ in transgenic mice models induced neurodegeneration with morphological and biochemical evidences of apoptosis (LaFerla et al., 1995;Bartley et al., 2012). On the other hand, in APP/PS1 transgenic mice, neuronal autophagy was early induced, before Aβ plaque deposition (Yu et al., 2005). In 3xTg-AD mice, induction of autophagy significantly reduced plaques, tangles and cognitive deficits (Majumder et al., 2011), indicating the importance of autophagic clearance in AD. Indeed, autophagy impairment induces intraneuronal and extracellular Aβ accumulation (Pickford et al., 2008). Importantly, neurons exhibiting high levels of autophagosome formation under Aβ toxicity did not show apoptotic features, suggesting that activation of autophagy by Aβ may precede apoptosis (Cheung et al., 2011). This last hypothesis was raised by the increased apoptosis in APP/PS1 mice after impairment of autophagic pathways (Yang et al., 2008).

In APP transgenic mice, Aβ-mediated apoptotic activation occurs through ER stress, endosomal/lysosomal leakage and mitochondrial dysfunction (Umeda et al., 2011). In cortical neurons, Aβ was shown to trigger apoptotic cell death, in part through ER stress activation (Ferreiro et al., 2006) which leads to ER caspase 12 activation (Nakagawa et al., 2000). One of the mechanisms implicated downstream of these alterations appears to be linked with the activation of c-Jun N-terminal kinase (JNK) (Yao et al., 2005;Ramin et al., 2011) since JNK phosphorylation inhibitors prevent apoptosis (Yao et al., 2005). Moreover, apoptosis may be a consequence of Aβ-induced oxidative stress (Kadowaki et al., 2005;Bartley et al., 2012). In this respect, the apoptosis signal regulating kinase 1 (ASK1) is required for ROS-induced JNK activation and further cell death (Kadowaki et al., 2005). Cytoskeleton disruption is also involved in Aβ-induced apoptosis; indeed, Aβ induced the proteolysis of microtubule-associated proteins, which is followed by apoptosis (Fifre et al., 2006). Importantly, intraneuronal Aβ downregulates the Akt survival pathway and blunts the stress response (Magrane et al.,

2005). These data suggest that apoptosis is a consequence of previous dysfunctional pathways induced by Aβ. Interestingly, in serum deprived neurons, a condition favoring apoptosis, the APP non-amyloidogenic pathway is decreased, whereas the levels of Aβ are increased (LeBlanc, 1995), suggesting that apoptosis may also increase Aβ levels, exacerbating the apoptotic pathways.

Despite its controversy, evidences of Aβ-induced apoptosis are numerous. Interestingly, in early studies Mattson and colleagues demonstrated that cytosolic extracts of synaptosomes exposed to Aβ are able to trigger DNA fragmentation in isolated nuclei, suggesting a "synaptic apoptosis" independently of the cell body, which can contribute to synaptic dysfunction and loss (Mattson et al., 1998).

1.4. Objectives of the present study

The first description of AD dates 1906. Intensive research in AD has made possible the identification of a series of cellular and molecular events occurring in AD. However, until now, we are still unable to determine the initial event(s), as well as the exact sequence of mechanisms underlying AD progression.Accumulation of Aβ peptide, a histopathological hallmark of AD, is one of the fundamental steps relevant for disease progression. Several studies suggest that Aβ activates NMDARs, leading to NMDAR overactivation (Alberdi et al., 2010) and further activation of many dysfunctional processes believed to be involved in AD (Alberdi et al., 2010;Costa et al., 2012b;Ferreira et al., 2012). In contradiction with the initial hypothesis that NMDARs activation could occur at late-stage of AD, recent studies suggested an early activation of NMDARs (Parameshwaran et al., 2008). Therefore, we hypothesized that NMDARs participation in AD pathological process initiates in a preclinical phase of AD, wherein changes in biomarkers are measurable and reflect very early signs of the disease.

Thus, the aims of this thesis were to better understand the importance of NMDARs in AD progression and to evaluate possible biomarkers of pathological progression related with oxidative stress, using Aβ 1-42 treated neuronal cultures, hippocampal and cortical brain samples from the 3xTg-AD mice and peripheral blood mononuclear cells (PBMCs) derived from AD patients. For this purpose, the following specific objectives were pursued:

1. To determine the involvement of NMDARs, and more particularly the GluN2A and GluN2B subunits, in microtubule deregulation and toxic processes in rat hippocampal neuronal cultures exposed to Aβ.
2. To evaluate alterations in NMDARs expression and activation in the 3xTg-AD mice and its correlation with Src activity and possible regulation through the reelin-Dab pathway.
3. To investigate the relationship between the different stages of cognitive impairment and peripheral cell injury markers, namely changes in oxidative stress involving increased calcium levels, ROS production and Nrf2 transcription regulation in PBMCs from human subjects with different stages of cognitive impairment.

Our findings provide evidence that NMDARs and more particularly extrasynaptic GluN2B-containing NMDARs are involved in $A\beta_{1-42}$-induced microtubule deregulation

and that in 3xTg-AD mice there is a tissue specific and age- and gender-dependent alterations of GluN2B subunit and also early alterations in proteins involved in cytoskeleton stabilization. Moreover, we demonstrate that antioxidant dysfunction associated with deregulated Nrf2 transcription factor may underlie oxidative stress in early stages of AD pathogenesis, namely in mild cognitive impairment, considered a pre-clinical stage of AD.

Chapter 2

Materials and Methods

2.1. Materials

Neurobasal medium, B27 supplement, gentamicin and trypsin were from Gibco-BRL, Life Technologies (Paisley, Scotland, UK). The synthetic Aβ 1-42 peptide was from from American peptide (Sunnyvale, CA, USA). BioRad protein assay reagent was from Bio-Rad (Munich, Germany). BCA protein assay was obtained from Pierce Thermo Fisher Scientific (Rockford, IL, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore Chemicon (Billerica, MA, USA). ECF reagent was obtained from Amersham (Buckinghamshire, UK). NMDA was purchased from Tocris (Bristol, UK). Memantine was a kindly gift from Lundbeck Portugal. (+)-MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate) was from Calbiochem (Darmstadt, Germany). Heparinized tubes for blood collection were from BD Bioscience (San Jose, CA, USA). FicollHistopaque, was obtained from GE Healthcare (Buc, France). Fluorescent probes Fura-2-AM, dichlorodihydrofluoresceindiacetate (DCFH2-DA) and fluorescente dye Hoechst 33342 were purchased from Molecular Probes-Invitrogen (Eugene, OR, USA). VECTASTAIN Elite ABC Kit was purchased from Vector Lab (Burlingame, CA, USA). Glutamate, ifenprodil, glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), protease cocktail inhibitors, RPMI 1640, H_2O_2 and 3,3′-diaminobenzidine (DAB) as well as other analytical grade reagents were from Sigma Chemical and Co. (St. Louis, MO, USA).

2.2. Antibodies

Primary antibodies	**Application (dilution)**	**Source**
Aβ $_{1-16}$, 6E10	IHC (1:5000)	Covance (New Jersey, USA)
Aβ $_{1-42}$ (clone G2-G13)	IHC (1:1000)	Millipore Chemicon (Peniecula, California, USA)
Actin	WB (1:20000)	Sigma (St. Louis, MO, USA)
APP (clone Y188)	IHC (1:1000)	Abcam (Cambridge, UK)
Alpha-tubulin	WB (1:20000)	Sigma (St. Louis, MO, USA)
AT8	ICC (1:200)	Pierce Thermo Fisher Scientific (Rockford, IL, USA)
Bak	WB (1:2500)	Abcam (Cambridge, UK)
Bax	WB (1:1000)	Cell Signalling (Denvers, MA, USA)
Bcl-2	WB (1:500)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Beta-tubulin III	WB (1:20000)	Covance (New Jersey, USA)
Beta-tubulin III	ICC (1:200)	Millipore Chemicon (Peniecula, California, USA)
CBP	WB (1:200)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Cortactin	WB (1:750)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Cortactin pTyr421	WB (1:200)	Biosource-Invitrogen (Leiden, Netherlands)
CREB	WB (1:1000)	Cell Signalling (Denvers, MA, USA)
GCLc	WB (1:1000)	Abcam (Cambridge, UK)
GFAP	ICC (1:200)	Santa Cruz Biotechnology (Santa Cruz, USA)
GluN1	WB (1:1000)	Cell Signalling (Denvers, MA, USA)
GluN2A	WB (1:1000)	Millipore Chemicon (Peniecula, California, USA)
GluN2A pSer1232	WB (1:500)	Tocris (Bristol, UK)
GluN2B	WB (1:1000)	BD Biosciences (Franklin Lakes, NJ, USA)
GluN2B pTyr1472	WB (1:500)	ProSci Incorporated (Poway, CA,

		USA)
MAP-2	ICC (1:200)	Sigma Chemical and Co. (St. Louis, MO, USA)
Neu-N	ICC (1:200)	Millipore Chemicon (Peniecula, California, USA)
Nrf2	WB (1:1000)	Abcam (Cambridge, UK)
PCG-1α	WB (1:500)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Pro-caspase 3	WB (1:1000)	Cell Signalling (Denvers, MA, USA)
SOD1	WB (1:1000)	Abcam (Cambridge, UK)
Synaptophysin	ICC (1:200)	Millipore Chemicon (Peniecula, California, USA)
Secondary antibodies		
Alexa Fluor-594 goat anti-mouse	ICC (1:200), IHC (1:200)	Molecular Probes-Invitrogen (Eugene, OR, USA)
Alexa Fluor-488 goat anti-rabbit	ICC (1:200), IHC (1:200)	Molecular Probes-Invitrogen (Eugene, OR, USA)
Anti-mouse	WB (1:20000)	Amersham (Buckinghamshire, UK)
Anti-rabbit	WB (1:20000)	Amersham (Buckinghamshire, UK)
Biotinilated anti-mouse	IHC (1:200)	Vector Lab (Burlingame, CA, USA)
Biotinilated anti-rabbit	IHC (1:200)	Vector Lab (Burlingame, CA, USA)

WB – Western blot, ICC – Immunocytochemistry, IHC – Immunohistochemistry

2.3. Methods

2.3.1 Primary hippocampal cell culture

Primary hippocampal cells were prepared as described previously (Ambrosio et al., 2000), with some minor modifications. Briefly, brains were dissected out from Wistar fetal rats at embryonic 18-19 day and hippocampus were then digested with 0.6 mg/mL trypsin. for 5 min at 37°C in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution containing (in mM) 137 NaCl, 5.36 KCl, 0.44 KH_2PO_4, 0.34 $Na_2HPO_4.2H_2O$, 4.16 $NaHCO_3$, 5 glucose, 1 sodium pyruvate and 10 HEPES, pH 7.2. Cells were then plated at a density of 9×10^4 cells/cm^2 in poly-D-lysine coated 24- or 6-well plates for MTT assay and Western blotting, respectively, or at a density of 5×10^4 cells/cm^2 in poly-D-lysine coated glass coverslips for immunocytochemistry. Cells were cultured for 17 days in vitro (DIV) in 95% air and 5% CO_2, in serum-free Neurobasal medium supplemented with 2% B27, 25 µM glutamate, 0.5 mM glutamine and 0.12 mg/mL gentamicin. Once a week, one half of the medium was changed with fresh medium without added glutamate; the resulting culture medium at 17 days in culture was used for incubation with Aβ or for the 'recovery period', designated thereafter by conditioned culture medium. All animal experiments were carried accordingly to the care and use of laboratory animals and guidance of CNC, University of Coimbra, with care to minimizing the number of animals and their suffering.

2.3.2 Aβ preparation

Aβ peptide preparation, previously described as ADDLs (Aβ-derived diffusible ligands), was made from synthetic Aβ 1–42 peptide, as previously described (Ferreira et al., 2011;Resende et al., 2008;Dahlgren et al., 2002). Briefly, synthetic Aβ was dissolved in HFIP to a final concentration of 1 mM. HFIP was then removed in a Speed Vac, and dried HFIP film was stored at -20ºC. 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 4ºC. The preparation was centrifuged at 15,000*g* for 10 min at 4ºC to remove insoluble aggregates, and the supernatant containing soluble oligomers and monomers was transferred to clean tubes and stored at 4ºC. Protein concentrations of Aβ were then determined using the Bradford protein assay reagent. 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 stained with Coomassie G-250. Aβ 1-42 preparation contained a high percentage of low-n oligomers (about 60%) and monomers (by about 40%), as described previously (Ferreira et al., 2011).

2.3.3 Aβ and NMDA treatment

Hippocampal cells were exposed to 500 nM of soluble Aβ for 6 h (or 2 h, when indicated) in conditioned culture medium. In some conditions, neurons were also exposed for 15 min to 100 µM NMDA/10 µM glycine (NMDA/gly) in Mg^{2+}-free Na^{+} medium containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 10 Glucose, 10 Hepes, pH 7.4/NaOH for 15 min. Pharmacological inhibition was achieved by using the NMDARs antagonists MK-801 (5 µM) or memantine (10 µM), or the selective GluN2B subunit antagonist ifenprodil (10 µM). In this case, a 5 min pre-incubation with the antagonists was performed and maintained during treatment with Aβ and/or NMDA/gly. In some experiments, after acute exposure cells were washed with Hank's balanced salt solution and incubated for further 18 h ('recovery period') in conditioned culture medium (i.e. medium where the cells were cultured) without the injury stimuli, in order to evaluate the delayed effects of Aβ and/or NMDA/gly treatments. In all experiments, control or basal conditions represent cells without any treatment.

2.3.4 Immunocytochemistry

In Chapter 3, hippocampal cells grown in glass coverslips were washed three times with phosphate saline buffer (PBS) containing (in mM): 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 $Na_2HPO_4.2H_2O$, pH 7.4, fixed for 15 min in 4% paraformaldehyde (in PBS) at room temperature (RT) and washed three times with PBS. After permeabilization with 0.2% Triton X-100 in PBS for 2 min, cells were washed three times in PBS, and further incubated with a blocking buffer containing 3% BSA in PBS, for 1 h. Cells were then incubated with primary antibodies for 1 h, at RT. Primary antibodies were visualized by using the secondary antibodies Alexa Fluor-594 anti-mouse IgG or Alexa Fluo-488 anti-rabbit IgG. Cells on the glass coverslips were washed three times with PBS and mounted with DakoCytomation fluorescent mounting solution on a microscope slide

Cells were visualized by using an epifluorescence microscope (Axiovert Microscope 200 Zeiss).

2.3.5 Analysis of apoptotic nuclei

The nuclear morphology of hippocampal cells exposed to 500 nM Aβ for 6h and/or 100 µM NMDA/gly for 15 min, in the absence or in the presence of MK-801, memantine or ifenprodil was analyzed by fluorescence microscopy, by using the fluorescent dye Hoechst 33342. After immunocytochemistry procedure, cells were incubated with 1 µg/mL Hoechst 33342, in the dark, for 5 min at RT, washed three times in PBS, mounted with DakoCytomation fluorescent solution and further examined and scored using an epifluorescence microscope (Axiovert Microscope 200, Zeiss). Nuclei were considered apoptotic if they were fragmented and showed intense blue color when labelled with Hoechst 33342; conversely, cells were considered viable when nuclei appeared round with an homogeneous blue color. Approximately 40 cells per field were counted and 5-6 fields per coverslips were analyzed. The number of apoptotic cells was expressed as the percentage of the total number of cells in the microscope field.

2.3.6 Preparation of cell extracts containing polymeric tubulin

According to the procedure previously described by Joshi and Cleveland (Joshi and Cleveland, 1989), cells were washed twice very gently with a microtubule stabilizing buffer (0.1 M *N*-morpholinoethanesulfonic acid, at pH 6.75, 1 mM $MgSO_4$, 2 mM EGTA, 4 M glycerol). For analysis of polymerized tubulin cells were extracted with a scraper in 200 µl of 25 mM Tris (pH 6.8) containing 0.5% SDS; then, samples were frozen 3 times. Extracted proteins were submitted to Western blotting, as described next.

2.3.7 MTT Assay

MTT colorimetric assay is based on the reduction of MTT by intracellular dehydrogenases (present in living cells) to the insoluble blue formazan salt, being the intensity of color produced dependent on the cell reducing activity (Mosmann, 1983). Thus, loss of MTT reduction may be used as an indicator of cell dysfunction. After treatment with or without recovery, cells were washed three times with PBS and incubated with MTT (0.5 mg/mL) prepared in Na^+ medium containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 10 mM Hepes, pH 7.4/NaOH,

in the dark, for 2 h at 37°C. At the end of the incubation with MTT, an equal volume of isopropanol-acid (0.04 M HCl in isopropanol) was added and mixed thoroughly to dissolve the formazan crystals. The mixture was then collected from the wells and the extent of MTT reduction was measured spectrophotometrically at 570 nm. The results were expressed as a percentage of control cells.

2.3.8 Isolation of 3xTg-AD and WT mice tissues

The triple transgenic mice (3xTg-AD) were obtained through the micro-injection of human APP cDNA harboring the swedish mutation (KM670/671NL) and human four-repeat tau harboring the P301L mutation into single cell embryos of homozygous PS1m146v knock-in mice (Oddo et al., 2003b;Oddo et al., 2003a), originally generated as a hybrid 129/C57BL6 background. 3xTg-AD and Non-transgenic (WT) mice were obtained from Prof. LaFerla (Univ. California, Irvine, USA) and were bred and maintained in our local colony. Animals were housed under a constant temperature, humidity and a 12 h light/dark cycle in a pathogen-free environment and all studies were performed according to the principles and procedures outlined in the EU guidelines (86/609/EEC). Before sacrifice, mice were weighed, then sacrificed by cervical dislocation and brain was removed. Brain were weighed after remotion; then, cortex and hippocampus were isolated from 3-, 12- and 15-month-old male and female 3xTg-AD mice (and age- and gender-matched WT mice). Tissues were frozen and stored at -80ºC. 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.

3.9 Immunohistochemistry

In Chapter 4, 3xTg-AD or WT mice were deeply anesthetized with 15 µl/g of body weight of a mixture of ketamine (18.3%, Imalgene 1000) and xylazine (10.2%, Rompun 2%) prepared in 0.9% NaCl pH 7.4. Mice were further perfused intracardially with 0.9% NaCl pH 7.4, and then with 4% paraformaldehyde in 0.9% NaCl, at pH7.4. Brain was removed and cerebral hemispheres were further fixed for 2 h at 4 ºC in 4% paraformaldehyde, washed with PBS overnight and immersed in 30 % (w/v) saccharose overnight, at 4ºC. Hemispheres were frozen in liquid nitrogen and sliced with a criostat (Leica, Mannheim, Germany) to obtain 40 µm coronal sections.

3.9.1 Fluorescent immunohistochemistry

Free floating sections were washed with PBS (three times 5 min) and incubated with blocking solution containing 3% BSA and 0.2% Triton X-100 in PBS for 1 h at RT. Slices were then incubated with primary antibodies (anti-APP and anti-Aβ 1-42), diluted in blocking solution, overnight at 4 ºC. Sections were further washed with PBS (three times 10 min) and incubated for 2 h at RT with the secondary antibodies Alexa Fluor goat anti-mouse 594 and Alexa Fluor goat anti-rabbit 488 prepared in PBS containing 0.2% Triton X-100. After washing three times 10 min with PBS, sections were counterstained with Hoechst (1:10,000) and mounted with fluoroshield mounting medium. Fluorescent images of hemisphere sections were obtained using an epifluorescence microscope (Axiovert Microscope 200, Zeiss).

3.9.2 DAB immunohistochemistry

Free floating sections were washed with PBS (three times 5 min) and blocked for endogenous peroxidase with 0.5% H_2O_2 (in PBS), for 30 minutes, in the dark, at RT. Sections were then washed three times 10 min with PBS and incubated with blocking solution containing 3% BSA and 0.2% Triton X-100 in PBS for 1 h. Slices were then incubated with primary antibody (anti-Aβ 1-16, 6E10 or anti-PHF-tau, AT8), diluted in blocking solution, overnight at 4 ºC. Sections were further washed with PBS (three times 10 min) and incubated for 2 h at RT with the secondary antibody biotinilated anti-mouse diluted in PBS containing 0.2% Triton X-100. The secondary antibody was rinsed from the sections three times 10 min with PBS. 30 minutes prior incubation, reagents A + B were combined (5 µL A + 5 µL B in 1 mL PBS) and incubated with the sections for 1h at RT. Sections were washed two times 10 min with PBS and with Tris.HCl 0.05 M, pH 7.6. Sections were the incubated with 0.025% DAB in PBS containing 0.003% H_2O_2 for the necessary time to reveal the staining. Section were washed with Tris.HCl 0.05 M, followed by three times 10 min with PBS. Sections were mounted on microscope slide, dehydrated with 5 min 70%, 80%, 96% ethanol, 2 × 10 min 100% ethanol, 10 min xylene and mounted in DPX. Brightfield images of DAB staining were obtained using the Axiovert Microscope 200 from Zeiss.

2.3.10 Participants for the human study

A total of 104 subjects participated in this study, including 20 healthy controls, 24 clinically confirmed MCI, 27 mild AD and 33 moderate/severe AD patients from

Portuguese families. Patients were recruited at the dementia outpatient clinics at Hospital Center of Coimbra University (CHUC), in accordance with the Ethical Committee from this institution. Age-matched controls were volunteers, usually spouses or friends of patients who were requested to participate in the study. MCI and AD cases were subjected to clinical history, neurological examination, laboratorial evaluation and brain imaging (computed tomography or nuclear magnetic resonance scan). Inclusion criteria for AD were based on the 4th edition of The Diagnostic and Statistical Manual of Mental Disorders (DSM IV – TR) (American Psychiatric association, 2000). MCI criteria were those proposed by the European Alzheimer's Disease Consortium (Portet et al., 2006). Cognitive impairment was also quantified using the Minimental-State Evaluation (MMSE) (Folstein et al., 1975). Patients were followed during several years with periodic clinical evaluations including specific staging scales, providing objective information about the evolution of the disease and the severity of dementia in various domains. Control subjects did not present evidence of cognitive deterioration or cognitive complaint and had a MMSE above cut-off and their value in the Clinical Dementia Rating Scale (CDR) was zero. The exclusion criterion for all groups was the presence of other neurological, psychiatric or medical pathologies that could cause cognitive impairment, or a history of alcohol or drug abuse. All participants signed an informed consent before any study procedure. For AD patients, informed consents from respective caregivers were also obtained. Demographic and clinical characteristics of the participants are shown in Table 5.1 (Chapter 5).

2.3.11 Isolation and culture of human peripheral blood mononuclear cells

Peripheral whole blood from MCI, AD or healthy age-matched individuals was drawn in heparinized tubes and cells separated by gradient centrifugation in order to discard erythrocytes and granulocytes. Briefly, 10 ml of blood were carefully layered onto 8 ml of Ficoll solution and tubes centrifuged at 1500 *g* for 20 minutes at 18°C in a swing-out rotor, without break. After centrifugation, the ring containing mononuclear cells was carefully removed from the interface using a sterile Pasteur pipette and the harvested fraction diluted in 45 ml of sterilized PBS containing (in mM): 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 $Na_2HPO_4.2H_2O$, pH 7.4. An aliquot of serum was removed from the upper layer of the gradient and kept at RT until use. Cells were pelleted by centrifugation at 530 *g* for 10 minutes at 18°C, ressuspended in RPMI 1640 without serum. Cells were immediately extracted (as described in 3.14) for Western blot analysis or alternatively

cultured for 1 day in RPMI 1640 medium plus 10% (v/v) autologous serum in 25 cm^2 culture flasks at a concentration of $2x10^6$ cells/ml in a humidified incubator chamber with 95% air and 5% CO_2 at 37ºC, for intracellular Ca^{2+} measurements or ROS production experiments.

2.3.12 Intracellular free Ca^{2+} recording

Cells were washed in Na^+ medium containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4/NaOH and then incubated in Na^+ medium containing the fluorescent probe Fura-2/AM (10 µM) for 40 min at 37ºC. After a washing step, cells were placed in Mg^{2+}-free Na^+ medium in the presence of 10 µM glycine in order to potentiate putative effects of glutamate stimulation. $Ca^{2+}{}_i$ levels were measured using $0.5x10^6$ cells per experimental condition, in a microplate reader Spectrofluorometer Gemini EM (Molecular Devices, USA) (340/380 nm excitation wavelength, 510 nm emission wavelength) for 5 min (basal values) and 15 min after being stimulated with 1 mM H_2O_2 or 1 mM glutamate. All plotted values were normalized for baseline levels.

2.3.13 Measurement of reactive oxygen species production

Cells were washed in Na^+ medium containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4/NaOH and then incubated with the fluorescent probe $DCFH_2$-DA (20 µM) in Na^+ medium for 30 min at 37 ºC. After a washing step, cells were processed as described in previous section. The fluorescence signals, corresponding to intracellular ROS production were monitored for 5 min (basal values) and for 30 min after stimulus addition (1 mM H_2O_2 or 1 mM glutamate) in a microplate reader (SpectraMax Gemini EM) at 480 nm excitation and 550 nm emission. All plotted values were normalized for baseline levels.

2.3.14 Sample preparation and Western blotting

Cells were extracted in RIPA buffer (Chapters 3 and 5) containing 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, supplemented with 1 mM DTT, 1 mM PMSF and 1 µg/mL protease inhibitors (chymostatin, pepstatin A, leupeptin and antipain). For experiments described in chapter 4, cortex and hippocampus isolated from mice were homogenized at 280 rpm (20 strokes) in a tissue homogeneizer in 22.5 ml of ice-cold supplemented RIPA buffer

(described previously) per gram of tissue. Homogenates were then centrifuged at 14,000xg for 10 min at 4ºC and the pellet was discarded. Supernatant was collected and protein content was determined using the BioRad protein assay reagent, using the Bradford dye-binding procedure. For analysis of GluN2A and GluN2B subunits (Chapter 3), cells were washed with PBS and extracted in 1% SDS, boiled for 10 min and further sonicated for 15 min. In this case, protein content was determined by the BCA protein assay. Samples were prepared with denaturating buffer containing 50 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 100 mM DTT, 0.01% bromophenol blue, for 5 min, at 95ºC. Equivalent amounts of protein were separated by electrophoresis on a 6-15% SDS-PAGE gel and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were further blocked with 5% fat-free milk or 5% BSA before incubation with the specific antibody overnight, at 4ºC. An anti-mouse IgG secondary antibody or anti-rabbit was used. Immunoreactive bands were visualized by alkaline phosphatase activity after incubation with ECF reagent on a BioRad Versa Doc 3000 Imaging System.

2.3.15 Statictical analysis

Data were expressed as mean ± SEM of the number of experiments indicated in the figure legends. Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA) followed by the Dunnett's Multiple Comparison post-hoc test or by two-way ANOVA followed by the Bonferroni's post-hoc test in the case of 3xTg-AD *vs* WT comparisons (Chapter 4). Student's *t*-test was also performed for comparison between two Gaussian populations, as described in figure legends. Significance was defined as $p < 0.05$.

Chapter 3

Amyloid-beta peptide 1-42 causes microtubule deregulation through *N*-methyl-D-aspartate receptors in mature hippocampal cultures

3.1. Summary

AD is the most common age-related neurodegenerative disorder among the elderly. NMDAR overactivation has been implicated in early synaptic dysfunction that precedes late neurodegeneration in AD. Moreover, oligomers of Aβ $_{1-42}$ are considered the most synaptotoxic forms, responsible for early cognitive deficits in AD. In this work we evaluate the role of NMDARs on Aβ-evoked neuronal dysfunction and cell death through changes in microtubule polymerization in mature hippocampal cultures. Exposure to Aβ $_{1-42}$ caused a decrease in total and polymerized levels of beta-III tubulin and polymerized alpha-tubulin, suggesting microtubule disassembly. Furthermore, Aβ induced DNA fragmentation in neuronal and non-neuronal cells. Indeed, the effects of Aβ on beta-III tubulin polymerization were significantly correlated with reduced neurite length and neuronal DNA fragmentation. Interestingly, these effects were prevented by MK-801 and memantine, largely suggesting the involvement of extrasynaptic NMDARs on Aβ toxicity, and by ifenprodil, further indicating the involvement of GluN2B-containing NMDARs. Nevertheless, exposure to Aβ did not potentiate the effects caused by selective activation of NMDARs. Data largely suggest that Aβ-induced hippocampal neuronal dysfunction occurs through NMDAR-dependent microtubule disassembly associated to neurite retraction and DNA fragmentation in mature hippocampal cells.

3.2. Introduction

AD is the main cause of dementia in the elderly. Extensive research has emerged regarding the role of Aβ in AD pathogenesis (Selkoe, 2000), since mutations in the APP or in PS, proteases that promote the production of Aβ from APP, have been shown to cause familial forms of AD (LaFerla and Oddo, 2005). Recent findings posit that Aβ induces an increase in cytosolic Ca^{2+} levels that may underlie mitochondrial Ca^{2+} dyshomeostasis and ultimately damaging the neurons, namely by activating NMDARs, a subtype of ionotropic glutamate receptors (Ferreira et al., 2010).

Oligomeric forms of Aβ have been shown to be more toxic than monomers or unsoluble forms (Resende et al., 2008a;Shankar et al., 2007), being $Aβ_{1-42}$ the most toxic form of the peptide (Selkoe, 1996). Recently, Jan and colleagues (Jan et al., 2011b) evidenced that crude $Aβ_{1-42}$ preparations, consisting of monomeric and oligomeric Aβ forms, were more toxic than purified monomeric, protofibrillar fractions or fibrils in different cell lines and primary neurons (Jan et al., 2011b). Moreover, Aβ peptides 1-40, 1-42 and 25-35 were able to block long term potentiation in hippocampal slices (Chen et al., 2000) and in the CA1 region of rat hippocampus (Freir et al., 2001). Indeed, $Aβ_{1-42}$ oligomers led to impairment in long-term potentiation (Li et al., 2011;Ronicke et al., 2011), reducing baseline synaptic transmission and neuronal spontaneous network activity (Ronicke et al., 2011). Concomitantly, Aβ has been linked to retraction of synaptic contacts, which can be associated to microtubule deregulation, long before major cytotoxic effects are visible in both primary hippocampal cell culture and hippocampal slices from rat and mouse (Ronicke et al., 2011). Recently, it was shown that Aβ leads to a dramatic decrease in alpha-tubulin acetylation, suggesting a decrease in microtubule stability in primary neuronal cultures (Henriques et al., 2010). Furthermore, Aβ oligomers caused microtubule and mitochondrial depletion, tau missorting and loss of spines, which were prevented by taxol, a microtubule stabilizer (Zempel et al., 2010), largely suggesting Aβ-induced microtubule destabilization in AD. Moreover, $Aβ_{1-42}$ oligomers induce a massive entry of Ca^{2+} in cortical and hippocampal neurons, promote mitochondrial Ca^{2+} overload and induce the mitochondrial permeability transition pore, cytochrome c release, apoptosis and cell death, showing large similarities with the excitotoxic process (Sanz-Blasco et al., 2008). Interestingly, physiological amounts of $Aβ_{1-42}$ oligomers are not sufficient to induce apoptosis in human primary neurons (from fetal brains with 13-17 weeks), but are able to increase their vulnerability by depleting the levels of the anti-apoptotic protein Bcl-2 (Sanz-Blasco et al., 2008).

NMDARs play a pivotal role in synaptic mechanisms of learning and memory. Functional NMDARs are heterotetramers composed of two glycine-binding GluN1 subunits and two glutamate-binding GluN2 (GluN2A-D) subunits, or in some cases GluN3 (GluN3A-B) subunits, the later replacing the GluN2 subunits (Cull-Candy and Leszkiewicz, 2004). The most widely expressed NMDARs contain the obligatory subunit GluN1 plus either GluN2B or GluN2A or a mixture of the two. The role of NMDARs in Aβ-mediated synaptic dysfunction and cell death has been investigated in the last few years. Indeed, neurodegeneration and synaptic dysfunction due to massive entry of Ca^{2+} induced by $A\beta_{1-42}$ oligomers involve overactivation of NMDARs (Alberdi et al., 2010). By using an $A\beta_{1-42}$ preparation enriched in oligomers we recently showed that the peptide disturbs intracellular Ca^{2+} homeostasis through activation of GluN2B-containing NMDARs in primary cortical cultures (Ferreira et al., 2012). Moreover, Aβ oligomers negatively impact on axonal transport by a mechanism initiated by NMDARs activation (Decker et al., 2010) and both Aβ-induced spine pathology and tau-dependent neurodegeneration were shown to be mediated by downstream NMDARs activation pathways (Tackenberg and Brandt, 2009), suggesting that NMDARs mediate Aβ toxicity. It was also demonstrated that $A\beta_{1-42}$ modifies NMDAR trafficking and activation, leading to GluN1 and GluN2B endocytosis through dephosphorylation of GluN2B subunit at Tyr1472 (Snyder et al., 2005).

Activation of NMDARs was initially hypothesized to occur at late-stage AD since memantine, an uncompetitive open channel blocker of NMDARs, is prescribed as a memory-preserving drug for moderate- to late-stage AD patients (Reisberg et al., 2003). However, more recent reports suggest that activation of NMDARs by Aβ accumulation occurs at early stages of the disease (Parameshwaran et al., 2008). Early trapping of NMDAR at synapses was observed in the hippocampus of a conditional knock out mouse for PS1 and 2, which may underlie the reduced synaptic plasticity (Aoki et al., 2009). Ronicke and colleagues (Ronicke et al., 2011) demonstrated that the early neuronal dysfunction induced by Aβ is mediated by an activation of GluN2B subunits in primary neuronal culture and hippocampal slices from rat and mouse. More recently, it has been suggested that the enhancement of GluN2A activity and/or the reduction of GluN2B activity can halt manifestation of a key early-stage event in AD (Liu et al., 2010).

In the present study we investigated the role of NMDARs on Aβ-induced early cellular dysfunction through alterations in beta-III tubulin (a microtubule element expressed

exclusively in neurons) and polymerized tubulin as an approach to evaluate microtubule deregulation using mature rat hippocampal primary cultures. We found that $A\beta_{1-42}$ enriched oligomer preparation induce neuronal microtubule deregulation in a process regulated by the NMDARs, which is unrelated with massive cell death.

3.3. Results

3.3.1 Characterization of mature hippocampal culture

Expression of GluN2A and GluN2B subunits of the NMDARs depends on the developmental stage of the neurons, since GluN2B is expressed during embryonic phase, whereas GluN2A expression is only evident after birth (Monyer et al., 1994;Zhou and Baudry, 2006). Furthermore, established synapses in cultured mature hippocampal cells have been defined at > 10 days *in vitro* (DIV) (Jun et al., 2007). In order to assess the stage of hippocampal cells in culture, we examined total expression levels of GluN2A and GluN2B-containing NMDARs subunits with increasing time in culture. Data depicted in Figure 3.1A show that protein levels of GluN2A and GluN2B subunits increase with age in culture (up to 21 DIV) when compared with fresh whole hippocampus obtained from 18-19-day-old embryos (0 DIV). Our results also demonstrate that GluN2B and, at a less extent GluN2A subunits, are expressed in E18-19 embryos (0 DIV), as previously described (Thomas et al., 2006;Grabrucker et al., 2009;Basarsky et al., 1994). Furthermore, the protein levels of GluN2A and GluN2B were shown to be similar in hippocampal cells cultured for 17-21 DIV. Hippocampal cultures at 17 DIV were further characterized by using both the neuronal markers Neu-N and MAP-2, the synaptic marker synaptophysin, as depicted in Figure 3.1B, confirming the presence of synapses and a neuronal network. Altogether, these results indicate the presence of mature, synapse-forming neurons expressing both GluN2A and GluN2B NMDARs subunits in our hippocampal cultures. The glial cell marker GFAP (glyceraldehyde 3-phosphate dehydrogenase) was further used to confirm the presence of astrocytes (Fig 3.1 B).

According to this characterization, the percentage of neurons in our culture was about 43%, indicating the presence of a co-culture of neuronal and non-neuronal cells. Rat mature hippocampal cells cultured for 17 DIV were used in the following experiments.

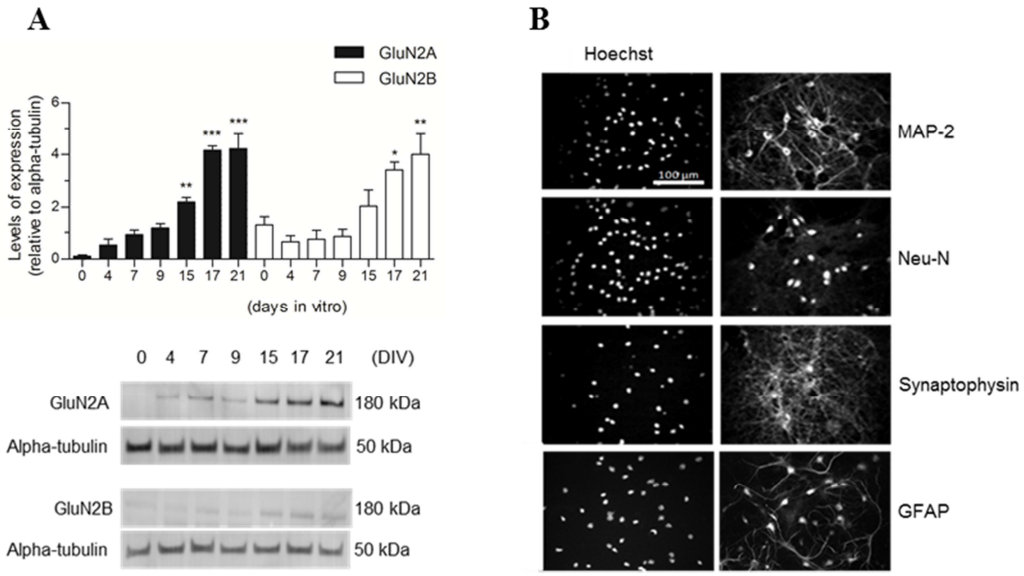

Figure 3.1: Characterization of rat hippocampal neuronal culture. A. Time-dependent analysis of GluN2A and GluN2B subunit protein expression levels by Western blotting of total extracts derived from whole rat brain before dissociation (0 days *in vitro*, DIV) and in hippocampal neurons maintained in culture (at 4, 7, 15, 17 and 21 DIV). **B.** Cells were stained with selective antibodies for the neuronal markers MAP-2, Neu-N, synaptophysin and GFAP, at 17 DIV. Nuclei were stained with Hoechst 33342 and visualized by epifluorescence microscopy. Data are the mean ± SEM of 3-4 independent experiments. Statistical analysis: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different when compared with subunit expression at 0 DIV. (Dunnett's *post-hoc* test).

3.3.2 Toxic effects of Aβ

The toxic profile of Aβ was then evaluated in 17 day-old mature hippocampal cultures. In previous studies Puzzo and colleagues (Puzzo et al., 2008) showed that pM concentrations of $A\beta_{1-42}$ monomers and oligomers cause a marked increase, whereas nM concentrations reduce hippocampal long-term potentiation. Moreover, while the concentration of $A\beta_{1-42}$ oligomers in the cerebrospinal fluid of AD patients is in the nM range (Tomic et al., 2009), the concentration of soluble $A\beta_{1-42}$ is about 3 µM in pyramidal neurons from the hippocampus of AD patients (Hashimoto et al., 2010). Taking into account these studies and recent characterization of a similar Aβ preparation in primary cortical neurons (Ferreira et al., 2012), in the present work we analysed the toxic profile caused by 500 nM Aβ.

We analysed acute hippocampal cell toxicity after exposure for 6 h to Aβ (Fig. 3.2 A). Our results show that Aβ induced a small but significant decrease in cell viability (7%; $p<0.05$ by *t*-test) when compared to the control and that this effect was counteracted by MK-801, the potent non-competitive antagonist of the NMDARs (Fig. 3.2 A).

Treatment of hippocampal cells with 100 µM NMDA plus 10 µM glycine, in the absence of added Mg^{2+}, for 15 min, triggered about 20% decrease in cell viability. Additional exposure to NMDA/gly, in cells previously subjected to Aβ for 6 h, did not significantly potentiate the effect exerted by NMDA/gly alone (25%, $p<0.001$). However, acute toxicity induced by Aβ plus NMDA/gly was not completed prevented by MK-801 (Fig. 3.2 A).

We next analysed the delayed toxicity boosted by the previous described conditions, evaluated after 18 h ('recovery period'), as depicted in Figure 3.2 B. In this case, after each treatment, cells returned to the incubation chamber in the conditioned cultured medium (collected before each treatment). As shown in Figure 3.2 B, cells appear to have recovered from Aβ-induced toxicity; however, cell toxicity induced by NMDA/gly was increased by about 43% ($p<0.001$) upon the 'recovery period' and again it was not potentiated by a pre-exposure to Aβ. Under these conditions, MK-801 fully prevented the effects caused by Aβ plus NMDA/gly on mature hippocampal cells.

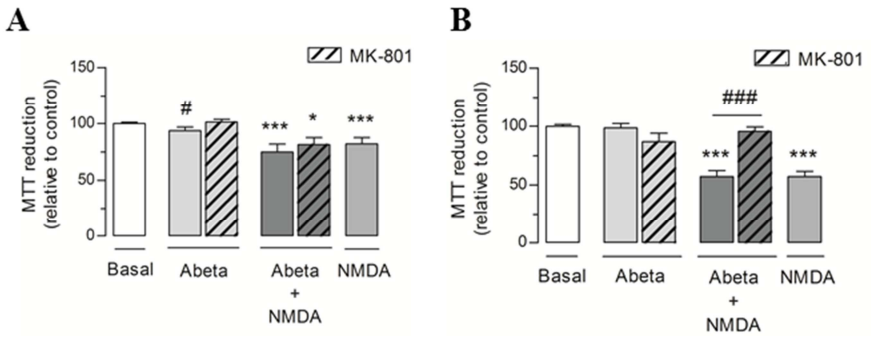

Figure 3.2: Effect of Aβ on cell viability. To analyze early toxicity, cells were incubated with 500 nM Aβ during 6 h in the absence or presence of 5 µM MK-801, 10 µM memantine or 10 µM ifenprodil, followed by incubation with 100 µM NMDA/gly in the absence of Mg^{2+}, for 15 min, with or without antagonists (**A**). In **B** incubations were followed by a recovery period of 18 h in conditioned cell culture medium, in order to study delayed toxicity. In **A, B,** cell reducing capacity by the MTT test was expressed in percentage of control. Results were expressed as the mean ± SEM of 3-6 distinct experiments performed in triplicate or quadruplicate. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* control (one way ANOVA followed by Dunnett's post-hoc test), $^{\#}p < 0.05$ *versus* control (*t*-test).

Since acute or delayed exposure of hippocampal cells to 500 nM Aβ did not exhibit massive cell death, as evaluated by the MTT assay, we further analyzed the effect of delayed exposure to Aβ and/or NMDA/gly on DNA condensation/fragmentation, using the fluorescent dye Hoechst 33342 (Fig. 3.3 A and B). Our results demonstrate that exposure to 500 nM Aβ (6 h incubation plus 18 h 'recovery period') alone significantly increased DNA condensation/fragmentation in mature hippocampal cells (14% of total

cells, $p<0.01$). Moreover, NMDA/gly treatment in the absence or presence of Aβ induced a significant increase in DNA condensation/fragmentation (by about 17% and 21% of total cells, $p<0.01$ and $p<0.001$, respectively). Our results show that both Aβ and Aβ plus NMDA effects were completely prevented by the NMDARs antagonists MK-801 and memantine (Fig. 3.3 A and B). Since previous unpublished results showed that ifenprodil (a selective antagonist of GluN2B subunit), but not NVP-AAM077 (a GluN2A selective antagonist), reverted the decrease in hippocampal cells viability induced by Aβ, the effect of ifenprodil was also tested in our conditions. Results depicted on Figures 3.3 A and B also indicate that ifenprodil completely prevent the effects induced by both Aβ and Aβ plus NMDA, suggesting that Aβ cause delayed cell death by activating GluN2B containing NMDARs in mature hippocampal cells.

We further evaluated the contribution of both neuronal and non-neuronal hippocampal cells regarding chromatin condensation/fragmentation, as depicted in Figures 3.3 C and D. Interestingly, Aβ alone or in the presence of NMDA/gly induced a significant increase in DNA condensation/fragmentation in both neuronal and non-neuronal cells and this effect was completely counteracted by MK-801, memantine or ifenprodil (Fig. 3.3 C and D). Moreover, NMDA/gly treatment alone induced a significant increase in DNA fragmentation in neuronal cells and non-neuronal cells, when compared to basal conditions, which was not potentiated by Aβ. Altogether, these results suggest that Aβ cause DNA condensation/fragmentation, commonly related with apoptotic cell death, in neuronal and non-neuronal cells, the latter having a fundamental role in maintaining neuronal function (Ben and Pascual, 2010) and being implicated in modulation of neurotoxic injury (Aschner et al., 2002).

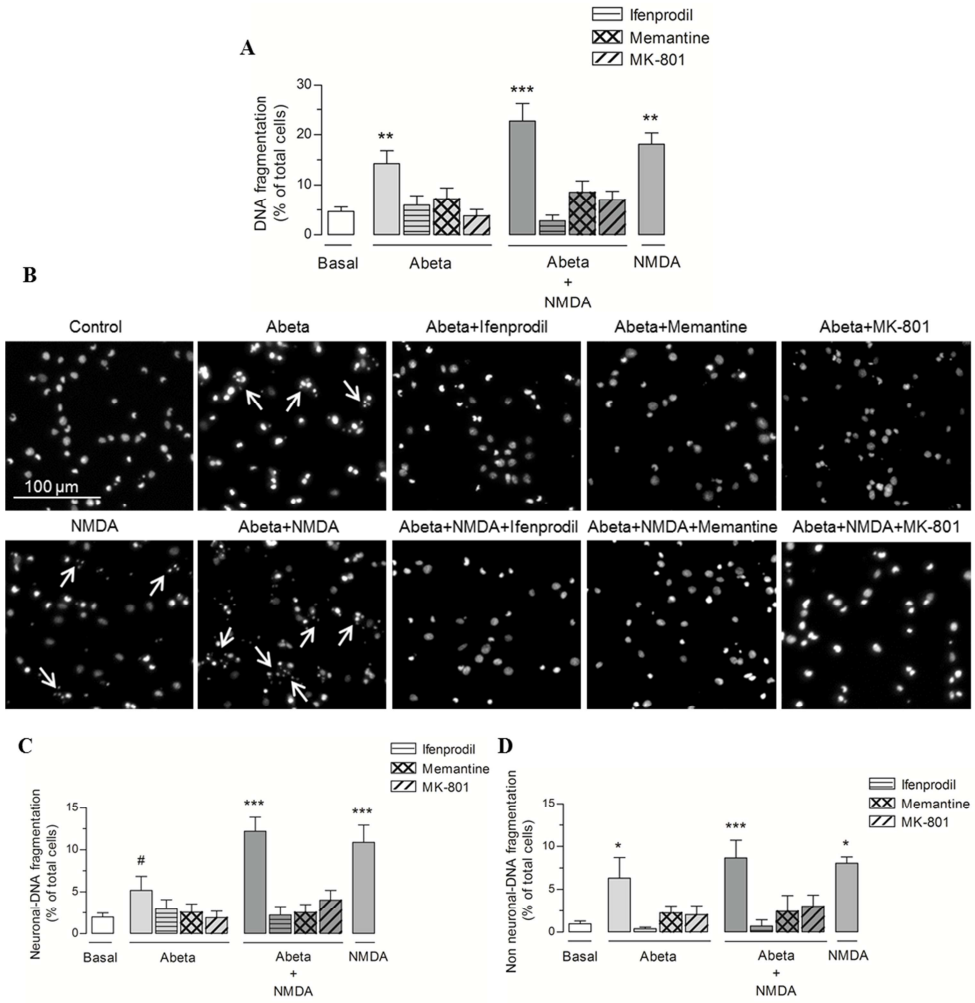

Figure 3.3: Effect of Aβ on cell viability and DNA fragmentation. Cells were incubated with 500 nM Aβ during 6 h in the absence or presence of 5 µM MK-801, 10 µM memantine or 10 µM ifenprodil, followed by incubation with 100 µM NMDA/gly in the absence of Mg^{2+}, for 15 min, with or without antagonists and incubations were followed by a recovery period of 18 h in conditioned cell culture medium, in order to study delayed toxicity. Cells were then stained with Hoechst 33342 and nuclei showing DNA condensation/fragmentation were counted. In **A**, cell viability is expressed as a percentage of the total number of cells (approximately 40 cells per field were counted and 5-6 fields per coverslips were analyzed). **B** shows representative images of Hoechst 33342 staining of data depicted in **A**. **C** and **D** show DNA fragmentation in neuronal and non-neuronal cells, respectively, as a percentage of the total number of cells, as defined upon labeling for beta-III tubulin. Results are expressed as the mean ± SEM of 3 to 7 distinct experiments performed in duplicates. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* control (one way ANOVA followed by Dunnett's post-hoc test), $^{\#}p < 0.05$ *versus* control (*t*-test).

3.3.3 Aβ induces neuronal dysfunction through microtubule disassembly

AD is characterized by the presence of aggregates of Aβ and hyperphosphorylated tau, a modification of a microtubule-associated protein that may alter cytoskeletal dynamics in neurons (Zempel et al., 2010). Thus, we further analysed the levels of the neuronal marker, beta-III tubulin protein, and microtubule assembly upon Aβ exposure. Results depicted in Figure 3.4 A show that acute exposure of hippocampal neurons to 500 nM

Aβ for 2 h did not affect cytoskeletal beta-III tubulin total protein levels, in contrast with 6 h exposure in which a decrease of about 40% ($p<0.05$) was observed. In the presence of memantine and ifenprodil, Aβ-induced decrease in beta-III tubulin levels was not significant, although did not completely prevent the toxic effect (Fig. 3.4 A). We further analyzed the delayed effect of Aβ in the presence or in the absence of NMDA/gly on beta-III tubulin labeling (Fig. 3.4 B and C). In these conditions Aβ, and at a higher extent NMDA/gly and Aβ plus NMDA/gly, significantly decreased the number of beta-III tubulin positive cells by about 40% ($p<0.001$) and 95% ($p<0.001$), respectively. Moreover, pre-incubation with ifenprodil, memantine and MK-801 completely prevented the decrease in beta-III tubulin labelling following exposure to Aβ alone. Interestingly, the decrease on beta-III tubulin positive cells achieved by Aβ plus NMDA/gly was fully counteracted by pre-incubation with ifenprodil and memantine, but partially counteracted by MK-801 pre-incubation (Fig. 3.4 B and C). Exposure to NMDA/gly alone caused a similar drastic decrease in beta-III tubulin labelling ($p<0.001$) as that achieved by NMDA/gly in the presence of Aβ (Fig. 3.4 B and C).

In order to investigate whether the observed decrease in neuronal specific beta-III tubulin was due to a decrease in polymerized tubulin, the polymerized tubulin fraction was extracted from hippocampal cells (Fig. 3.5). Our results demonstrate that acute effects evoked by 6 h exposure to 500 nM Aβ does not affect significantly the levels of polymerized beta-III tubulin (Fig. 3.5 A), but decrease the levels of polymerized alpha-tubulin (about 25% relatively to the control, $p<0.05$), an effect counteracted by MK-801 (Fig. 3.5 C). We further analyzed the effects induced by delayed exposure to Aβ (Fig. 3.5 B and D). Interestingly, Aβ in the absence or in the presence of NMDA/gly significantly decreased polymerized beta-III tubulin by about 25% (Fig 3.5B, $p<0.01$) in hippocampal cultures. Importantly, MK-801 rescued polymerized beta-III tubulin in cells treated with Aβ alone or Aβ plus NMDA/gly, and memantine or ifenprodil prevented the effect of Aβ on polymerized beta-III tubulin. Furthermore, our results show a tendency, although not statistically significant, for a decrease in polymerized alpha-tubulin in cells treated with Aβ alone and a significant decrease (20%; $p<0.05$ by *t*-test) in cells treated with Aβ plus NMDA/gly (Fig. 3.5 D). These results suggest that exposure to Aβ affects the levels of polymerized tubulin in neuronal cells in a process that depends on GluN2B-containing NMDARs. Interestingly, early effects of Aβ (after 6 h incubation) accounted for changes in polymerized alpha-tubulin, whereas the

neuron-specific tubulin isoform was significantly affected following delayed Aβ incubation.

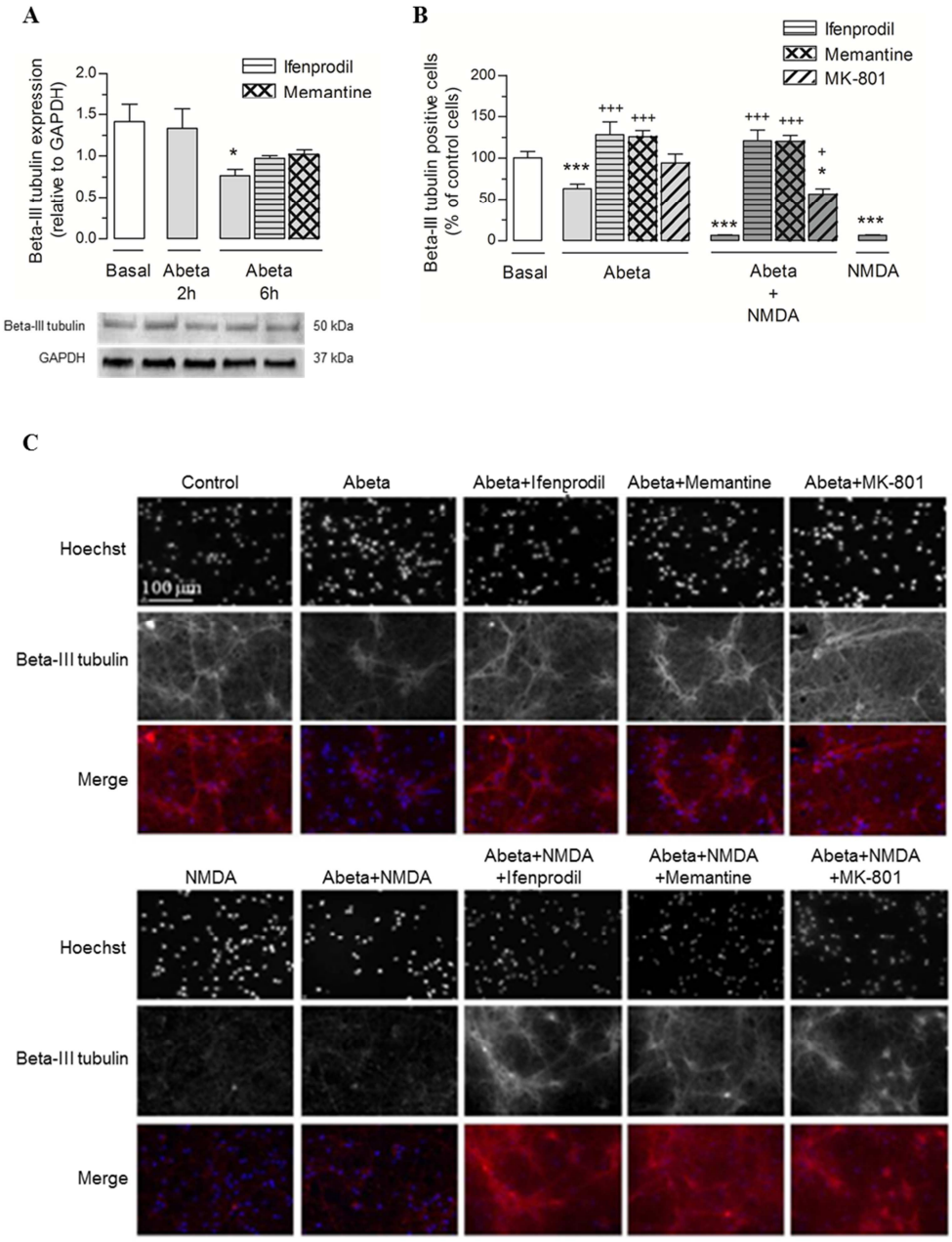

Figure 3.4: Effect of Aβ on beta-III tubulin protein levels. A. Cells were incubated with 500 nM Aβ during 2 or 6 h in the absence or presence of 5 µM MK-801, 10 µM memantine or 10 µM ifenprodil and extracted in Ripa buffer and immunoblotted, as described in Methods. Beta-III tubulin protein levels were expressed in arbitrary units relative to GAPDH. **B** and **C**. Cells were incubated with 500 nM Aβ during 6 h, in the absence or presence of 5 µM MK-801, 10 µM memantine or 10 µM ifenprodil, followed by incubation with 100 µM NMDA/gly for 15 min ± antagonists. Then, the cells returned to the incubation chamber for 18 h in culture conditioned medium. Cells were immunolabeled with anti-tubulin beta III and nuclei were stained with Hoechst 33342. In **B** beta-III tubulin positive cells are expressed relative to the total of cells labeled with Hoechst 33342 (approximately 40 cells per field and five fields per coverslips were counted). **C** shows representative images of data depicted in B. Results are the mean ± SEM of 4-7

distinct experiments. Statistical significance: $^{*}p < 0.05$, $^{***}p < 0.001$ *versus* control (one way ANOVA followed by Dunnett's post-hoc test).

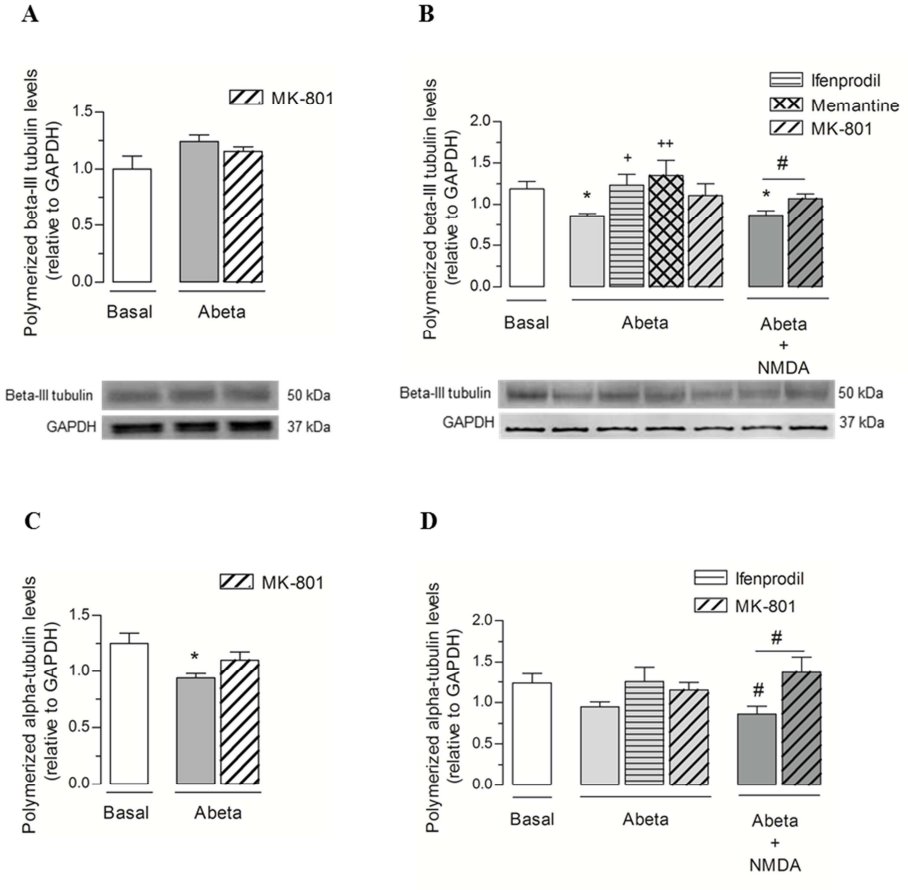

Figure 3.5: Influence of Aβ on beta-III tubulin and alpha-tubulin polymerization. In **A** and **C**, cells were treated with 500 nM Aβ during 6 h, in the absence or presence of 5 µM MK-801. In **B** and **D**, cells were incubated with 500 nM Aβ during 6 h, in the absence or presence of 5 µM MK-801, 10 µM ifenprodil or 10 µM memantine, followed by incubation with 100 µM NMDA/gly for 15 min ± antagonists. Then, cells returned to the incubation chamber for 18 h in culture conditioned medium. Polymerized tubulin fraction was extracted and immunoblotted for beta-III tubulin (**A**, **B**) or alpha-tubulin (**C**, **D**). Results are the mean ± SEM of 4-7 distinct experiments. Statistical significance: $^{\#}p < 0.05$ *versus* control (*t*-test), $^{*}p < 0.05$ *versus* control (one way ANOVA followed by Dunnett's post-hoc test).

We further analysed MAP-2 labelling in cells exposed to Aβ (Fig. 3.6 A and B). Although cell body labelling for MAP-2 did not vary significantly upon exposure to Aβ, treatment with NMDA/gly or Aβ plus NMDA/gly significantly decreased cell body size as depicted by the arrows in Figure 6A. In addition, incubation with the Aβ peptide or NMDA/gly alone significantly ($p<0.001$) decreased neurite length by about 30% and 65% respectively (Fig. 3.5 B). Moreover, Aβ effects were largely ameliorated by MK-801, memantine or ifenprodil (Fig. 3.6 A and B). Aβ pre-exposure did not account for an additional decrease in neurite length exerted by NMDA/gly, although it was only

partially prevented by the NMDAR antagonist MK-801, similarly to what was observed for the delayed effects of Abeta plus NMDA/gly on beta-III tubulin positive cells (Fig. 3.4 B). Interestingly, both memantine and ifenprodil completely counteracted the effects of Aβ plus NMDA/gly on neurite length (Fig. 3.6 A and B).

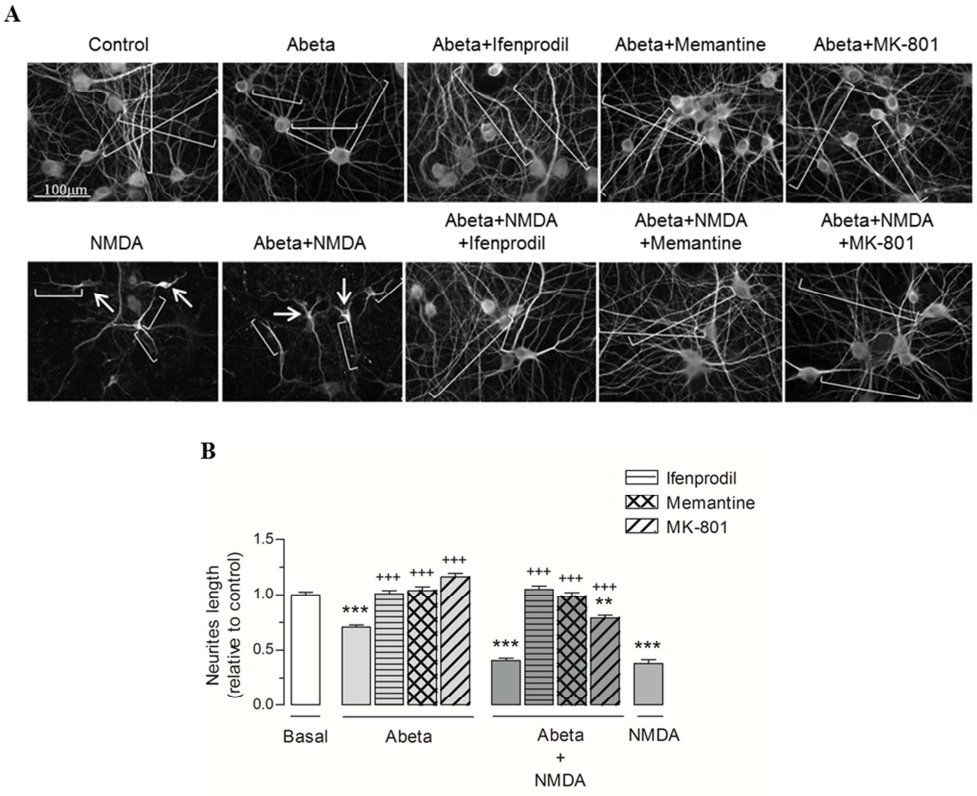

Figure 3.6: Effect of Aβ on neurite length. Cells were incubated with 500 nM Aβ during 6 h, in the absence or presence of 5 µM MK-801, 10 µM memantine or 10 µM ifenprodil, followed by incubation with 100 µM NMDA/gly for 15 min ± antagonists. Then, the cells returned to the incubation chamber for 18 h. Cells were immunolabeled for MAP-2 and nuclei were stained with Hoechst 33342. Measurement of neurite length was performed with the software Axiovision, as shown in **A**. In **B**, data were expressed as the mean ± SEM of 3-7 distinct experiments performed in duplicates. Statistical significance: $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* control (one way ANOVA followed by Dunnett's post-hoc test).

For better understanding the relationship between depolymerized neuron-specific beta-III tubulin and decreased neurite length in cells exposed to Aβ and/or NMDA/gly and the protective effect achieved by MK-801, memantine and ifenprodil, data from Figures 3.5 B and 3.6 B were replotted, as shown in Figure 3.7A. Interestingly, data suggest that there is a significant ($p<0.05$) and strong positive correlation between neurite length and polymerized beta-III tubulin ($r= + 0.797$), suggesting that Aβ-induced microtubule disassembly is closely linked to neurite retraction in mature hippocampal neurons. In the same way, data from beta-III tubulin polymerization (Fig 3.5 B) and neuronal DNA

fragmentation (Fig 3.3 C) were replotted as shown in Figure 3.7B. We found a significant ($p<0.05$) negative correlation between neuronal DNA fragmentation and polymerized neuron-specific beta-III tubulin ($r= – 0.725$), suggesting that Aβ-induced microtubule disassembly is highly linked to DNA fragmentation in cultured neurons.

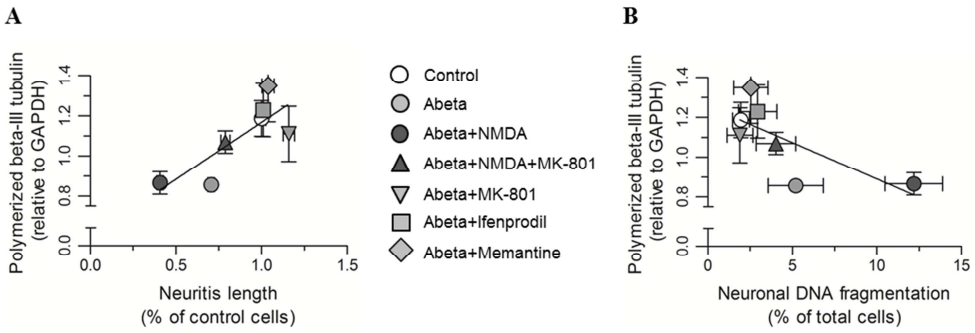

Figure 3.7: Correlations between tubulin depolymerization and neuritis length or DNA fragmentation. A. Correlation analysis between neurite length and polymerized beta-III tubulin. Data in the graph are from experiments showing polymerized tubulin (Fig 3.5B) and neurite length (Fig 3.6B) in mature hippocampal neurons exposed to all experimental conditions. B**.** Correlation analysis between neuronal DNA fragmentation and polymerized beta-III tubulin. Data in the graph are from experiments showing polymerized tubulin (Fig 3.5B) and DNA fragmentation (Fig 3.3C) in mature hippocampal neurons exposed to all experimental conditions.

Collectively, these results suggest that $A\beta_{1-42}$ leads to microtubule disassembly due to a decrease in polymerized tubulin, which parallels a decrease in neurite length and DNA fragmentation, involving GluN2B-containing NMDARs.

3.4. Discussion

AD is characterized by synapse and neuronal loss in the brain. Recently, the importance of soluble species of Aβ in triggering synaptic dysfunction long before neuronal death, in early stages of the disease, has become apparent (Sakono and Zako, 2010). Moreover, there is growing consensus regarding the involvement of NMDAR in neurodegeneration in AD. In the present study we provide evidence that the toxic effects induced by 500 nM $A\beta_{1-42}$-enriched oligomer preparation in hippocampal cells, are counteracted by MK-801, memantine and ifenprodil, evidencing the involvement of NMDAR and, particularly, the GluN2B subunit. Aβ did not trigger massive cell death, but caused delayed DNA fragmentation. Interestingly, our results show that Aβ induces microtubule disassembly, as evidenced by a decrease in polymerized alpha-tubulin and beta-III tubulin and reduced neurite length, in a process mediated by NMDARs.

NMDARs blockade with memantine, a memory-preserving drug, usually prescribed for treatment of moderate to severe AD patients and which exerts an effective antagonism of excessive chronic NMDARs activity (Reisberg et al., 2003), was previously shown to reduce neurodegeneration induced by $A\beta_{1-40}$ (Miguel-Hidalgo et al., 2002). More recently, memantine prevented the deleterious effect of $A\beta_{1-42}$ on synaptic plasticity and learning behavior in rats (Klyubin et al., 2011). A decrease in synaptic responses due to a reduction of the PSD-95 and NMDARs levels was also observed in response to Aβ oligomers exposure in hippocampal neurons (Dinamarca et al., 2008). Furthermore, still in hippocampal neurons, Aβ oligomers stimulated excessive formation of reactive oxygen species, which was shown to be dependent on a mechanism requiring NMDAR activation, since the effect of Aβ was completely blocked by an antibody for the extracellular domain of GluN1, an obligatory subunit of NMDARs, and by memantine (De Felice et al., 2007). Recently, Decker and colleagues demonstrated that Aβ oligomers-induced disruption of trafficking is prevented by NMDARs antagonists MK-801, D-AP5 and memantine (Decker et al., 2010). In AD brain and human cortical neurons, Aβ oligomers co-localize with synaptic markers, being this effect counteracted by the NMDARs antagonists memantine and ifenprodil (Deshpande et al., 2009), and ifenprodil prevented Aβ-mediated synaptic plasticity disruption in the hippocampus *in vivo* (Hu et al., 2009). Other studies evidenced that Aβ oligomers may induce an activation of NMDARs directly by binding to the receptors (De Felice et al., 2007) or indirectly by increasing glutamate release (Brito-Moreira et al., 2011), suggesting an interaction between Aβ oligomers and NMDARs in neuronal dysfunction observed in

AD. In the present study we demonstrate the involvement of NMDARs in the toxicity induced by $A\beta_{1-42}$ since all its effects were partially or completely prevented by distinct NMDAR antagonists. Interestingly, consistent protection was achieved with memantine and ifenprodil, but not with MK-801, stressing the importance of extrasynaptic NMDARs, mainly composed of GluN2B subunits (Stocca and Vicini, 1998;Tovar and Westbrook, 1999). Indeed, the concentration of memantine used in this study has been shown to mainly block extrasynaptic NMDARs (Xia et al., 2010).

Studies on AD transgenic mice also support the importance of Ca^{2+} homeostasis perturbation in AD (LaFerla, 2002), which may result from NMDARs overactivation. In previous studies, we showed that exposure to fibril $A\beta_{1-40}$ mediated necrotic cell death through changes in Ca^{2+} homeostasis in HEK293 cell line expressing GluN1/GluN2A subunits (Domingues et al., 2007). The ability of Aβ oligomers to affect synapse composition, structure and abundance has been thoroughly investigated. Very recently, $A\beta_{1-42}$ oligomers were shown to induce mitochondrial and synaptic dysfunction due to massive Ca^{2+} entry through NMDA and AMPA receptors in rat cortical neurons (Ferreira et al., 2012;Alberdi et al., 2010); importantly synaptic impairment occurs before neurodegeneration in hippocampal neurons treated with Aβ (Kelly and Ferreira, 2006). This sustained NMDAR-induced Ca^{2+} influx activates calpain, which in turn induces dynamin 1 degradation, a protein that plays a critical role in synaptic vesicle recycling and in synapse signaling properties (Kelly and Ferreira, 2006). Furthermore, chronic administration of low doses of memantine in Tg 2576 mouse was associated with a significant decrease in Aβ plaque deposition and increase in synaptic density (Dong et al., 2008).

Aβ oligomers were also shown to cause a decrease in mitochondrial transmembrane potential, cytochrome c oxidase activity and ATP levels in hippocampal neurons, followed by apoptosis due to an increase in pro-apoptotic proteins (Bax, Bid and cytochrome c) and a decrease in Bcl-2 (Yang et al., 2009). Our data obtained in mature hippocampal cells evidence that 500 nM Aβ cause a delayed toxic profile, exhibiting a small but significant DNA fragmentation in both neuronal and non-neuronal cells. Furthermore, we demonstrate that Aβ-induced toxic effects involve the activation of NMDARs and more particularly the GluN2B subunit. The effects of Aβ on non-neuronal cells may be accounted by the fact that astrocytes have been reported to express NMDARs (Lee et al., 2010;Zhou et al., 2010). Nevertheless, we can not exclude the possibility that glial apoptotic cell death is driven by the degenerating neurons,

which can produce and release reactive oxygen species (Butterfield, 2002), affecting the neighbouring cells. Accordingly, Deb and colleagues (Deb et al., 2003) showed that Aβ induces secretion of several matrix-degrading proteases in rat astrocyes corresponding to the activation of mononuclear phagocytes and reactive astrocytes. Due to the important crosstalk between neurons and glial cells (Ben and Pascual, 2010), effects on non-neuronal cells may lead to deregulation of neuronal function, possibly exacerbating neurite retraction and synaptic dysfunction. Interestingly, memantine was shown to promote the release of glial-derived neurotrophic factor (GDNF) from the astrocytes (Caumont et al., 2006), which can also account for by the neuroprotective effect of NMDAR blockage .

In primary neuronal cultures, microtubule destabilization caused by Aβ was previously evidenced through decreased alpha-tubulin acetylation (Henriques et al., 2010). Also in primary hippocampal neurons, Zempel and colleagues (Zempel et al., 2010) demonstrated that Aβ oligomers induce spine loss, tau missorting and microtubule depletion. In pyramidal neurons from AD patients both number and total length of microtubules were shown to be significantly and selectively reduced when compared to control subjects (Cash et al., 2003). Nevertheless, Aβ oligomers were shown to disrupt organelle transport in primary hippocampal neurons, without affecting microtubule stability (Tackenberg and Brandt, 2009). Furthermore, early reactive oxygen species-dependent cytoskeleton disruption, preceding caspase activation, was observed in cortical neurons treated with soluble Aβ (Domingues et al., 2007;Sponne et al., 2003). Exposure of hippocampal neurons to Aβ oligomers also results in abnormal spine morphology, ultimately causing a significant decrease in spine density (Selkoe, 2008;Shankar et al., 2008) and in drebrin levels, a neuron-specific F-actin-binding protein (Lacor et al., 2007). Moreover, in primary cortical neurons, memantine treatment significantly protected cultured neurons against Aβ by attenuating tau phosphorylation and associated signaling mechanisms (Song et al., 2008). Our results show that Aβ-induced decrease in polymerized alpha-tubulin is an early event, whereas decreased polymerized beta-III tubulin is a delayed event, being the latter correlated with reduced neurite length in a process dependent on NMDARs. The implication of ATP in microtubule polymerization/depolymerization is well known, since ATP binding to microtubules stimulates polymerization (Zabrecky and Cole, 1982). Indeed, Aβ-evoked tubulin depolymerization may be associated with mitochondrial dysfunction (and thus ATP decrease), which is also known to occur through NMDAR activation

(Zempel et al., 2010). Indeed, an early decrease in MTT reduction (although very slight) is suggestive of decreased activity of mitochondrial dehydrogenases, and possibly other cellular dehydrogenases responsible for maintaining high ratios of $NADH/NAD^+$, essential for ATP generation. Furthermore, our results showed a high correlation between delayed depolymerized beta-III tubulin and DNA fragmentation in a process dependent on NMDARs. Indeed, several previous studies evidenced that cytoskeleton disruption is a key factor to induce cell death after several toxic stimuli (Cabado et al., 2004;Miura et al., 1999;Tsukidate et al., 1993).

Altogether, these results suggest that $A\beta_{1-42}$ oligomers (in the presence of monomers) induce microtubule deregulation, neurite retraction and DNA fragmentation in mature hippocampal neurons, in a process dependent on NMDARs, namely extrasynaptic GluN2B-containing NMDARs. Thus, microtubule depolymerization appears to have a primal role in exacerbating synaptic and neuronal dysfunction in AD. In addition, GluN2B-containing NMDARs can be assigned as early therapeutic targets to counteract AD progression.

Chapter 4

NMDA receptor and Src related signaling in Alzheimer's disease

4.1. Summary

Early cognitive deficits in Alzheimer's disease (AD) are thought to be related to NMDA receptor (NMDAR) dysregulation and synaptic dysfunction, in response to amyloid-beta peptide accumulation. Thus, we analyzed age-and gender-dependent changes in NMDAR subunit protein levels and activation using cortical and hippocampal homogenates from the 3xTg-AD *versus* age-matched WT mice. In hippocampus, GluN2B Tyr1472 phosphorylation increased in 3xTg-AD females at 15 months of age, but decreased in 3xTg-AD males at 3 months of age; importantly, the latter was correlated with modified activity of Src Tyr kinase. Moreover, early decreased Src activation in hippocampus and cortex of 3xTg-AD mice was associated with decreased Dab1 activation, a target of Src linked to cytoskeleton stability. Reelin protein levels were also diminished in the cortex of young 3xTg-AD mice. In addition, cortactin, another protein linked to cytoskeleton stability, was significantly decreased in 3 month-old 3xTg-AD hippocampus and cortex. Our results evidence early reduced Src activity in hippocampus, accompanied by decreased activation of Dab1 and GluN2B-composed NMDARs, favoring early NMDAR-related signaling modifications in AD.

4.2. Introduction

N-methyl-D-aspartate receptors (NMDARs) have critical roles in excitatory synaptic transmission, neuronal plasticity and excitotoxicity in the central nervous system. In human *post-mortem* brains of late Alzheimer's disease (AD) patients, mRNA and protein levels of NMDARs subunits were previously shown to be altered, correlating with disease-related cognitive deficits (Sze et al., 2001;Mishizen-Eberz et al., 2004). Nevertheless, changes in NMDARs subunits were not the same in the affected brain areas. In the hippocampus, the levels of GluN1 and GluN2B subunits were decreased, whereas GluN2A levels decreased in the entorhinal cortex (Sze et al., 2001). Activation of NMDARs linked to excitotoxicity in AD was initially hypothesized to occur at a late-stage of the disease; concordantly, memantine, an uncompetitive NMDARs open channel blocker, has been prescribed as a memory-preserving drug for moderate- to late-stage AD patients (Reisberg et al., 2003). However, recent reports suggest that overactivation of NMDARs may also occur at early stages of the disease. In young 3xTg-AD mice, NMDARs-induced calcium signals were higher than in non-transgenic mice and led to aberrant calcium release from endoplasmic reticulum compartment in dendrites, suggesting early alterations in NMDARs activity (Goussakov et al., 2010). Moreover, we previously demonstrated the involvement of GluN2B-containing NMDARs activation in $A\beta_{1-42}$-induced intracellular Ca^{2+} rise in cortical neurons (Ferreira et al., 2012), and microtubule deregulation (Mota et al., 2012) as well as $A\beta_{1-42}$-induced ER stress (Costa et al., 2012) in hippocampal neurons. Interestingly, evaluation of GluN1 or GluN2 subunit levels in *post-mortem* AD brains at an early stage of the disease showed no differences compared with control individuals (Sze et al., 2001), suggesting that any modification in NMDAR function at early stages of AD is not be linked to altered NMDAR protein levels.

Phosphorylation at Tyr1472 of GluN2B subunit enhances NMDAR activity by increasing their number at the synapse (Goebel et al., 2005; Goebel-Goody et al., 2009). On the other hand, phosphorylation at Tyr1336 of GluN2B is associated with extrasynapse NMDARs enrichment (Goebel-Goody et al., 2009). Thus, Src and Fyn Tyr kinases are involved in the upregulation of GluN2B-containing NMDARs at the membrane (Sinai et al., 2010; Xu et al., 2006). On the contrary, dephosphorylation of Tyr1472 by $STEP_{61}$, a Tyr phosphatase, leads to decreased NMDAR activity and trafficking (Braithwaite et al., 2006) through internalization of GluN1/GluN2B receptor complexes from the neuronal surface (Kurup et al., 2010a). Moreover, disruption of the

interaction between GluN2B and the scaffolding proteins PSD-95 and SAP102 after phosphorylation of Ser1480GluN2B by casein kinase 2 (CK2) led to decreased GluN2B surface expression (Chung et al., 2004).

Interestingly, cortactin, a protein that promotes actin polymerization, indirectly linking the NMDAR to actin cytoskeleton (Naisbitt et al., 1999), also associates with Src kinase (Okamura and Resh, 1995). Phosphorylated cortactin binds neuronal Wikott-Aldrich Syndrome protein (N-WASP) to activate the actin-related protein (Arp)2/3 complex (Tehrani et al., 2007) thus promoting actin polymerization (Uruno et al., 2001).

Reelin, a secreted glycoprotein involved in synaptic plasticity modulation in the adult, was previously shown to be depleted in AD brain (Herring et al., 2012) and mediates enhanced NMDARs activity through Src (Qiu et al., 2006) and Dab1 (Chen et al., 2005) activation. Stimulation of neurons by reelin leads to Dab1 phosphorylation (Howell et al., 1999) by Fyn (Arnaud et al., 2003) and Src (Kuo et al., 2005). Once activated, Dab1 induces actin polymerization through directly binding to N-WASP and subsequent activation of the Arp2/3 complex (Suetsugu et al., 2004), similarly as described for activated cortactin. On the other hand, phosphorylated Dab1 is able to activate Src kinase family through phosphorylation (Ballif et al., 2003;Bock and Herz, 2003), suggesting a link between actin polymerization modulators and NMDARs. In agreement, inhibition of reelin pathway significantly decreased the availability of GluN2B subunit at the synapse (Groc et al., 2007).

Taking into account the importance of NMDARs in AD and their role in the initial stages of the disease, we evaluated the age and gender-dependent changes in NMDAR subunits (GluN1, GluN2A and GluN2B) in AD affected brain areas, hippocampus and cortex, in an *in vivo* model of the disease, the 3xTg-AD mice (Oddo et al., 2003b). Previous studies demonstrated a higher susceptibility of 3xTg-AD females to the disease (Hirata-Fukae et al., 2008) in part due to the expression of sex steroid hormones (Carroll et al., 2010). GluN2B Tyr phosphorylation and the correlation with the activity of Src kinase and the phosphatase $STEP_{61}$ were further studied. In addition, the signaling pathways that link NMDAR and Src kinase to actin cytoskeleton polymerization, namely cortactin, reelin and Dab-1 were also analyzed. Our study shows a clear correlation between decreased GluN2B activation and a decrease in Src activation in early stages of AD, which is accompanied by alterations in Dab1 and cortactin, two proteins implicated in actin cytoskeleton stabilization.

4.3. Results

4.3.1. Characterization of WT and 3xTg-AD mice colonies

In order to initially characterize the mice colonies used in this study, we measured the body weight and the brain weight, as well as Aβ deposition and tau accumulation in young and old 3xTg-AD *versus* WT mice. Taking into account previous reports that 3xTg-AD females could be more susceptible to the disease compared to age-matched 3xTg-AD males (Carroll et al., 2010;Hirata-Fukae et al., 2008), we studied both females and males.

We observed that in old 3xTg-AD (12 and 15 months) body weight is significantly decreased compared to WT mice, both in females and males (supplementary Fig. 4.1 A, B). Moreover, evaluation of brain weight in males evidenced a significant decrease in brain weight in 3xTg-AD mice already in the initial stages of the disease, i.e., at 3 months of age (supplementary Fig. 4.1 C).

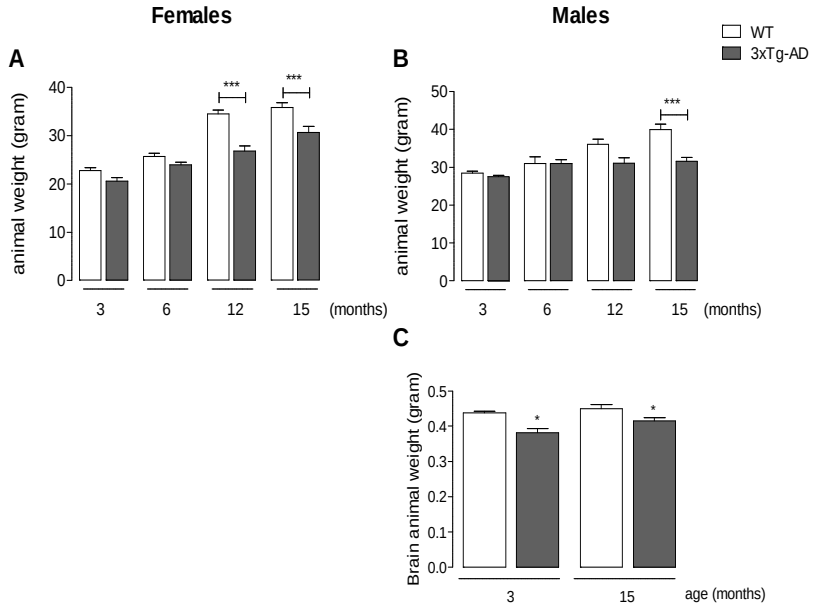

Figure 4.1: Animal and brain weight of 3xTg-AD *versus* WT mice. Animals were weighed before sacrifice, at 3, 6, 12 and 15 months of age for females (**A**) and males (**B**); male brains were also weight after sacrifice at 3 and 15 months of age (**C**). Results are the mean ± SEM of 7-37 animals per group for the corporal weight and 5-13 animals per group for the brain weight. Statistical analysis: $^{*}p<0.05$, $^{***}p<0.001$ (two way ANOVA), compared to WT mice.

We found evidences for intracellular Aβ accumulation in the hippocampus and cortex of 3 month-old 3xTg-AD males (Fig. 4.2 A) and females (Fig. 4.2 B) and extracellular Aβ plaques in both hippocampus and cortex of 15 month-old 3xTg-AD males (Fig. 4.2 D)

and females (Fig. 4.2 F) which were not observed in age-matched WT mice (Fig. 4.2 C, E). Moreover, analysis of tau accumulation showed hippocampal and cortical deposits of tau protein in 3xTg-AD males (Fig. 4.3 B) and females (Fig. 4.3 D), which were not observed in WT mice (Fig. 4.3 A, C). Although no differences in Aβ or tau pathologies were detected between males and females, in the following studies we still examined the alterations seen in males and females, taking into account the fact that females 3xTg-AD might be more susceptible to the disease compared to age-matched 3xTg-AD males (e.g. (Carroll et al., 2010;Hirata-Fukae et al., 2008)).

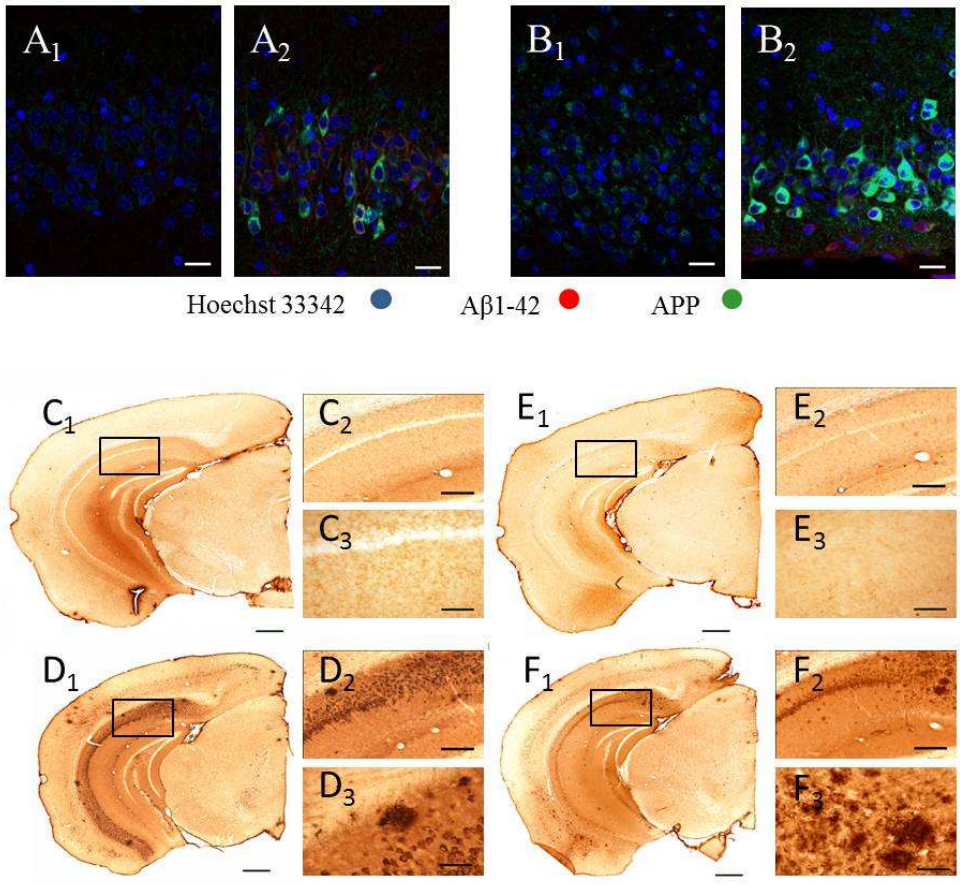

Figure 4.2: Aβ deposition in 3xTg-AD *versus* WT mice. (**A**, **B**) Coronal sections from 3 month-old WT (**A_1**, **B_1**) and 3xTg-AD (**A_2**, **B_2**), males (**A_1**, **A_2**) and females (**B1**, **B_2**), were evaluated with Aβ $_{1\text{-}42}$- (red) and APP-specific antibody (green). Nuclei were stained with Hoechst 33342 (blue). (**A_2**, **B_2**) Intraneuronal Aβ immunoreactivity appears within the hippocampus (CA3) of 3xTg-AD. (**C-F**) Coronal sections from 15 month-old WT (**C**,**E**) and 3xTg-AD (**D**, **F**) males (**C**, **D**) and females (**E**, **F**) were evaluated with a specific 6E10 antibody. Extracellular Aβ plaques appear in the hippocampus and the cortex of 15 month-old 3xTg-AD males (**D**) and females (**F**). Aβ immunoreactivity is not observed in 3 month-old (**A**) or 15 month-old (**C**, **E**) WT mice.A,B scale=50 μm; C_1-F_1 scale=500 μm; C_2-F_2 scale= 200 μm; C_3-F_3 scale=50 μm.

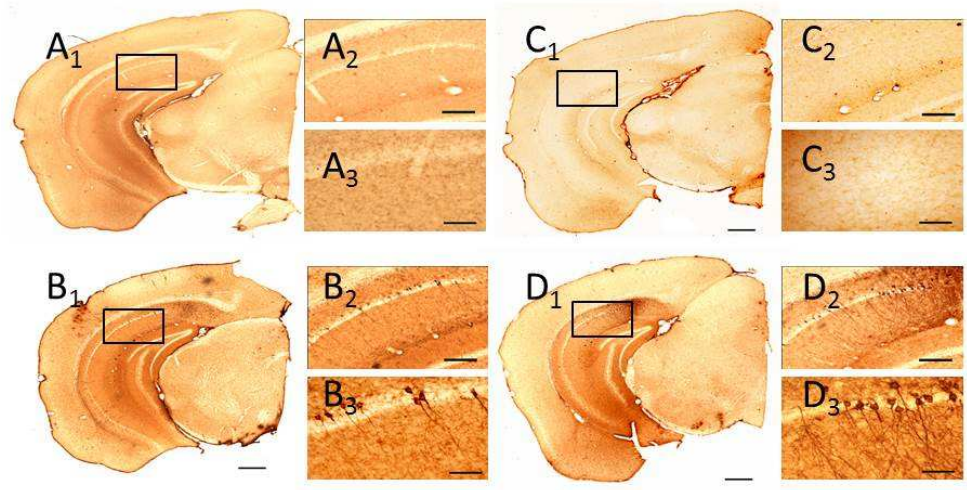

Figure 4.3: Tau deposition in 3xTg-AD *versus* WT mice. (**A-D**) Coronal sections from 15 month-old WT (**A**,**C**) and 3xTg-AD (**B**,**D**) males (**A**,**B**) and females (**C**,**D**) were evaluated with a specific anti-phosphorylated tau antibody. Intracellular tau deposits appear in the hippocampus and the cortex of 15 month-old 3xTg-AD males (**B**) and females (**D**). Tau immunoreactivity is not observed in 15 month-old (**A**,**C**) WT mice. A_1-D_1 scale=500 µm; A_2-D_2 scale= 200 µm; A_3-D_3 scale=50 µm.

4.3.2. Age and gender-dependent modifications of GluN2B subunit Tyr phosphorylation in 3xTg-AD mice hippocampus

In *post-mortem* human brain samples, alterations of GluN1 and GluN2A/B mRNA and proteins levels correlated with cognitive deficits (Sze et al., 2001;Mishizen-Eberz et al., 2004). Therefore, we analyzed the total levels of NMDARs subunits GluN1, GluN2A and GluN2B and the levels of PSD-95, a scaffold protein that anchors NMDARs to the cellular membrane in hippocampal and cortical 3xTg-AD mice brains extracts. We found no significant alterations in the total levels of these proteins in the hippocampus (Fig. 4.4) or cortex (Fig. 4.5) of both 3xTg-AD mice males (Figs. 4.4-4.5 A-D) and females (Figs. 4.4-4.5 E-H), when compared to age-matched WT mice.

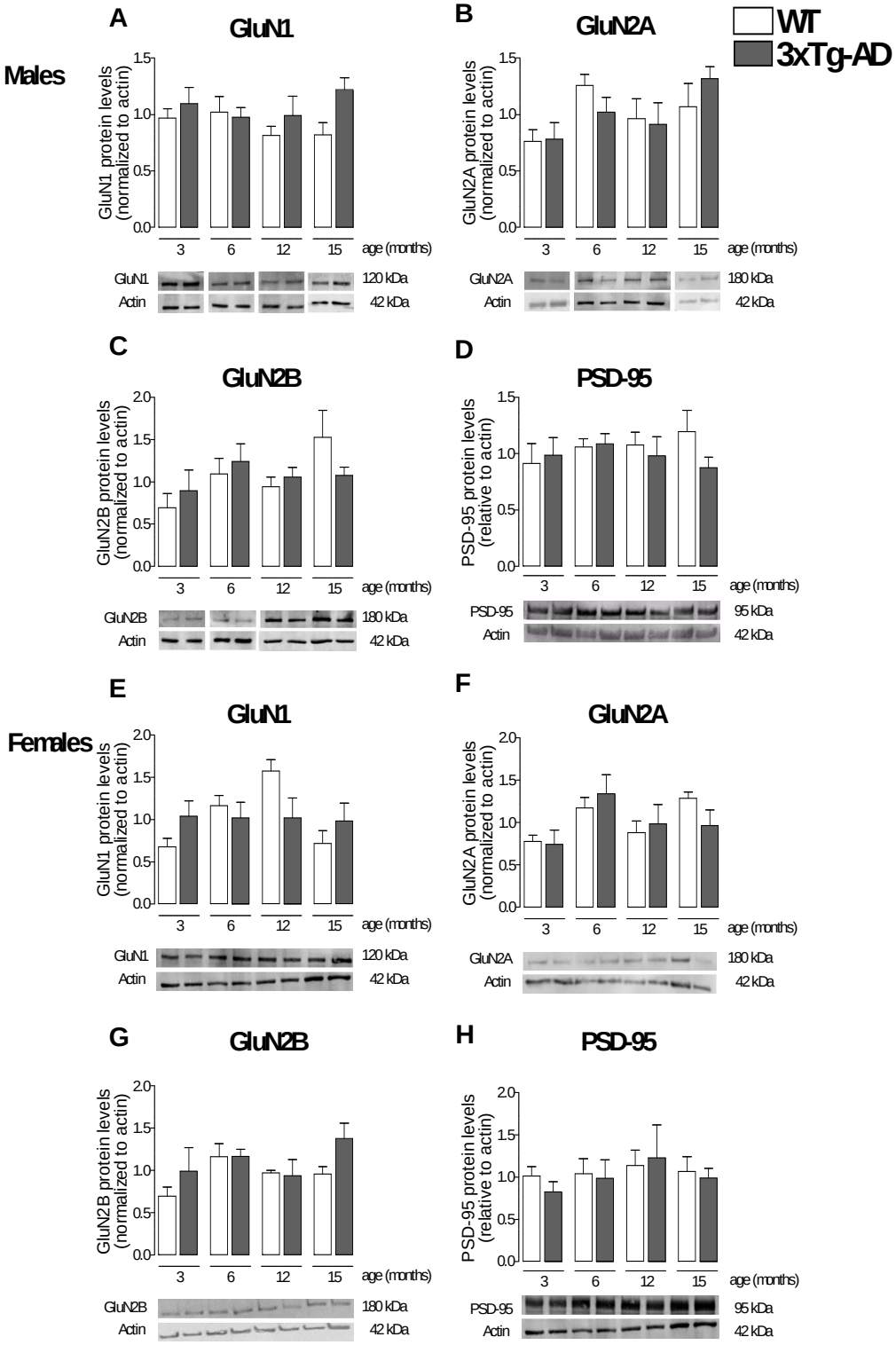

Figure 4.4: Hippocampal levels of GluN1, GluN2/B subunits and PSD-95 in 3xTg-AD *versus* WT mice. Mice were sacrificed at 3, 6, 12 and 15 months of age and hippocampi were isolated. Total extracts were performed in RIPA buffer and levels of GluN1 (**A**, **E**), GluN2A (**B**, **F**), GluN2B (**C**, **G**) and PSD-95 (**D**, **H**) were analyzed by western blotting in males (**A**-**D**) and females (**E**-**H**). Results, expressed in arbitrary units relative to actin, are the mean ± SEM of 6-7 animals per group.

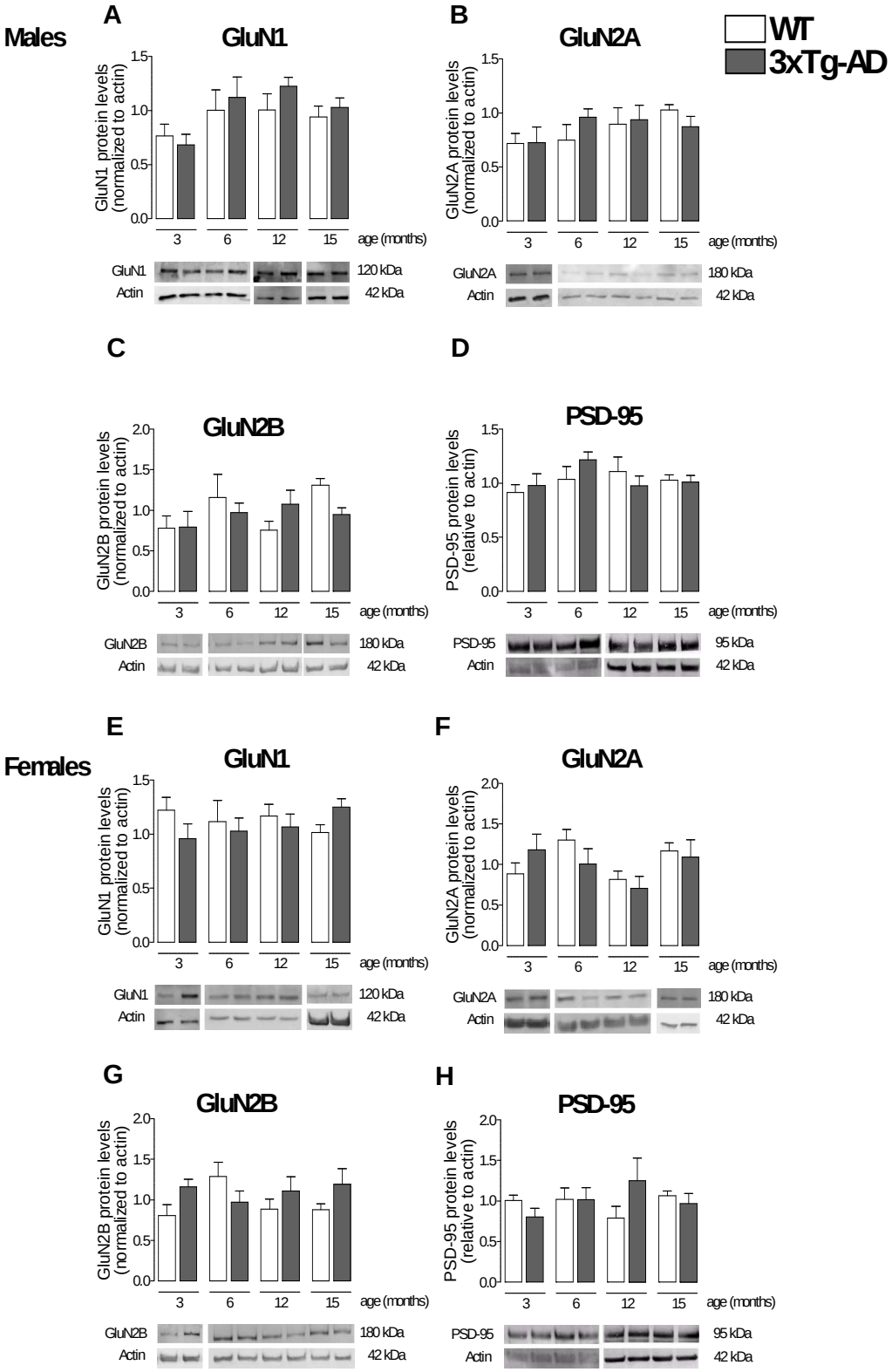

Figure 4.5: Cortical levels of GluN1, GluN2/B subunits and PSD-95 in 3xTg-AD *versus* WT mice. Mice were sacrificed at 3, 6, 12 and 15 months of age and cortex were isolated. Total extracts were performed in RIPA buffer and levels of GluN1 (**A**,**E**), GluN2A (**B**,**F**), GluN2B (**C**,**G**) and PSD-95 (**D**,**H**) were analyzed by western blotting in males (**A**-**D**) and females (**E**-**H**). Results, expressed in arbitrary units relative to actin, are the mean ± SEM of 6-7 animals per group.

Since AD-associated neurodegeneration has been linked, in part, to overactivation of NMDARs, we further evaluated the activation of GluN2A and GluN2B by analyzing the phosphorylation status of these two subunits at residues Ser1232 and Tyr1472, respectively (Li et al., 2001;Goebel et al., 2005). Results depicted in Fig. 4.6 show that there are no alterations in GluN2A phosphorylation at residue Ser1232 in the hippocampus along age in 3xTg-AD mice, when compared to age-matched WT mice neither in males (Fig. 4.6 A) nor in females (Fig. 4.6 C). Interestingly, we found age- and gender-dependent alterations in GluN2B subunit phosphorylation in 3xTg-AD hippocampus. Indeed, results show decreased P(Tyr1472)GluN2B at 3 months of age in males (Fig. 4.6 B), suggesting decreased NMDAR activity, and a late increase in GluN2B activity in 15 month-old females, as suggest by an increase in P(Tyr1472)GluN2B relatively to WT (Fig. 4.6 D). Interestingly, we did not find significant alterations in phosphorylation status of GluN2A/B subunits in the cortex, neither in males nor in females (Fig. 4.7).

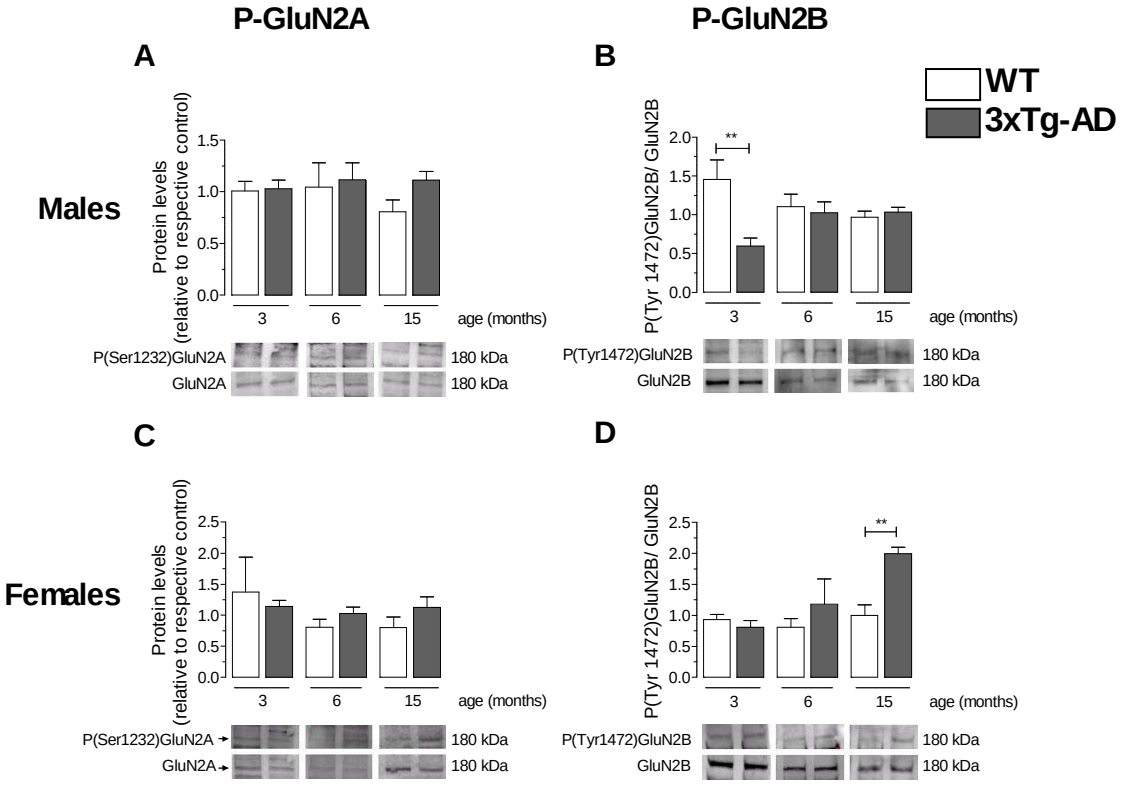

Figure 4.6: Hippocampal GluN2A/B subunit activation in 3xTg-AD *versus* WT mice. Mice were sacrificed at 3, 6 and 15 months of age and hippocampi were isolated. Total extracts were performed in RIPA buffer and levels of P(Ser1232)GluN2A/GluN2A (**A,C**) and P(Tyr1742)GluN2B/GluN2B (**B,D**)

were analyzed by western blotting in males (**A,B**) and females (**C,D**). Results are the mean ± SEM of 6-7 animals per group. Statistical analysis: $^{**}p<0.01$ (two way ANOVA), compared to WT mice.

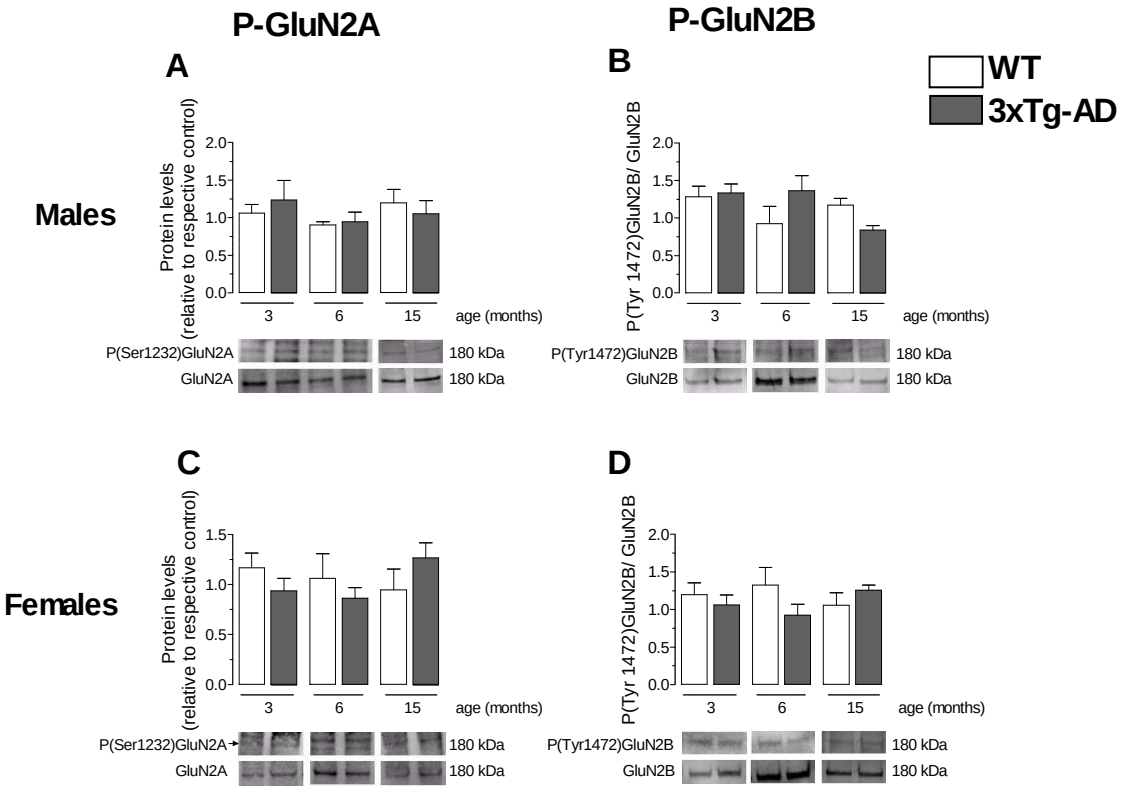

Figure 4.7: Cortical GluN2A/B subunit activation in 3xTg-AD *versus* WT mice. Mice were sacrificed at 3, 6 and 15 months of age and cortex were isolated. Total extracts were performed in RIPA buffer and levels of P(Ser1232)GluN2A/GluN2A (**A,C**) and P(Tyr1742)GluN2B/GluN2B (**B,D**) were analyzed by western blotting in males (**A,B**) and females (**C,D**). Results are the mean ± SEM of 6-7 animals per group.

These results suggest that there is an age- and gender-dependent alteration in GluN2B activation in hippocampus of 3xTg-AD mice, which is not observed in the cortex. Differences observed between males and females suggest activation of different mechanisms during disease progression. Moreover, considering that Aβ deposits start approximately at 6 month-old in the hippocampus of 3xTg-AD mice (Oddo et al., 2003a), changes in hippocampal GluN2B activity observed in this study may be considered early AD alterations.

4.3.3. Modified activity of GluN2B subunit correlate with Src activation in 3xTg-AD mice hippocampus

GluN2B Tyr phosphorylation is known to be regulated by Src and $STEP_{61}$. Indeed, Src kinase phosphorylates GluN2B at Tyr1472 (Cheung and Gurd, 2001) and the phosphatase $STEP_{61}$ dephosphorylates this residue, leading to internalization of GluN2B-containing NMDARs (Kurup et al., 2010b). Thus, we analyzed the levels of total and activated Src and $STEP_{61}$ in 3xTg-AD compared to WT mice, in females and males at 3, 6 and 15 months of age, in hippocampus (Fig. 4.8) and cortex (Fig. 4.9). Results show that total levels of Src protein are not altered in 3xTg-AD males (Fig. 4.8 A) or females (Fig. 4.8 E) along disease progression in hippocampus. The same result was found in cortex (Fig. 4.9 A and E). We were also unable to detect changes in total levels of $STEP_{61}$ in both hippocampus (Fig. 4.8 C and G) and cortex (Fig. 4.9 C and G) of males or females. Activation of Src and $STEP_{61}$ proteins were evaluated by analyzing levels of P(Tyr418)Src and non-P(Ser221)$STEP_{61}$. Our data demonstrated that there are no gender- or age-alterations of $STEP_{61}$ activation in hippocampus (Fig. 4.8 D and H) or cortex (Fig. 4.9 D and H) in 3xTg-AD mice. Interestingly, in hippocampus, we found early decreased Src activity in 3xTg-AD males, as shown by decreased levels of P(Tyr418)Src at 3 months of age (Fig. 4.8 B), which closely correlates with early decreased GluN2B Tyr phosphorylation in the same animals. Moreover, higher levels of P(Tyr418)Src were observed in 15 month-old 3xTg-AD females, relatively to WT age-matched females (Fig. 4.8 F), occurring concomitantly with increased levels of P(Tyr1472)GluN2B in these animals. Surprisingly, results depicted in Fig. 4.9 B show a decrease in Src activation in the cortex of 3 month-old 3xTg-AD males, when compared to age-matched WT mice, which was not correlated with alterations in GluN2B phosphorylation status.

Altogether, our results indicate that alterations in GluN2B phosphorylation observed in hippocampus of 3xTg-AD involve changes in Src kinase activation and are unrelated with $STEP_{61}$ modified protein levels or activation. Moreover, we found an early alteration in Src activity in the cortex of 3xTg-AD mice, apparently unrelated with GluN2B subunit modifications, suggesting other pathological mechanisms in cortex involving Src kinase.

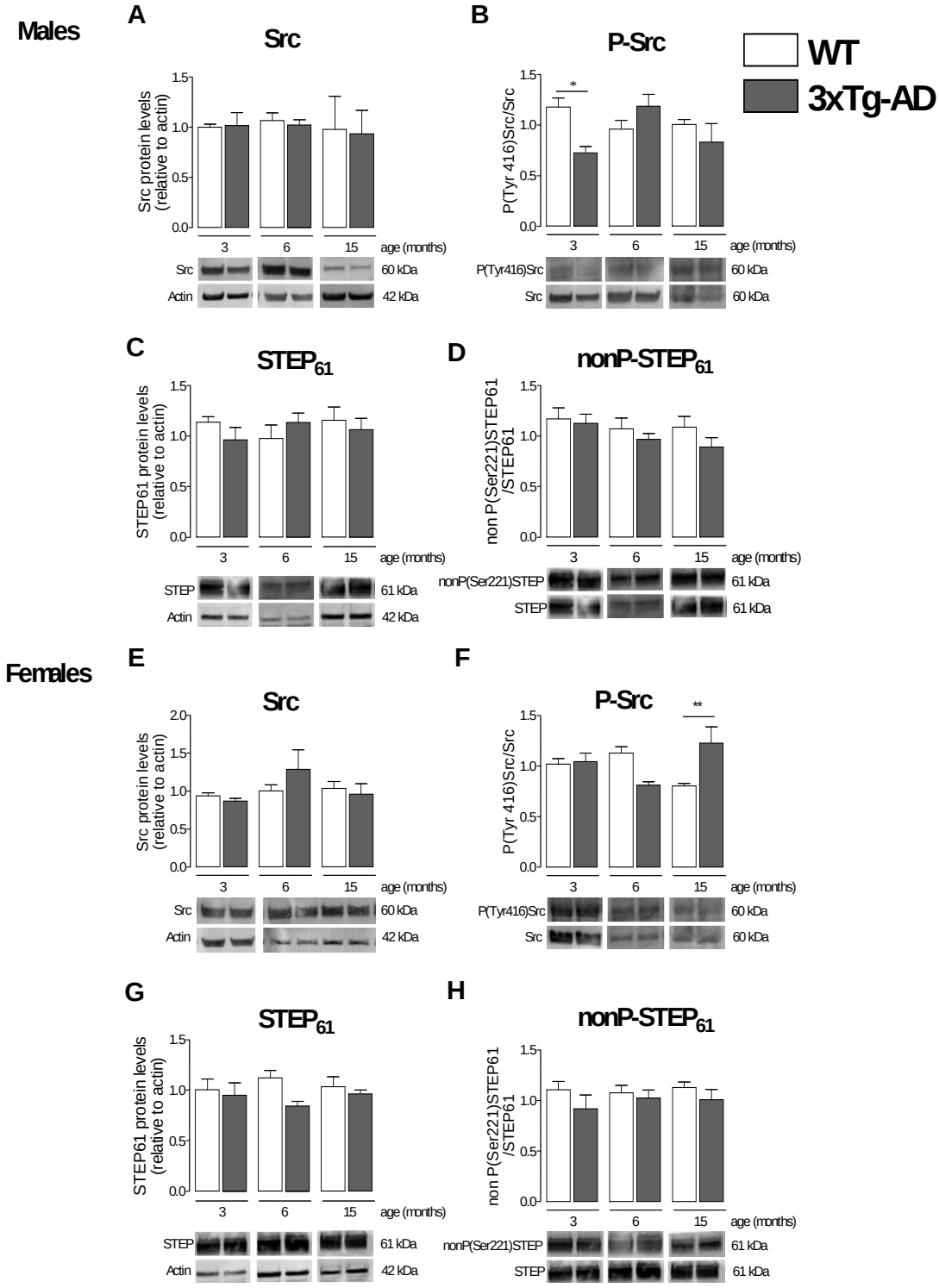

Figure 4.8: Hippocampal Src family and $STEP_{61}$ activation in 3xTg-AD *versus* WT mice. Mice were sacrificed at 3, 6 and 15 months of age and hippocampi were isolated. Total extracts were performed and levels of Src (**A,E**), P(Tyr416)Src/Src **(B,F**), $STEP_{61}$ (**C,G**) and non-P(Ser221)$STEP_{61}$/$STEP_{61}$ (**D,H**) were analyzed in males (**A-D**) and females (**E-H**) (n=6-7 animals for each experimental group). Statistical analysis: $^{*}p<0.05$, $^{**}p<0.01$ (two way ANOVA), compared to WT mice.

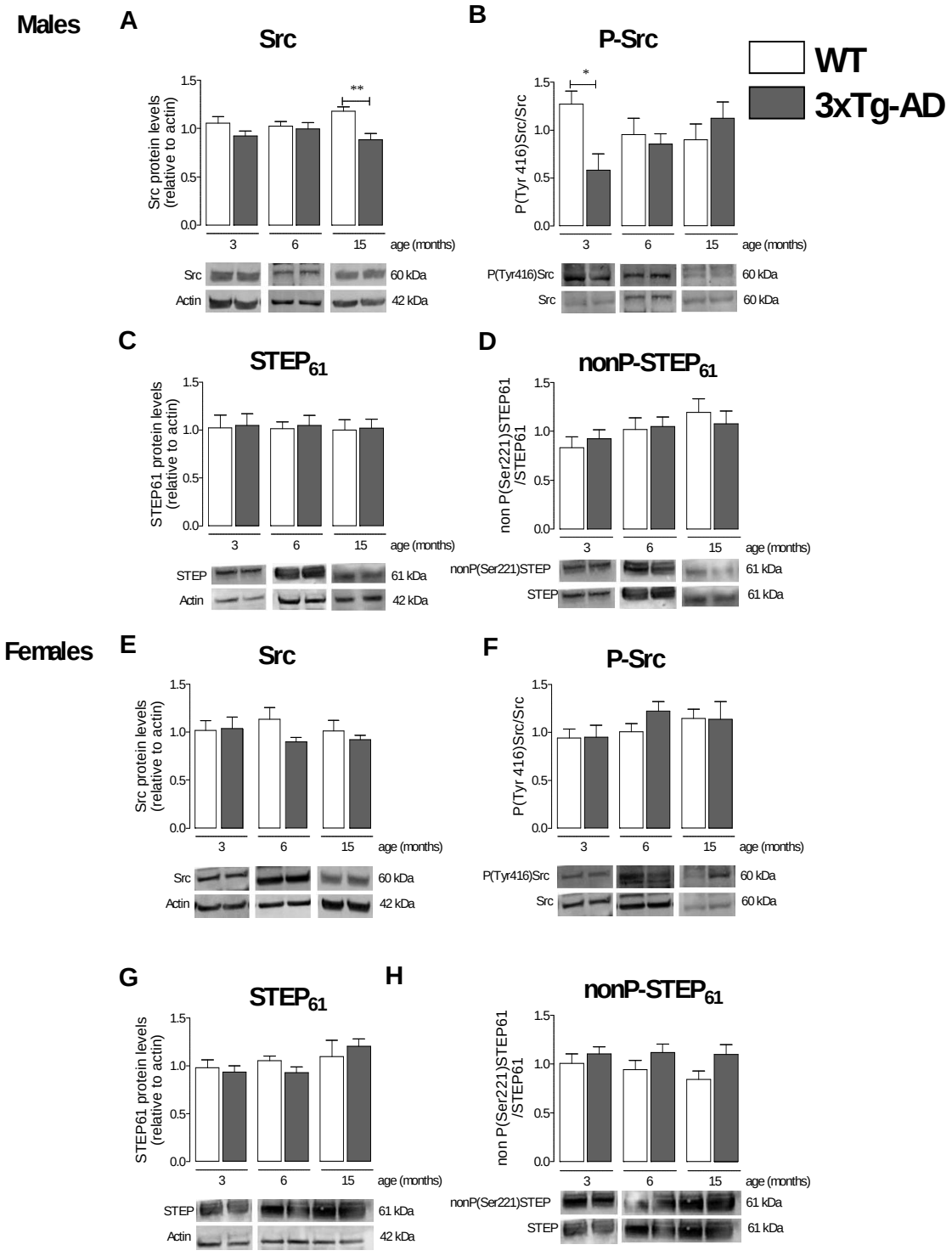

Figure 4.9: Cortical Src family and STEP$_{61}$ activation in 3xTg-AD *versus* WT mice. Mice were sacrificed at 3, 6 and 15 months of age and cortex were isolated. Total extracts were performed and levels of Src (**A,E**), P(Tyr416)Src/Src **(B,F**), STEP61 (**C,G**) and non-P(Ser221)STEP$_{61}$/STEP$_{61}$ (**D,H**) were analyzed in males (**A-D**) and females (**E-H**) (n=6-7 animals for each group). Statistical analysis: $^{*}p<0.05$, $^{**}p<0.01$ (two way ANOVA), compared to WT mice.

4.3.4. Src activation modifications are accompanied by alterations in Dab1 and cortactin phosphorylation

Reduction in Src activation in young 3xTg-AD males occurring in the hippocampus (Fig. 4B) and cortex (Fig. 5B) was related with NMDAR activity modification in the case of hippocampus only. Src kinase has been also related to regulation of cortactin phosphorylation (Huang et al., 1997) and reelin signaling pathway by inducing Dab1 phosphorylation (Kuo et al., 2005), both involved in cytoskeleton stabilization (Chai et al., 2009;Weaver et al., 2001) and subsequent NMDAR surface expression (Chen et al., 2005). In order to determine if alterations of GluN2B and Src activation may be correlated with alternative signaling pathways, we determined the protein levels of reelin as well as Dab1 and cortactin phosphorylation in hippocampus and cortex of young (3 month-old) 3xTg-AD male mice (Figs. 6 and 7). In the hippocampus, we found no alterations in total levels of reelin in 3xTg-AD (Fig. 6A). However, we detected decreased P(Tyr220)Dab1 in 3xTg-AD mice (Fig. 6 C), which can be correlated to the decreased in Src activation observed in these mice. Nonetheless, this was not accompanied by decreased P(Tyr421)cortactin (Fig. 6E). Data depicted in Fig. 6D further showed decreased total cortactin levels (Fig. 6D) in the hippocampus of young AD mice males. In the cortex, we found a decrease in reelin protein levels (Fig. 7A), correlated with decreased levels of total Dab1 and P(Tyr220)Dab1 (Fig. 9 B, C). As found in hippocampus, levels of cortical phosphorylated cortactin were not changed in young 3xTg-AD, when compared to WT age-matched mice (Fig. 7E), but we detected a lower amount of cortactin in the same animals (Fig. 7D).

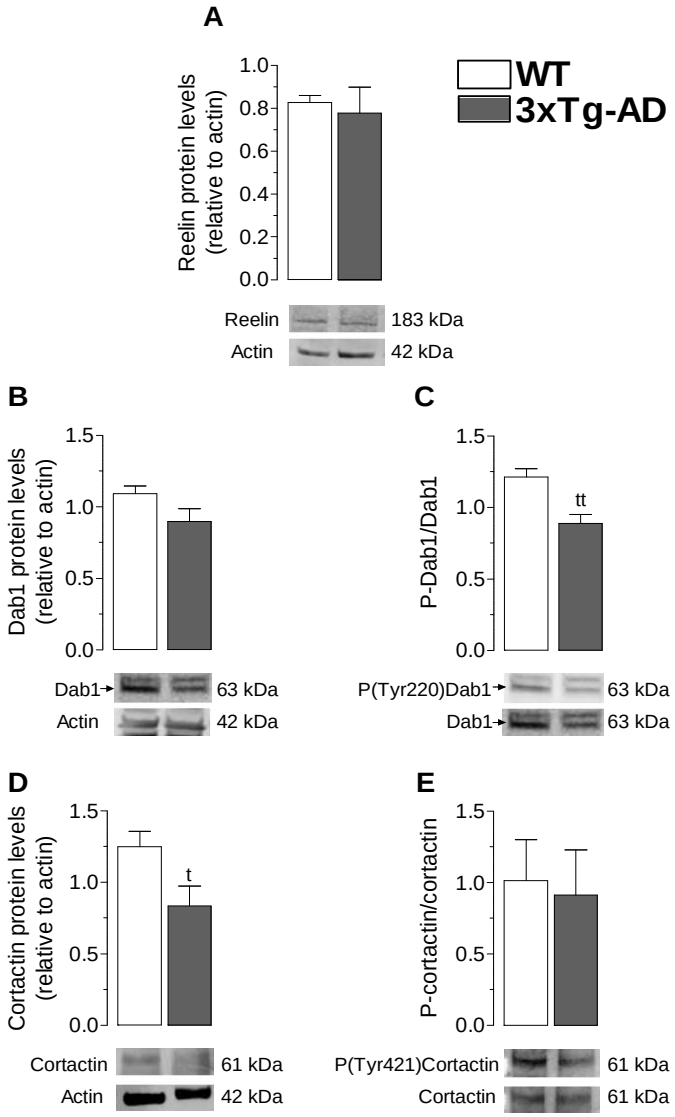

Figure 4.10: Hippocampal levels of reelin and levels and activation of Dab1 and cortactin in 3xTg-AD *versus* WT mice. Male mice were sacrificed at 3 months of age and hippocampi were isolated. Total extracts were performed in RIPA buffer and levels of reelin (**A**), Dab1 (**B**) and cortactin (**D**) were analysed by western blotting. Activation of Dab1 and cortactin were evaluated through analysis of P(Tyr220)Dab1/Dab1 (**C**) and P(Tyr421)cortactin/cortactin (**E**), respectively, by western blotting. Results are the mean ± SEM of 6-7 animals per group. Statistical analysis: $^{t}p<0.05$, $^{tt}p<0.01$ (Student's *t*-test).

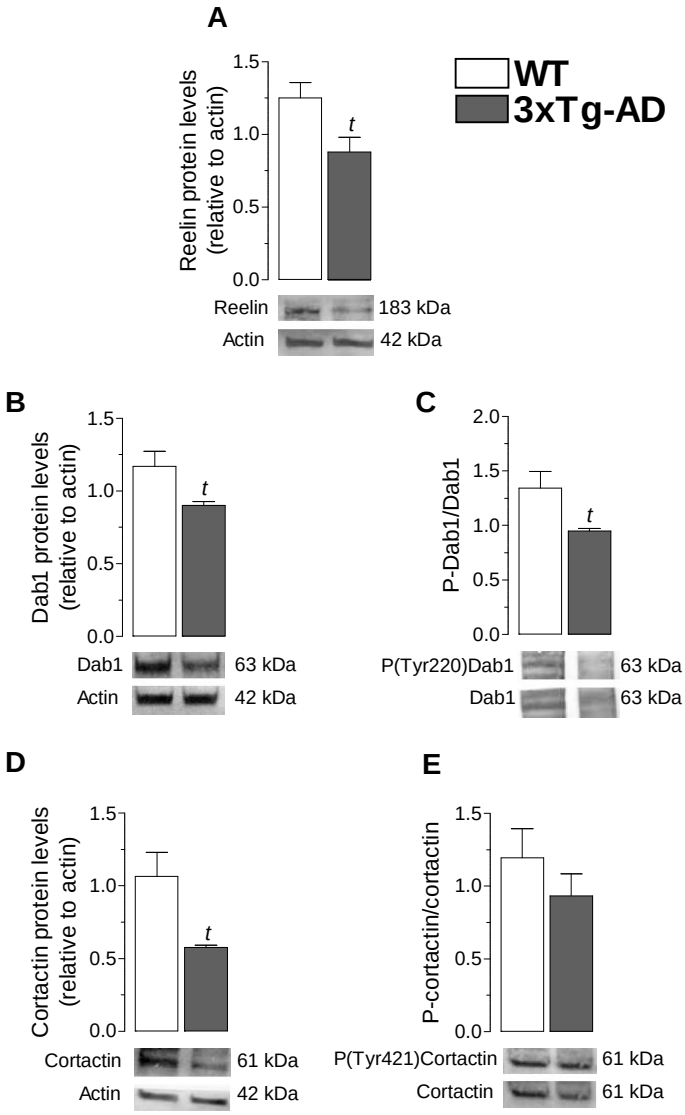

Figure 4.11: Cortical levels of reelin and levels and activation of Dab1 and cortactin in in 3xTg-AD *versus* WT mice. Male mice were sacrificed at 3 months of age and the cortex were isolated. Total extracts were performed in RIPA buffer and the levels of reelin (**A**), Dab1 (**B**) and cortactin (**D**) were analysed by western blotting. Activation of Dab1 and cortactin were evaluated by the analysis of P(Tyr220)Dab1/Dab1 (**C**) and P(Tyr421)cortactin/cortactin (**E**), respectively. Results are the mean ± SEM of 6-7 animals per group. Statistical analysis: $^{t}p<0.05$ (Student's *t*-test).

4.4. Discussion

Neurodegeneration in AD has been largely associated to extracellular Aβ deposits; moreover, the role of intracellular species in triggering synaptic dysfunction before cell death, in early stages of the disease, has become apparent (Reddy et al., 2012;Du et al., 2010). Furthermore, there is growing consensus regarding the involvement of NMDARs in AD progression. In the present study we evaluated activation of GluN2A and GluN2B subunits in a transgenic mice model of AD, the 3xTg-AD mice. This AD mouse model shows early intracellular and late extracellular accumulation of Aβ, the later concomitant with tau phosphorylation, which seems to recapitulate the human AD condition. We observed early decreased GluN2B activation in the hippocampus of 3xTg-AD males, before Aβ plaque formation, and late increase in females, after Aβ extracellular deposits in the hippocampus, being these alterations correlated with Src kinase activity. Our data also indicate that in the hippocampus, early decreased Src activation may be linked to decreased Dab1 activation. In this brain area diminished Src and Dab1 activation is not directly related to changes in reelin levels; however, they are accompanied by a decrease in P(Tyr1472)GluN2B, which has been described as a signal for NMDAR internalization (e.g. Kurup et al., 2010a). Conversely, in the cortex, we found decreased protein levels of reelin that correlates with decreased levels and activation of Dab1 and Src, but are not linked to modified GluN2B phosphorylation. Notably, both reduced Dab1 and cortactin may be implicated in less actin cytoskeleton stabilization in young 3xTg-AD mice, as described previously by Huang et al. (1997) and Kuo et al. (2005).

Direct activation of NMDARs by Aβ has been described (Texido et al., 2011;Costa et al., 2012) and we previously demonstrated that $A\beta_{1-42}$ disturbs intracellular calcium homeostasis (Ferreira et al., 2012) namely through ER stress (Costa et al., 2012) and causes microtubule deregulation (Mota et al., 2012) through GluN2B-containing NMDARs activation, as observed in cortical and hippocampal neurons. In the present study, analysis of NMDAR subunits (GluN1, GluN2A or GluN2B) protein levels showed no modifications in young or old 3xTg-AD mice. However, in *post-mortem* AD brain, NMDAR subunits protein and mRNA were reported to be decreased (Hynd et al., 2001;Hynd et al., 2004b;Hynd et al., 2004a;Bi and Sze, 2002;Mishizen-Eberz et al., 2004). Although our findings are apparently in opposition with observations made in *post-mortem* AD human brains derived from old patients (more than 70 years-old), the fact that the 3xTg-AD animal model does not exhibit massive neuronal death (Oddo et

al., 2003b), thus not reflecting a complete AD human late stage, may explain this difference. Nonetheless, 3xTg-AD mice develop both Aβ and tau pathologies (Oddo et al., 2003b) as in AD patients, being Aβ pathology the first to appear (Oddo et al., 2003a). Furthermore, this model reproduces numerous mechanisms involved in AD, such as impairment in intracellular Ca^{2+} homeostasis (Lopez et al., 2008), oxidative stress (Resende et al., 2008), spine degeneration (Bittner et al., 2010) and early cognitive deficits (Billings et al., 2005).

Our data further indicates no changes in GluN2A-containing NMDARs in the progression of the disease in the 3xTg-AD model. Decreased levels of phosphorylated GluN2A subunit were previously observed in *post-mortem* AD human cortex (Sze et al., 2001), whereas increased levels were observed in rat hippocampus treated with Aβ (Wu and Hou, 2010). The difference regarding our study may be explained by the fact that we analyzed the phosphorylation levels of Ser1232, while other authors studied the phosphorylation of Tyr residues of GluN2A subunit. Thus, we may hypothesize that phosphorylation of Ser1232 of GluN2A is not required for AD progression linked to Aβ and tau deposition. Interestingly, results evidenced age- and gender-dependent modifications of GluN2B subunit activation, particularly in the hippocampus. We show early decreased P(Tyr1472)GluN2B levels in 3xTg-AD males hippocampus at 3 months of age, when intracellular Aβ is present. GluN2B subunit activation is regulated by phosphorylation of Tyr1472 induced by Src kinase (Cheung and Gurd, 2001), whereas its dephosphorylation is promoted by $STEP_{61}$ (Kurup et al., 2010a). $STEP_{61}$ was previously implicated in AD progression: $STEP_{61}$ increased in AD transgenic mice and in prefrontal human cortex, leading to dephosphorylation of Tyr1472GluN2B and internalization of NMDARs composed by the GluN2B subunit (Kurup et al., 2010b). Our results demonstrated that $STEP_{61}$ is not involved in the regulation of GluN2B phosphorylation in 3xTg-AD hippocampus; nevertheless, the latter highly correlates with modified activity of Src kinase in the hippocampus, namely an early decrease in males and a late increase in females. Dephosphorylation of GluN2B subunit on Tyr1472 has been correlated with internalization of GluN1/GluN2B-composed NMDARs (Kurup et al., 2010a) and subsequent decreased activity. Indeed, decreased GluN2B activation due to decreased Src activity seems to be correlated with early cognitive impairment observed in 4 month-old 3xTg-AD mice (Billings et al., 2005). On the other hand, the GluN2B subunit, which is highly present in extrasynaptic sites, has been involved in

cell death processes (e.g. Liu et al., 2007), suggesting that decreased GluN2B subunit activity may be an attempt to prevent excitotoxicity.

Differences between men and women in AD patients have been reported and the incidence of the disease appears to be higher in post-menopausal women than in age-matched men (Fratiglioni et al., 1997;Bonomo et al., 2009). Mice females are considered to initiate menopause at 12-14 months of age (Ison and Allen, 2007); thus, changes in 15 month-old mice females may be extrapolated to post-menopausal women. In previous studies (Resende et al., 2008) showed evidences for early oxidative stress in 3-5 month-old 3xTg-AD females total brain homogenates, although no comparison has been made with males. Our results showed increased phosphorylated GluN2B subunits in the hippocampus of 3xTg-AD females, indicating increased GluN2B-containing NMDARs activity in post-menopausal females. Differences in mitochondrial susceptibility to Aβ has been suggested to be one of the elements explaining sex differences in AD (Vina and Lloret, 2010;Lloret et al., 2008). In fact, mitochondria from young female mice seem to be more protected from Aβ toxicity than young male mitochondria and produces less reactive oxygen species (Lloret et al., 2008). Evaluation of spontaneous alternation behavior, which is dependent on the working memory and visual attention, showed larger behavioral deficits in females, which also exhibited increased Aβ, compared to age-matched males (Carroll et al., 2010). Moreover, these differences were shown to be dependent on the expression of sex steroid hormones during development, because treatment of 3xTg-AD males at postnatal days with feminine hormones led to increased Aβ accumulation and decreased behavioral abilities at 7 months of age; inversely, treatment of females with masculine hormones caused decreased Aβ accumulation and improved behavior (Carroll et al., 2010). The late activation of GluN2B subunit in old 3xTg-AD females may implicate the activation of different pathways for disease progression in 3xTg-AD mice males and females.

In 3xTg-AD mice, progression of tau and Aβ pathologies are tissue specific. In fact, Aβ deposits initiate in the cortex and progress to the hippocampus, in contrast to tau pathology, which is first apparent in the hippocampus, particularly in pyramidal neurons of the CA1 area (Oddo et al., 2003a). Importantly, our results also show a brain tissue-specificity since we were unable to detect alterations in GluN2B subunit activation in the cortex. As described before in this study, Src kinase is involved not only in GluN2B subunit phosphorylation (Sinai et al., 2010), but also in the regulation of proteins involved in actin cytoskeleton stabilization, namely cortactin (Huang et al., 1997) and

Dab1 (Kuo et al., 2005). In this context, the reelin pathway has been involved in the regulation of synaptic plasticity in normal adult (Levenson et al., 2008). Reelin stimulates neurons through the very-low-density-lipoprotein receptor (VLDLR) and apolipoprotein E receptor-2 (ApoER-2) (Weeber et al., 2002), leading to Dab1 phosphorylation in a Src-dependent manner; indeed, once activated, Dab1 may cause NMDAR activation through Src (Kuo et al., 2005). Moreover, Dab1 has been involved in actin polymerization through direct binding to N-WASP, which further activates the Arp2/3 complex (Suetsugu et al., 2004), enhancing actin nucleation (Mullins et al., 1998). In the present study we found decreased total and phosphorylated Dab1 levels in both hippocampus and cortex. In the cortex, Dab1 downregulation was directly correlated to a decrease in reelin. Moreover, in both cortex and hippocampus, data suggest an impairment in reelin receptor-mediated signaling pathways linked to reduced Dab1 phosphorylation. Since activated Dab1 phosphorylates Src (Ballif et al., 2003) and, on the other hand, Src can phosphorylate Dab1 (Kuo et al., 2005), we are unable to determine if diminished Src phosphorylation is the starting point or the consequence of dysfunctional reelin pathway. Notably, decreased Src activity found in young males hippocampus was also found in the cortex, although it did not correlate with P(Tyr1472)GluN2B. Thus, decreased Src activation in early stages of AD appears to be linked to different pathways in the hippocampus and in cortex, respectively the GluN2B-containing NMDARs and the reelin pathway. Furthermore, NMDARs are attached to cytoskeleton via the PSD complex, Shank and GKAP proteins (Naisbitt et al., 1999). Shank protein links PSD-95 to cortactin, which also regulates actin polymerization (Naisbitt et al., 1999). Interestingly, our results evidence low cortactin levels in young 3xTg-AD males. Based on modified levels of Dab1 and cortactin, our data suggest early decreased actin polymerization in 3 month-old 3xTg-AD males.

In conclusion, we clearly demonstrate tissue specific, age- and gender-dependent alterations in GluN2B subunit of NMDARs in 3xTg-AD mice. These findings point out the probable existence of different pathways for NMDARs-mediated toxicity in AD in males and females and the activation of tissue-specific pathological mechanisms. Furthermore, we evidenced early alterations in Dab1 and cortactin, two proteins implicated in actin cytoskeleton stabilization, suggesting an involvement of cytoskeleton deregulation in early phases of AD progression, which may be responsible for spine degeneration, synaptic dysfunction and cognitive deficits observed in AD.

Chapter 5

Changes in oxidative stress in peripheral blood mononuclear cells from Alzheimer's disease patients

5.1. Summary

Oxidative stress and deregulation of Ca^{2+}_i homeostasis have been suggested to be upstream of neuronal loss in AD. In this work we used human PBMCs isolated from mild, moderate and severe AD patients and individuals with MCI *versus* non-demented age-matched control subjects to study markers of peripheral cell injury related with oxidative and its correlation with the severity of cognitive impairment. We found evidences that deregulation of Ca^{2+}_i homeostasis in PBMCs from MCI subjects and mild AD patients is unrelated with changes in pro- or anti-apoptotic proteins. However, these alterations occurred concomitantly with increased ROS levels in MCI PBMCs. Concordantly, in MCI PBMCs, we demonstrated the downregulation of Nrf2, a ROS-related transcription factor, and SOD1, one of the Nrf2 target genes. However, no changes in GCLc (glutamate-cysteine ligase catalytic subunit), CREB (*cAMP response element-binding)*, CBP (CREB-binding protein) or PGC-1α were detected. In conclusion, these results show that PBMCs obtained from individuals diagnosed with pre-clinical and mild AD exhibit changes in Ca^{2+}_i and redox homeostasis that intimately correlate with decreased Nrf2-dependent transcription, implicating these events in cell dysfunction occurring in the initial stages of AD.

5.2. Introduction

AD is considered a neuropathological process with a spectrum of clinical presentations ranging from presymptomatic to full-blow dementia (Dubois et al., 2007); in this respect, MCI has been considered as an intermediate stage between clinically normal individuals and patients with AD (Grundman et al., 2004). Therefore, alterations occurring in MCI individuals represent an important stage to understand early basic mechanisms responsible for the neurodegenerative process in AD. Previous studies indicate that 10-20% of people aged 65 and older have MCI and that approximately 15% of these individuals may progress to dementia each year (Whalley et al., 2006).

Numerous studies suggest that neuronal death in AD occurs downstream of oxidative stress (Butterfield, 2002), impaired Ca^{2+} homeostasis and ER stress, all linked to mitochondrial dysfunction (Ferreiro et al., 2006a;Ferreiro et al., 2008a). Oxidative stress markers in AD have been shown in MCI brains (Pratico et al., 2002) and in the brain of transgenic AD mice before plaque deposition (Resende et al., 2008b), suggesting that these events occur in early stages of the disease. Aβ peptide has been demonstrated to induce oxidative stress in cultured hippocampal and cortical neurons (De Felice et al., 2007;Ferreiro et al., 2008a), which associates with the disorganization of the cytoskeleton and neurite retraction (Chapter 3), Ca^{2+} deregulation (Ferreira et al., 2012a), and apoptosis (Ferreiro et al., 2008a;Paradis et al., 1996). In APP/PS1 mice, induction of Nrf2, a transcription factor that regulates the antioxidant response following low levels of ROS, prevents or delays AD-like pathology (Kanninen et al., 2011). However, Nrf2 levels are decreased in the nucleus of hippocampal neurons of human *post-mortem* AD brains (Ramsey et al., 2007), suggesting decreased Nrf2-mediated transcription linked to oxidative stress. Aβ has been shown to induce apoptotic cell death both *in vitro* and *in vivo*, in a ER stress-dependent pathway (Ferreiro et al., 2006b;Umeda et al., 2011;Costa et al., 2012b).

Although these pathological mechanisms have been described in AD, it is still unclear whether they occur in pre-clinical stages before diagnosis of the disease and thus constitute early events in AD pathogenesis. The molecular mechanisms that lead to neurodegeneration, and which occur several decades before AD diagnosis, remain presently unclear. The assessment of tissue from patients with neurological disorders is limited by the inaccessibility of the organ and consequently, there is a long history of searching for peripheral markers capable of reflecting the pathology within the brain. Human PBMCs are easy to obtained from blood samples and share much of the

nonsynaptic biochemical environment of neurons, such as neurohormones, neuropeptides, cytokines, metabolites and medication blood levels (Gavin and Sharma, 2009), thus appearing as a valuable tool to understand AD pathogenesis.

In the present study we evaluated the relationship between the different stages of cognitive impairment and peripheral cell injury markers using human PBMCs obtained from MCI individuals, AD patients in mild and moderate plus severe stages, and age-matched controls. We analyzed changes in cytosolic Ca^{2+} levels and ROS production under basal conditions and after exposure to H_2O_2 or glutamate. Moreover, data were correlated with transcription regulation and expression of oxidative stress markers, and apoptosis-related proteins in PBMCs. We also evaluated how the treatment of MCI or AD patients with the most common molecules used in AD treatment, namely memantine, an uncompetitive NMDAR antagonist, and acetylcholinesterase (AChE) inhibitors, including donepezil, rivastigmine, and galantamine, altered the several studied parameters. Data provide evidence for Ca^{2+} dyshomeostasis and Nrf2-dependent oxidative damage constitute early events in AD development.

5.3. Results

In the present study, PBMCs were obtained from non-demented controls, individuals with MCI, corresponding to a pre-clinical stage of the disease, and also AD patients presenting different degrees of cognitive impairment (mild and moderate-severe stages). In this peripheral cell model we evaluated the hypothesis that markers of cell injury occurred early in the progression of AD-related cognitive impairment.

Table 5.1 summarizes the characteristics of the sample by diagnostic group. Controls, MCI, mild and moderate to severe AD patients were similar in sex, age and educational level. A significant difference in the MMSE was observed between patients groups ($p < 0.001$); indeed, worth results were observed from MCI to mild AD patients and from these to moderate-severe AD patients. The percentage of patients medicated with memantine, an uncompetitive antagonist of NMDARs, and/or AChE inhibitors, are also indicated in Table 5.1. Data show that memantine was predominantly prescribed in AD cases (mild and moderate-severe AD), whereas AChE inhibitors was similarly prescribed in MCI and moderate-severe AD patients, being also prescribed in a large number of mild AD patients. Patients were also medicated with psychopharmaceuticals (67% of MCI individuals, 89% and 100% of mild and moderate-severe AD patients, respectively), cholesterol-lowering drugs (30% of MCI individuals, 3.7% and 21.2% of mild and moderate-severe AD patients, respectively) and anti-coagulating drugs (approximately 20% in all groups) (data not shown).

Groups	Number of individuals (n)	Gender		Age (years)	Education (years)	Disease onset (years)	Cognition (MMSE Score)	Memantine -treated (%)	AChE inhibitors -treated (%)
		Male (n)	Female (n)						
Control (CDR - 0)	20	8	12	68±6.5 (55-79)	5.7±2.1 (3-9)	-	28.90 ±0.12 (23-30)	-	-
MCI (CDR- 0,5)	24	12	12	71.8±8.6 (48-93)	6.9±4.6 (0-15)	67.3±7.7 (46-78)	27.5±0.63 (21-30)	4.2	16.7
Mild AD (CDR - 1)	27	11	16	72.5±10.6 (53-92)	6.9±4.0 (0-17)	68.2±10.3 (51-89)	20.34±0.89 ***, ### (10-28)	22.2	66.7
Moderate-Severe AD (CDR – 2 and 3)	33	12	21	74.4±10.2 (55-90)	5.6±3.8 (0-15)	67.9±10.1 (50-85)	10.9±1.02 ***, ###,$$$ (0-22)	30.3	33.3

Table 5.1- Characterization of the sample population. Data represent the mean ± SEM of age, education, disease-onset and cognitive impairment (analyzed through MMSE, Mini Mental State Examination), as well as the percentage (%) of individuals under medication with memantine or acetylcholinesterase (AChE) inhibitors (donepezil, rivastigmine and galantamine) relatively to the number (n) of individuals per diagnostic group according to global staging (CDR – Clinical Dementia Rating); numbers in parentheses represent minimal and maximal values for each parameter. Statistical significance: $^{***}p< 0.001$ compared to control individuals, $^{###}p<0.001$ compared to MCI individuals, $^{$$$}p<0.001$ compared to mild individuals.

5.3.1. Deregulation of Ca^{2+}_i

Considering that deregulated Ca^{2+} homeostasis has been described in brain (Fedrizzi and Carafoli, 2011) and peripheral blood cells (Eckert et al., 1994) from AD patients, we evaluated the levels of Ca^{2+}_i in PBMCs from control, MCI and AD patients in basal conditions and following stimulation with two oxidative stress inducers, H_2O_2 and glutamate (Pereira and Oliveira, 1997) or thapsigargin, a selective, non-competitive, inhibitor of SERCA (Fig. 5.1). Basal Ca^{2+}_i levels were significantly higher in PBMCs obtained from mild AD patients (Fig. 5.1 A,B), indicating a peripheral cell deregulation of Ca^{2+}_i homeostasis in this possible initial stage of the disease. Stimulation of cells with H_2O_2 or glutamate caused an increase in Ca^{2+}_i in all groups tested (Fig. 5.1 C,F). However, the response was significantly lower in MCI ($p < 0.05$, Student's t-test) and apparently in AD PBMCs (although not statistically significant), when compared to control human cells (Fig. 5.1 D,E and G,H). These data suggest an inherent deficit in the regulation of Ca^{2+}_i in PBMCs from MCI individuals.

In order to evaluate the contribution of ER Ca^{2+}, PBMCs were also exposed to thapsigargin, a classical disruptor of ER Ca^{2+} homeostasis (Rogers et al., 1995) in the absence of external Ca^{2+}. In the presence of this ER Ca^{2+}ATPase inhibitor, Ca^{2+} entry to ER is blocked and the Ca^{2+} concentration gradient drives the release of its content to the cytosol, giving a good indication of initial ER Ca^{2+} content, as previously described by (Costa et al., 2010). Following exposure to thapsigargin, the levels of Ca^{2+}_i significantly decreased in both MCI and mild AD PBMCs ($p < 0.01$), suggesting decreased ER Ca^{2+} content in these cells (Fig. 5.1 I, J, K). Interestingly, no significant changes in Ca^{2+}_i levels were observed under basal or stimulation conditions in PBMCs obtained from moderate-severe AD patients, compared to control PBMCs. Moreover, no significant differences were observed between memantine- or AChE inhibitors-treated and non-treated patients within the same group (Fig. 5.1 B, E, H, K).

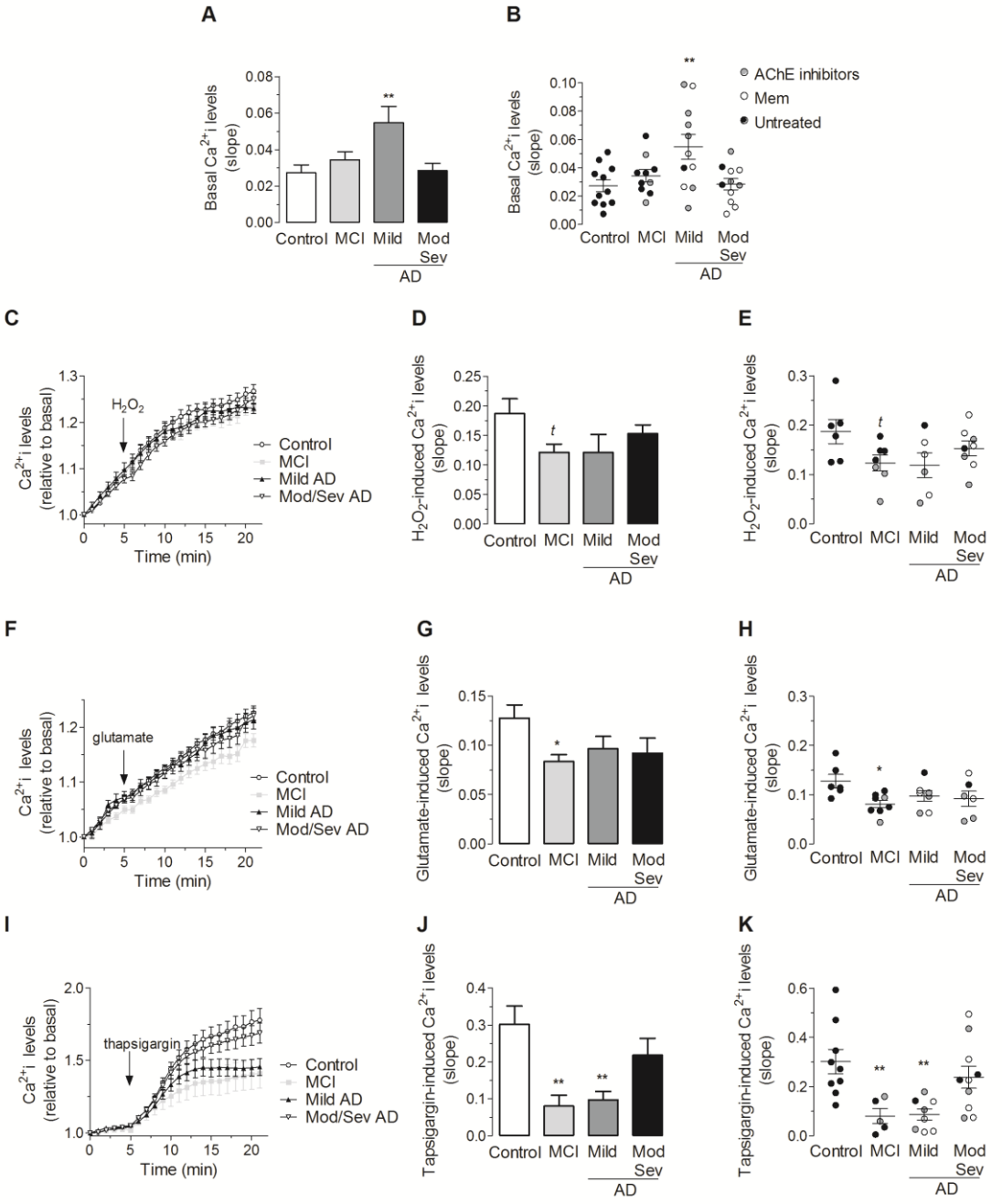

Figure 5.1: Cytosolic Ca^{2+} levels in PBMCs of control individuals, MCI and AD patients. Levels of Ca^{2+} in the cytosol and in ER stores were evaluated by monitoring the fluorescence of Fura-2 in the presence or in the absence of external Ca^{2+}, respectively, in PBMCs derived from control and MCI individuals and mild and moderate plus severe AD patients. (**A, B**) Basal Fura-2 fluorescence was recorded for 5 min. The effect of 1 mM H_2O_2 (**C, D, E**), 1 mM glutamate (**F, G, H**) or 2.5 μM thapsigargin (**I, J, K**) on intracellular Ca^{2+} levels were recorded for 15 min (***i***) and slope was calculated by analyzing the Fura-2 fluorescence ratio at 340/380 nm (***ii***). In **B**, **E**, **H** and **K**, patients medicated with memantine (Mem) appear in white circles and those medicated with acetylcholinesterase (AChE) inhibitors appear in grey circles, patients with none of these treatment (Untreated) appears in black. Data are the mean ± SEM from 8-11 per group individuals performed in triplicates. Statistical analysis: $^{*}p < 0.05$ and $^{**}p < 0.01$ significantly different when compared with control individuals (Dunnett's *post-hoc* test) and $^{t}p < 0.05$ *versus* control (student's *t-test*).

5.3.2. Levels of pro- and anti-apoptotic proteins

In order to ascertain the activation of apoptotic pathways, we further examined the levels of pro-apoptotic proteins, namely Bax, Bak and pro-caspase 3, and also the levels of the anti-apoptotic protein Bcl-2, in PBMCs from control, MCI and AD patients (Fig. 5.3). Our results demonstrate that the levels of these proteins are not significantly changed between PBMCs from AD patients or MCI subjects, relatively to control individuals. Altogether, these results indicate that apoptotic cell death pathways are not activated in PBMCs during disease progression, as demonstrated by the large dispersion of data points within each experimental group (Fig. 5.2).

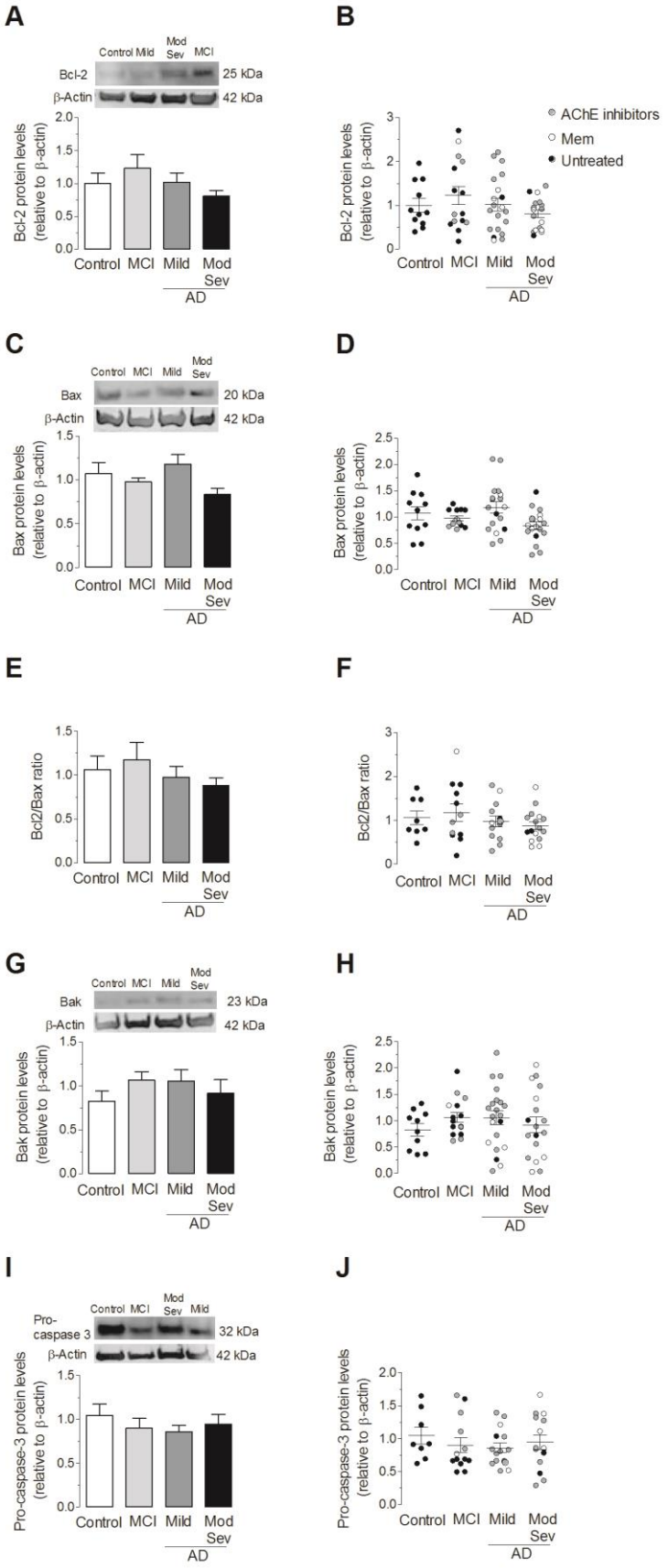

Figure 5.2: Levels of apoptotic proteins in PBMCs of controls, MCI and AD patients. Proteins were extracted in Ripa buffer and immunoblotted for Bcl-2 (**A, B**), Bax (**C, D**), Bak (**G, H**) and pro-caspase 3

(**I, J**). In **E and F** the ratio Bcl-2/Bax is represented. In **B**, **D**, **F**, **H**, **J** patients medicated with memantine (Mem) appear in white circles whereas those medicated with acetylcholinesterase (AChE) inhibitors appear in grey circles, patients with none of these treatment (Untreated) appears in black. Results are expressed in arbitrary units relative to actin as the mean ± of 8-20 individuals per group.

5.3.3. ROS production and transcriptional deregulation

Considering the relevance of oxidative stress in AD, production of ROS was evaluated in PBMCs obtained from MCI subjects or AD patients with different degrees of cognitive impairment (mild and moderate-severe stages, as depicted in Table 1) *versus* control individuals. Measurements were performed under basal conditions or after incubation with H_2O_2, glutamate or with thapsigargin (Fig. 5.3). Basal ROS production was significantly higher in MCI's PBMCs, compared to controls (Fig. 5.3 A,B), but no changes were observed in peripheral cells from established AD stages. Furthermore, treatment of PBMCs with H_2O_2 (Fig. 5.3 C,D,E) or glutamate (Fig. 5.3 F,G,H), induced a significant increase in ROS production in mild AD patients ($p < 0.05$), suggesting that increased susceptibility to oxidative stress occurs in AD cases. Exposure of MCI PBMCs to thapsigargin significantly increased ROS production ($p < 0.05$) (Fig. 5.3 I,J,K), implicating early ROS production possibly driven by ER dysfunction. No significant differences in ROS production were observed following exposure to H_2O_2, glutamate or thapsigargin in cells from moderate-severe AD patients. Furthermore, no protective effects of memantine or AChE inhibitors treatment were observed in MCI or AD patients (Fig. 5.3 B,E,H,K). Additionally, no significant differences between men and women were observed in our population (data not shown).

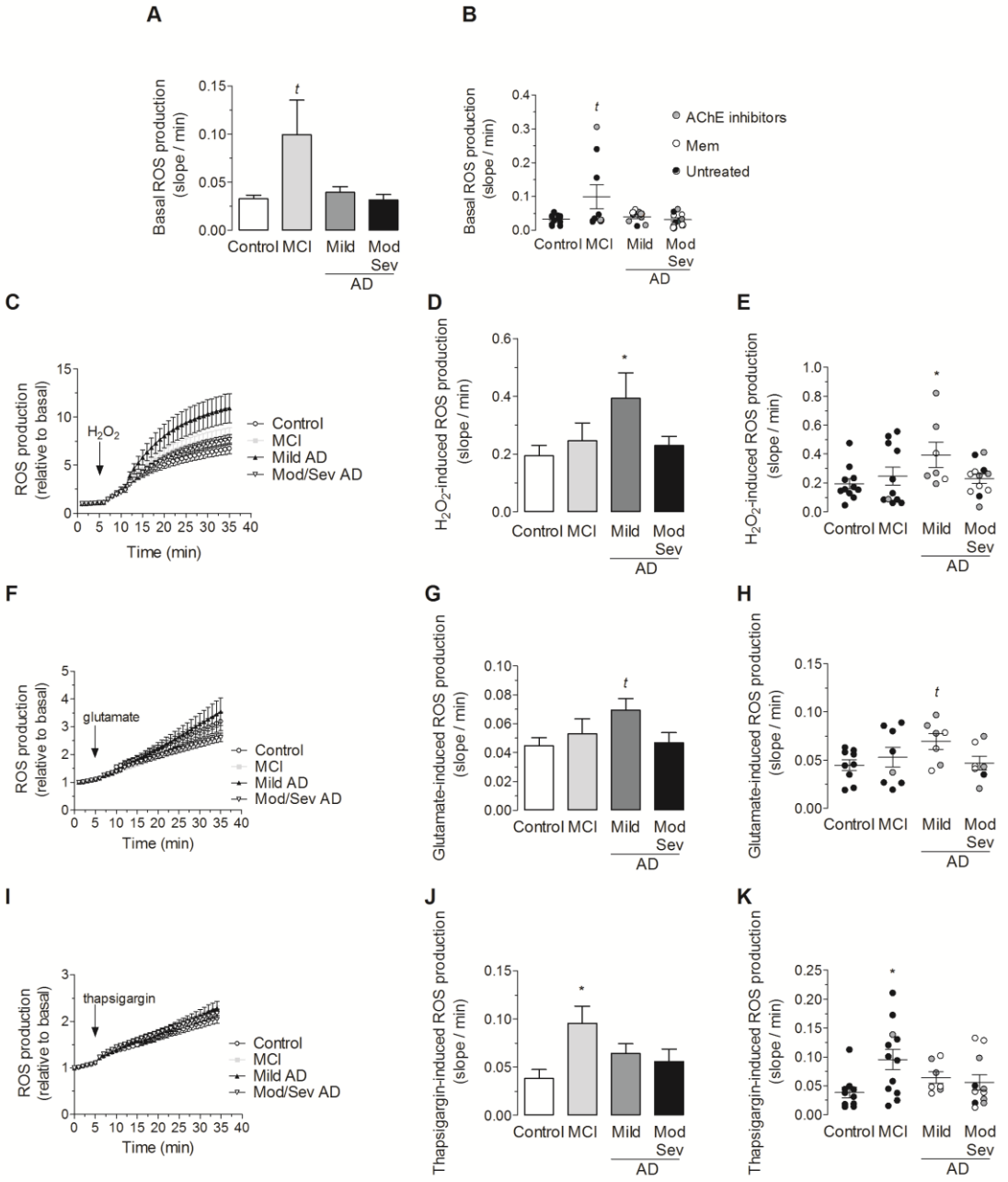

Figure 5.3: ROS production in PBMCs of controls, MCI and AD individuals. Basal DCF fluorescence was recorded for 5 min (**A, B**), cells were then stimulated with 1 mM H_2O_2 (**C, D, E**), 1 mM glutamate **(F, G, H)** or 2.5 µM thapsigargin (**I, J, K**) and fluorescence was recorded for further 30 min (***i***) and slope per minute was calculated (***ii***). In **B**, **E**, **H** and **K**, patients medicated with memantine (Mem) appear in white circles whereas those medicated with acetylcholinesterase (AChE) inhibitors appear in grey circles, patients with none of these treatment (Untreated) appears in black. Data are the mean ± SEM of triplicate from 8-12 individuals per group. Statistical analysis: $^{*}p < 0.05$ significantly different when compared with control individuals (Dunnett's *post-hoc* test) and $^{t}p < 0.05$ *versus* control (student's *t-test*).

In order to investigate if the observed increased in ROS production in PBMCs was due to a downregulation of defensive antioxidant pathways, we further investigated the

expression levels of Nrf2, a transcription factor that regulates a broad spectrum of protective genes, including GCLc and SOD1 (Han et al., 2008). Additionally, we analysed the levels of PGC-1α, a transcriptional co-activator that mediates antioxidant responses, apart from playing a central role in the regulation of cellular energy metabolism (St-Pierre et al., 2006). Finally, we investigated two other transcription factors linked to cell survival pathways, namely CREB and its co-activator CBP (Dragunow, 2004) in the human PBMCs (Fig. 5.4). Importantly, a significant decrease in Nrf2 levels was observed in MCI PBMCs (Fig. 5.4 A,B; $p < 0.05$ by Student's t-test), which was not accompanied by significant changes in other transcription factors or co-regulators, namely PGC-1α, CREB or CBP (Fig. 5.4 C-H). Interestingly, no significant changes were observed on GCLc levels, the catalytic subunit of the main enzyme responsible for glutathione synthesis; however, we detected a significant decrease in SOD1 levels in MCI PBMCs (Fig. 5.5 C,D; $p < 0.001$ by Student's t-test), closely linking ROS generation with decreased Nrf2 levels in pre-clinical AD. These results indicate a decrease in antioxidant defenses in PBMCs from MCI individuals that underlie the enhanced production of ROS. Peripheral cells isolated from mild AD patients showed a tendency for increased levels of SOD1, although not statistically significant in comparison with controls, which may indicate an attempt to compensate basal ROS generation. As described above for other investigated parameters, PBMCs from memantine- or AChE inhibitors-treated AD patients did not differ from non-treated patients, when considering the levels of transcription factors (Fig. 5.4 B,D,F,H) or the Nrf2-regulated target GCLc and SOD1 (Fig. 5.5 B,D); moreover, no changes were observed in PBMCs from men and women within each group (data not shown).

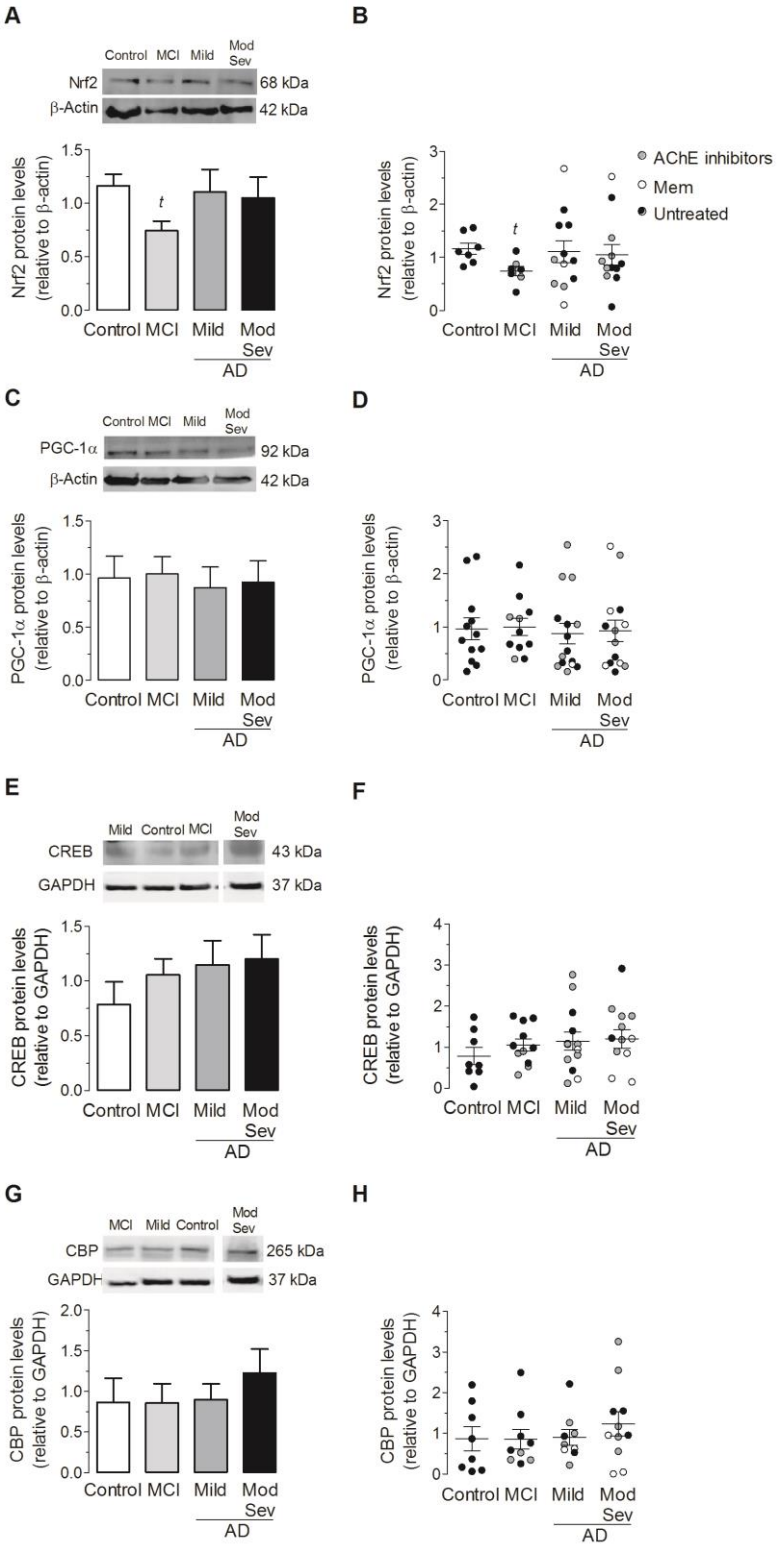

Figure 5.4: Levels of Nrf2, PGC-1α, CREB and CPB in PBMCs of controls, MCI and AD individuals. Proteins were extracted in Ripa buffer and immunoblotted for Nrf2 (**A, B**), PGC-1α (**C, D**), CREB (**E, F**) and CBP (**G, H**). In **B**, **D**, **F**, **H** patients medicated with memantine (Mem) appear in white circles whereas those medicated with acetylcholinesterase (AChE) inhibitors appear in grey circles, patients with none of these treatment (Untreated) appears in black. Results are expressed in arbitrary units relative to β-actin as the mean ± SEM of 7-15 individuals per groups. Statistical analysis: $^{t}p < 0.05$ significantly different when compared with control individuals (Student's *t-test*).

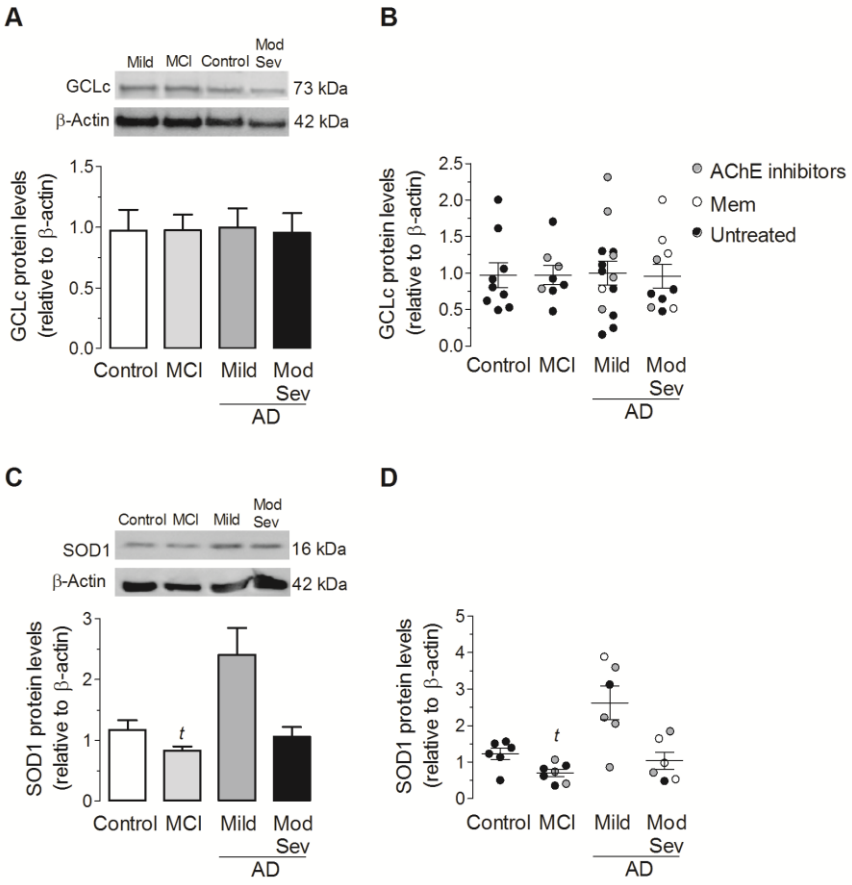

Figure 5.5: Levels of GCLc and SOD1 in PBMCs of controls, MCI and AD individuals. Proteins were extracted in Ripa buffer and immunoblotted for GCLc (**A, B**) and SOD1 (**C, D**). In **B**, **D** patients medicated with memantine (Mem) appear in white circles whereas those medicated with acetylcholinesterase (AChE) inhibitors appear in grey circles, patients with none of these treatment (Untreated) appears in black. Results are expressed in arbitrary units relative to β-actin as the mean ± SEM of 5-14 individuals per group. Statistical analysis: $^{t}p < 0.05$ significantly different when compared with control individuals (Student's *t-test*).

5.4. Discussion

The molecular mechanisms leading to neurodegeneration that occur several decades before diagnosis in AD remain presently unclear. Therefore, detection of early peripheral pathological changes appears to be a valuable tool to understand AD pathogenesis. In this study, we evaluated early peripheral cell injury markers in different stages of cognitive deterioration in AD. Importantly, several dysfunctional parameters were detected in PBMCs obtained from MCI individuals or mild AD patients. We found evidences for deregulation of $Ca^{2+}{}_i$ homeostasis in PBMCs from MCI p*a*tients. Concomitantly, we detected increased ROS levels in MCI patients PBMCs, concordant with downregulation of Nrf2 and SOD1 in MCI PBMCs. Interestingly, no evidences of apoptosis have been found in PBMCs from MCI and AD patients.

Accordingly to the 'amyloid cascade hypothesis', Aβ, particularly in the oligomeric form, triggers several pathological mechanisms leading to synaptic and neuronal dysfunction that underlie cognitive deficits and dementia (Blennow et al., 2006). This hypothesis is supported by numerous evidences including the recent discovery of an APP mutation that decreases Aβ levels *in vitro* and protects against AD-associated cognitive decline (Jonsson et al., 2012). Recently, we demonstrated that NMDA receptor-mediated Ca^{2+} dyshomeostasis is induced by Aβ in cultured neurons (Ferreiro et al., 2006b;Resende et al., 2008a;Costa et al., 2012a;Ferreira et al., 2012b). Furthermore, Aβ was shown to be an ER stressor that activates the UPR and deregulates ER Ca^{2+} homeostasis, increasing cytosolic Ca^{2+} levels and finally leading to apoptosis (Huang et al., 2000;Ferreiro et al., 2008b;Resende et al., 2008a). Previous reports described altered Ca^{2+} signaling in AD lymphocytes, which was assumed to underlie the loss of neuronal plasticity (Bondy et al., 1996). Nevertheless, different results have been found. Some authors revealed that basal cytosolic levels of Ca^{2+} in AD lymphocytes were increased compared to control individuals (Adunsky et al., 1991;Hartmann et al., 1994), whereas these alterations were not found in other studies (Bondy et al., 1994;Eckert et al., 1998). In the present work, we demonstrated that basal Ca^{2+} levels were significantly higher in the initial stage of cognitive deficits (mild AD patients), which may be related to the synaptic alterations previously observed in MCI subjects and mild AD patients (Scheff et al., 2007). Furthermore, we have previously demonstrated in PBMCs from MCI individuals decreased ER Ca^{2+} levels (Costa, 2011), which associated with increased $Ca^{2+}{}_i$ levels in the same individuals indicates a deregulation in ER Ca^{2+} buffering capacity, an early event implicated in ER stress

response (Kim et al., 2008). In the presence of oxidant toxic stimuli (H_2O_2 and glutamate), PBMCs from MCI individuals showed a reduced Ca^{2+} response, indicating increased susceptibility to Ca^{2+} dysregulation following a stressful insult. These results show that during the initial stages of the disease, PBMCs exhibit significant alterations of intracellular Ca^{2+} homeostasis regulation. Interestingly, we did not observe alterations in Ca^{2+} levels in PBMCs isolated from moderate-severe AD patients, suggesting that alternative mechanisms may occur in peripheral cells at later disease stages.

Previous Changes in ER stress Ca^{2+} content in PBMCs isolated from MCI subjects and in the early stages of the disease were followed by enhanced levels of GRP78 and XBP-1 (X box binding protein), valuable markers of ER stress that were found to be up-regulated in AD brains (Zhang and Kaufman, 2006;Yoshida, 2009).

Apoptotic cell death may occur through activation of the ER stress-induced transcription factor GADD153/CHOP (Schapansky et al., 2007;Pino et al., 2009). Previous results suggest the presence of ER stress in PBMCs from MCI and in the early stages of AD; however, no evidences for the involvement of GADD153/CHOP in peripheral cell damage have been found (Costa, 2011). In this work, we were unable to detect evidences of apoptosis activation in peripheral cells from AD and MCI patients. It was previously shown that lymphocytes from MCI subjects and AD patients exhibit increased basal ROS levels that are associated with oxidative DNA damage, as compared with lymphocytes obtained from control subjects (Mecocci et al., 1998;Leuner et al., 2007). Furthermore, Mórocz and colleagues found oxidized purines in nuclear DNA isolated from AD lymphocytes and a diminished repair capacity of H_2O_2-induced oxidized purines (Morocz et al., 2002). Our results are in accordance with these observations since PBMCs from MCI individuals exhibited an increase in ROS production under basal conditions. Importantly, this increase was accompanied by decreased levels of Nrf2, a ROS-related transcription factor, and SOD1, one of the Nrf2 target genes, suggesting an impairment of defensive oxidative stress pathways. These findings are in line with the decrease in Nrf2 observed in AD brains and its sequestration in the cytoplasm (Ramsey et al., 2007). Furthermore, Ca^{2+} released from ER leads to the generation of ROS and depletion of the antioxidant glutathione (GSH); in turn ROS activate the mitochondrial-mediated apoptotic cell death pathway in cultured cortical neurons (Ferreiro et al., 2008b). Therefore, our results obtained in human PBMCs suggest that during MCI, oxidative stress may result, at least in part, from deregulated ER Ca^{2+} homeostasis observed previously (Costa, 2011), correlating

with decreased Nrf2 and decreased antioxidant defenses. Importantly, evidences for deregulation of defensive oxidative pathways in the brain of 3xTg-AD transgenic mice and in $A\beta_{1-42}$-treated cortical neuronal cultures were also recently observed in our group (Caldeira GL, 2012). Mature $A\beta_{1-42}$-treated cortical neurons (maintained for 15 days in vitro) showed early decreased Nrf2 activation and subsequent decrease in nuclear Nrf2 levels; young (3 month-old) 3xTg-AD mice males exhibited increased levels of total Nrf2 in the nucleus, but decreased Nrf2 activity, which were concordant with diminished SOD1; moreover, a late (15 month-old) decrease in nuclear Nrf2 in the cortex of 3xTg-AD males, compared to WT mice (Caldeira GL, 2012), appeared to be in accordance with the decrease in Nrf2 levels observed in *post-mortem* human AD brains (Ramsey et al., 2007) and with our observations in blood peripheral MCI cells. Interestingly, GCLc levels were consistently unaffected in PBMCs and also in the described central models (Caldeira GL, 2012), indicating that early features of oxidative stress and ER stress in AD are unrelated with modified synthesis of glutathione.

Importantly, in all parameters evaluated, we did not observe differences between control individuals and moderate plus severe AD patients. We hypothesise that the similarity of PBMCs responses between controls and late AD patients may be due to the fact that moderate and severe AD patients are not only largely medicated for AD-associated compounds, but are also mediated with psychopharmaceutical, anti-cholesterol and anti-coagulation drugs and that this medication can interfere with the parameters evaluated in PBMCs. Moreover, data also indicate no major effects of memantine (NMDAR antagonist) and AChE inhibitors, the most commonly prescribed molecules in AD pathology, on the parameters evaluated in this study, suggesting that beneficial effects of these compounds may be restricted to the central nervous system.

In conclusion, results obtained in this study provide evidences for an early cell dysfunction arising from Nrf2 deficits and oxidative stress, along with Ca^{2+} dyshomeostasis found in PBMCs from subjects at different stages of cognitive impairment. Moreover, our results largely suggest a concordance between the evidences of oxidative stress markers occurring in human blood peripheral model of AD and in two central models of AD (the 3xTg-AD mice and $A\beta_{1-42}$-treated cortical neurons), suggesting that alterations occurring in peripheral blood cells during pre-clinical and initial stages of the disease might reflect brain changes and may thus be used to elucidate the molecular basis of synaptic dysfunction and neuronal loss in AD.

Therefore, studies conducted in PBMCs might be useful to identify possible targets for earlier detection and therapeutic intervention in AD.

Chapter 6

General Conclusions and Future Directions

AD is characterized by synapse and neuronal loss in the brain. There are increased evidences that synaptic dysfunction appears long before neuronal death in AD, in the early stages of the disease. The role of NMDARs on Aβ-induced neurotoxicity has been largely discussed. Interestingly, for a long time, NMDARs were proposed to be involved at late stages of the disease, as testified by the use of memantine as a memory-preserving drug in moderate- to late-stage AD patients (Reisberg et al., 2003). Currently, there are growing evidences for an early enrollment of NMDARs in AD pathogenesis (Parameshwaran et al., 2008). In accordance, soluble Aβ oligomers were demonstrated to disrupt synaptic transmission *via* NMDARs. In rat organotypic hippocampal slices, Aβ oligomers induced decreased density of dendritic spines and number of functional spines in a process dependent on NMDARs activity (Shankar et al., 2008). Moreover, in young 3xTg-AD mice, soluble Aβ led to disruption of ryanodine receptor signaling and subsequent Ca^{2+}-induced Ca^{2+} release, altering synaptic function in early phases of AD after NMDARs-mediated Ca^{2+} influx (Goussakov et al., 2010). Thus, in the present work we give a further step in this field of research by evaluating the involvement of NMDARs in AD progression using mature hippocampal cells in culture treated with Aβ oligomers and hippocampal and cortical extracts from the 3xTg-AD mice.

In previous studies, our group showed that exposure to fibril $A\beta_{1-40}$ (a less neurotoxic form of Aβ) mediated necrotic cell death through dysregulation of Ca^{2+} homeostasis in HEK293 cell line expressing NMDAR GluN1/GluN2A subunits (Domingues et al., 2007), thus independent of a neuronal context. Moreover, NMDARs overactivation was shown to be required for Aβ oligomers-induced inhibition of LTP in hippocampal slices of WT mice (Li et al., 2011), as well as for the formation of excessive ROS (De Felice et al., 2007); and memantine, a non-competitive antagonist of NMDAR, was recently shown to prevent expression of apoptosis markers and concomitant cognitive deficits triggered by $A\beta_{1-40}$ injections in rat (Javier Miguel-Hidalgo et al., 2012). Importantly, evidences for the role of GluN2B subunit in Aβ-mediated neurotoxicity are increasing. Indeed, our group previously demonstrated that Aβ increased intracellular Ca^{2+} through activation of GluN2B-containing NMDAR activation in cortical neuronal cultures and that, conversely, GluN2A antagonism potentiated Ca^{2+} dysregulation, suggesting a protective role for GluN2A-containing NMDARs (Ferreira et al., 2012). Other studies evidenced a direct activation of NMDARs by Aβ after binding to the receptor (De

Felice et al., 2007) or indirectly through the increase in glutamate release (Brito-Moreira et al., 2011). Furthermore, knock-out of GluN1 subunit of NMDAR in hippocampal neurons abolished Aβ oligomers binding to dendrites (Decker et al., 2010). Furthermore, there is now strong evidence that Aβ oligomers cause ER stress in hippocampal neurons after binding of Aβ to GluN2B-containing NMDARs (Costa et al., 2012). In the present thesis, we demonstrated the involvement of NMDARs in the toxicity induced by $A\beta_{1-42}$ since all its effect were partially or completely prevented by different NMDAR antagonists (Chapter 3). Interestingly, considering that the concentration of memantine used in our study has been shown to mainly block extrasynaptic NMDARs (Xia et al., 2010), and consistent protection was achieved with memantine and ifenprodil, but not with MK-801 (Chapter 3), our study reveal an important role of extrasynaptic NMDARs, mainly composed of GluN2B subunits (Stocca and Vicini, 1998;Tovar and Westbrook, 1999), in Aβ toxicity. These results are in accordance with a recent study demonstrating that Aβ oligomers preferentially potentiate extrasynaptic NMDAR-mediated responses and more particularly those containing the GluN2B subunit (Kervern et al., 2012), strengthening the potential role of NMDAR selective antagonists in the treatment of AD. In a second part of the thesis (Chapter 4) we were unable to detect significant alterations in total GluN1, GluN2A and GluN2B levels in both hippocampus and cortex from young and old 3xTg-AD mice (Chapter 4). These results appear in opposition with data from *post-mortem* AD patient brain that evidenced a decrease in NMDAR subunit protein and mRNA (Hynd et al., 2001;Hynd et al., 2004b;Hynd et al., 2004a;Bi and Sze, 2002;Mishizen-Eberz et al., 2004). This difference evidences a limitation of the 3xTg-AD mice as an AD animal model. 3xTg-AD mice develop both Aβ and tau pathologies (Oddo et al., 2003) and reproduces many functional processes thought to occur in AD, such as an impairment in $Ca^{2+}{}_i$ homeostasis (Lopez et al., 2008) and oxidative stress (Resende et al., 2008), among others; however, this animal model does not exhibit neuronal loss, indicating that Aβ deposition in 3xTg-AD does not reflect the AD patient late stage. Regarding the activation levels of GluN2A and GluN2B containing NMDARs, our data evidenced age- and gender-dependent modifications in GluN2B subunit activation in the hippocampus. We found a decrease in P(Tyr1472)GluN2B levels in young 3xTg-AD males hippocampus (3 month-old), reflecting decreased activity of GluN2B-containing NMDARs. Interestingly, 3xTg-AD present an early cognitive deficits at 4 months of age, which correlates with the presence of intracellular Aβ (Billings et al., 2005).

Importantly, we confirmed the presence of intracellular Aβ in our 3xTg-AD males at 3 months of age (Chapter 4), which suggests that early cognitive deficits observed in AD (Billings et al., 2005) may be linked to alterations of the GluN2B subunit activation status (Chapter 4), since GluN2B subunit is required for LTP (Bartlett et al., 2007). Moreover, regarding the involvement of the GluN2B subunit in excitotoxic processes (Ferreira et al., 2012;Costa et al., 2012), the hypothesis that decreased GluN2B activation may be an attempt to prevent neurotoxicity cannot be excluded. Conversely, in 3xTg-AD females, we found late (15 month-old) increase in GluN2B-containing NMDARs activity. Differences observed between males and females suggest activation of different pathways during disease progression depending on gender in 3xTg-AD mice, which seems to be in accordance with observations made in humans. In fact, the incidence of AD is higher in post-menopausal women than in age-matched men (Fratiglioni et al., 1997;Gao et al., 1998); whereas at young age, mitochondria from 3xTg-AD mice females are more resistant to Aβ than mitochondria derived from 3xTg-AD males (Lloret et al., 2008). Altogether, results obtained in rat hippocampal cells and in 3xTg-AD mice evidenced the importance of NMDARs and more particularly the GluN2B-containing NMDARs in AD progression.

Studies have demonstrated the negative effect of Aβ on cytoskeleton stability. Indeed, Aβ causes spine loss, tau missorting and microtubule depletion (Zempel et al., 2010), microtubule destabilization being in part associated to decreased alpha-tubulin acetylation (Henriques et al., 2010). Moreover, in human AD brain, the number and length of microtubules in pyramidal neurons were shown to be significantly reduced (Cash et al., 2003). Data obtained in mature hippocampal neurons in culture suggest that Aβ induces delayed decreased polymerized beta-III tubulin, which correlates with reduced neurite length in a process dependent on NMDARs, thus suggesting a destabilization of microtubule cytoskeleton by Aβ (Chapter 3). Moreover, our results showed a high correlation between delayed depolymerized beta-III tubulin and DNA fragmentation in a process dependent on NMDARs, which is in accordance with several studies wherein the cytoskeleton dysfunction was suggested to play a key role in cell death (Cabado et al., 2004;Miura et al., 1999;Tsukidate et al., 1993). In the 3xTg-AD mice, we found early Tyr1472GluN2B dephosphorylation in males (Chapter 4), which has been correlated in literature with the internalization of GluN1/GluN2B-composed NMDARs (Kurup et al., 2010), a process known to require actin cytoskeleton

rearrangement (Mandal and Yan, 2009). Moreover, in hippocampus, alterations in GluN2B activation were correlated with changes in Src kinase activation in our model (Chapter 4). Interestingly, Src kinase is also implicated in the regulation of proteins involved in actin cytoskeleton stabilization, namely cortactin (Huang et al., 1997) and Dab1 (Kuo et al., 2005). Dab1 is phosphorylated by Src after reelin-induced neuron stimulation and may lead further to NMDAR activation through Src phosphorylation (Kuo et al., 2005). In this work, we found significant decreased of total and phosphorylated Dab1 levels in both hippocampus and cortex of young 3xTg-AD males. Taking into account that Dab1 is involved in actin polymerization (Suetsugu et al., 2004), our data suggest a destabilization of actin cytoskeleton through the decrease in Dab1 total and activated levels in 3xTg-AD young males and, otherwise, an impairment in reelin signaling pathway. On the other hand, cortactin, a protein that regulates actin polymerization, is linked to NMDARs through PSD proteins (Naisbitt et al., 1999) and cortactin levels may reflect altered cytoskeleton stability. Although we did not detect changes in PSD-95 in both hippocampus or cortex of young 3xTg-AD males (Chapter 4), the decrease in GluN2B phosphorylation (without changes in total levels of NMDARs) may reflect an increase internalization of GluN2B-containing NMDAR and thus possible lessen binding to PSD-95. Furthermore, low levels of total and activated cortactin again suggest early decreased actin polymerization in AD. Altogether, our results suggest that Aβ triggers microtubule depolymerization in neurons, which can trigger cell death (Chapter 3) in a NMDARs-dependent manner. Moreover, we provided evidences for possible actin cytoskeleton destabilization in young 3xTg-AD mice expressing intracellular Aβ (Chapter 4). Since actin cytoskeleton destabilization may impact on the function of the post-synaptic terminal by remodeling the dendritic spine, AD-related changes in actin cytoskeleton ought to be thoroughly analysed in future studies.

In Chapter 3 we also evidenced deleterious effects of Aβ in non-neuronal cells. Our data obtained in mature hippocampal cells demonstrated that Aβ causes significant DNA fragmentation in glial cells; moreover, this effect was dependent on NMDARs and more particularly the GluN2B subunit. Astrocytes express NMDARs (Lee et al., 2010;Zhou et al., 2010), suggesting a possible effect of Aβ directly on astrocytic NMDARs. However, glial apoptotic cell death may be also driven by degenerating neurons, which can produce and release ROS (Butterfield, 2002), affecting proximate cells. Moreover,

considering the important crosstalk between neurons and glial cells (Ben and Pascual, 2010), we can not exclude that effects on non-neuronal cells may lead to deregulation of neuronal function, possibly exacerbating neurite retraction and synaptic dysfunction.

Data shown and discussed in Chapter 5 further support the hypothesis that early cellular alterations occurring in the AD brain may be reflected in peripheral cells. Previous reports described several cytogenetic alterations in AD human lymphocytes (Petrozzi et al., 2002). Dysregulation of Ca^{2+} signaling in AD lymphocytes was found and compared to Ca^{2+} dysregulation observed in neuronal death (Bondy et al., 1996). In this work, we demonstrated significant alterations in intracellular Ca^{2+} homeostasis in PBMCs involving the ER in the initial stages of the disease and increased susceptibility to Ca^{2+} dysregulation following a stress condition in PBMCs obtained from MCI individuals. Increased basal $Ca^{2+}{}_i$ levels in PBMCs from mild AD patients were also correlated with decreased ER Ca^{2+} levels, indicating a deregulation in ER Ca^{2+} buffering capacity, considered one of the earliest events implicated in ER stress response (Kim et al., 2008). We further observed increased basal ROS production in PBMCs from MCI individuals, accompanied by decreased levels of Nrf2, a ROS-related transcription factor, and SOD1 (one of the Nrf2 targets), suggesting an early impairment of defensive antioxidant pathways. Interestingly, this last observation has also been observed in central models of AD, namely brain samples from the 3xTg-AD and in $A\beta_{1-42}$-treated cultured cortical neurons (Caldeira GL, 2012). Our results are in accordance with evidences of oxidative stress in lymphocytes from MCI (Leuner et al., 2012;Mecocci et al., 1998) and AD subjects (Morocz et al., 2002). Importantly, our study suggests a role for Nrf2 transcription pathways in early stages of AD. In fact, Nrf2 was decreased in AD brains and sequestered in the cytoplasm of neurons in *post-mortem* human brain (Ramsey et al., 2007); here, and for the first time, we demonstrated that alterations in Nrf2 also occur in peripheral cells, at an early stage of the disease. Importantly, we did not observe differences between control individuals and moderate plus severe AD patients in the several studied parameters, which can be explain in part by the fact that late AD patients are not only largely medicated with AD-associated compounds, but are also medicated with other drugs that may interfere with the parameters evaluated in PBMCs, such as antidiabetic (Kanigur-Sultuybek et al., 2007) or anxiolytic (Nunez et al., 2011) compounds acting as protective agents.

In conclusion, in this thesis we have clearly demonstrated the involvement of NMDARs in AD progression and more particularly the GluN2B-containing NMDARs. Interestingly, consistent protection achieved by memantine stresses the importance of extrasynaptic NMDARs; nevertheless, the role of synaptic or extrasynaptic location of NMDAR subunits and the dynamic distribution of these receptors in and out the plasma membrane in AD remains unresolved. In this context, it would be interesting to characterize changes in GluN2A-B subunit NMDAR composition and specific localization in synaptic *versus* extrasynaptic sites, in association with modified NMDAR activation and their role in the toxicity induced by Aβ. Regarding the evaluation of injury markers in PBMCs form MCI and AD patients, results in MCI patients clearly indicate an early impairment of antioxidant response. Taking into account the increasing interest in early prediction of AD, before symptoms appearance, it would be interesting to evaluate in the next 3-5 years whether MCI individuals progressed or not into probable AD cases, and whether they show changes in oxidative stress -related parameters evaluated in the present study.

Chapter 7

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