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Neurodegenerative role of stress and glucocorticoid hormones through Alzheimer's disease Tau protein: a proteomic link between stress and brain pathology.

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Trabalho realizado sob a orientação de
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Anexo 3

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"Nothing in life is to be feared, only to be understood."

Marie Curie

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ABSTRACT

Neurodegenerative role of stress and glucocorticoid hormones through Alzheimer's disease tau protein: a proteomic link between stress and brain pathology.

The importance of mechanistic understanding of TAU-mediated neurodegeneration as well as of involvement of risk factors in Alzheimer's disease (AD) and related Tauopathies has been recently recognized towards prevention of tauopathy-associated dementias. Several lines of clinical investigation have identified different risk factors such as aging, genetic mutations, stressful environmental circumstances and gender, although their inter-significance to the onset and progression of TAU-related AD pathology is unclear. Previous studies have revealed that stress triggers APP misprocessing and Aβgeneration as well as abnormal tau hyperphosphorylation but there is lack of molecular evidence of stress role on TAU aggregation mechanisms and its significance on cognitive and the less studied emotional component of AD pathology. Using a newly generated transgenic model expressing P301L-TAU, this study shows that prolong stress triggers TAU pathology, aggravating levels of tau aggregates and associated behavioral impairments in a gender-specific manner, with female animals being more vulnerable to the deleterious effects of stress which influences both their emotional and cognitive status. Furthermore, molecular analysis revealed that stress deregulated chaperone-mediated TAU degradation machinery by altering molecular chaperones such as Hsp90 and Hsp70 while it resulted in increased generation of truncated TAU triggering apoptotic pathways. These Master project findings provide further molecular evidence of the neurodegenerative potential of stress providing further support of gender implication in stress-triggered brain pathology in AD and other Tauopathies.

RESUMO

O papel do stresse na neurodegeneração induzida pela Tau: ligação proteómica entre o stresse e a doença de Alzheimer.

Compreender os mecanismos moleculares bem como o envolvimento dos factores de risco no desenvolvimento das doenças neurodegenerativas, como a doença de Alzheimer ou as Tauopatias, tem sido um dos grandes focos de interesse da comunidade científica, de forma a identificar as estratégias preventivas destas demências. Várias linhas de investigação científica têm vindo a identificar alguns factores que contribuem para o desenvolvimento da Doença de Alzheimer, tais como a idade, mutações específicas ou parâmetros ambientais, no entanto a sua relevância no contexto destas patologias ainda é escasso. Estudos recentes demonstraram o envolvimento do stresse crónico e das hormonas de resposta ao stresse, os glucocorticóides, como potenciais agentes desencadeadores/promotores dos mecanismos neurodegenerativos da doença de Alzheimer, e com impacto tanto a nível cognitivo e na componente emocional da doença.

Neste trabalho estudamos um ratinho transgénico que expressa a Tau humana mutada que contém uma das mutações identificadas mais comuns (P301L). Demonstramos que o stresse crónico despoleta algumas das características patológicas associadas com as Tauopatias e que este efeito é dependente do género do animal. Enquanto que os machos apenas apresentam um fenótipo ansioso, nas fêmeas, o stresse crónico despoleta/agrava a formação dos agregados da Tau e induz o aparecimento de deficits cognitivos (além dos emocionais).

A análise molecular revelou ainda que o stresse desregula a maquinaria celular envolvida no folding/degradação da Tau, pois altera os níveis das Hsp90 e 70; e também parece ter um efeito substancial nos níveis envolvidos na apoptose (Bcl2,Bax).

Este estudo contribuiu para o melhor conhecimento dos mecanismos moleculares patogénicos induzidos pelo stresse no contexto de uma Tauopatia, e demonstrou que o género é um factor determinante no desenvolvimento destas patologias.

CONTENTS

ABREVIATIONS

µm – micrometer

a.a. – Aminoacid

ACTH – Adrenocorticotrophin Hormone

AD – Alzheimer's Disease

AICD - APP intracellular domain

ANOVA – Analysis of Variance

APP – Amyloid Precursor Protein

ATP – Adenosine Triphosphate

Aβ – Amyloid beta

BACE – β-secretase

Bcl-2 – B cell lymphoma 2

BSA – Bovine serum albumin

C99 – APP C-terminal fragment

CBD – Corticobasal Degeneration

CHIP – Carboxyl terminus of the Hsp70

CONT – Control

CORT – Corticosterone

EGTA – Ethylene glycol tetra-acetic acid

EPM – Elevated Plus Maze

FTD – Frontotemporal Dementia

FTDP-17 – Frontotemporal Dementia with Parkinson, linked to chromosome 17q21

g – Gram

GC – Glucocorticoids

GR – Glucocorticoid Receptor

HCl – Hydrochloride

HPA – Hypothalamic-Pituitary-Adrenal

HSF-1 – Heat shock factor 1

Hsp – Heat Shock Protein

kDa – kiloDalton(s)

LC – Locus coeruleus

MAP – Microtubule Associated Protein

MAPT – Microtubule-associated Protein Tau

MC – Mineralocorticoids

MCI – Mild Cognitive Impairment

mL - milliliter

mM – milimolar

mPFC – medial-PFC

MR – Mineralocorticoid Receptor

mRNA – messenger Ribonucleic Acid

MT – Microtubule

MW – Molecular Weight

MWM – Morris Water Maze

NFT – Neurofibrilary Tangle

NMDA – N-methyl-D-aspartate

NTh's – Neurophil threads

ON - overnight

PB – Phosphate Buffer

PBS – Phosphate buffered saline

PD – Pick's Disease

PFC – Prefrontal Cortex

PHF – Paired Helical Filament

PP1/2 – Protein Phosphatase ½

RNA – Ribonucleic acid

RT – Room Temperature

SEM – Standard Error Mean

SF – Straight Filaments

STR - Stress

Tau-C3 – Tau protein clone 3

TBS – Tris Buffer Saline

TEM – Transmission Electron Microscope

Tg - transgenic

T-test – Student's T test

WT–Wild-type

1. GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 ALZHEIMER´S DISEASE – A 100-YEARS OLD DISORDER WITH UNCLEAR ETIOLOGY

In 1906, Dr Alois Alzheimer described a surprising new clinical disorder after examining Auguste D, a 51 year old woman that exhibited a cluster of clinical symptoms, including progressing amnestic disorder, alexia, aphasia and agraphia, accompanied by disorientation, auditory hallucinations, paranoia, profound agitation and marked psychosocial impairment (Graeber & Mehraein, 1999). Auguste D died after four and a half years since the first symptoms appeared. This fast development of the disease and its pronounced symptoms made this clinical case unusual leading Dr Alzheimer to investigate the neuropathological features. The histological analysis of her brain revealed that "*cerebral cortex was severely damaged exhibiting numerous small miliary foci and neurons with several fibrils*". All of these histopathological findings, the early onset and the rapid progression of the disease were completely apart from all the mental disorders described so far considering this condition as a unique disease, named later as "Alzheimer's disease" (AD). (Feldman, 2007).

Today, AD is considered a progressive age-related neurodegenerative and irreversible disorder while it is the most common type of dementia with a parallel decline in language and learning functions, followed by apathy and severe mood deficits. In advanced stages of the disease, psychosis and agitation become part of the daily life of patients, affecting much of their activities. (Kelly, 2008). Among AD patients, 5-7% of them develop an early onset of the disease, after 40 years old (so-called familial AD) usually due to one or more mutations in genes related to the disease (see below) while more than 95% of AD cases (sporadic AD) are affected later in life (>65 years old), without clarified genetic mutations. In general, AD patients exhibit an age-dependent increased probability for developing the disease with individuals beyond the age of 70 exhibiting a dramatic increase (Feldman, 2007) (Morawe, et al., 2011). AD is currently affecting more than 26 million people worldwide while this number is expected to rise to 106 million by 2050 (Frisardi, et al., 2011) due to the increased longevity, raising AD one of the major health problem for modern societies. Thus, the importance of an early diagnosis and treatment are of extreme relevance for public health (Wehling & Groth, 2010).

Although the understandings of the molecular and cellular pathways underlying the disease have grown enormously in the last years, AD etiopathology is still unclear with many parameters which are involved in the development of the disease with aging being the main predominant risk factor. In addition, genetic mutations in specific genes have also been found to be responsible for the development of the disease (e.g. APP, PS1, PS2), especially in the familial AD (Turner, et al., 2003). Furthermore, gender differences are also quite pronounced in AD as women are more prone to develop AD; a difference commonly associated with the post-menopausal decline in levels of the protective estrogen hormones (see review in (Pike, et al., 2009)). In addition, other predisposing factors have been associated with the onset of AD, such as vascular risk factors for heart disease or stroke, (e.g. like diabetes, hypertension, smoking and obesity) (Reitz, et al., 2011). Recently, chronic psychological stress is also thought to have a detrimental role of AD since several studies describe elevated cortisol levels in AD patients (Swaab, et al., 1994) (Hartmann, et al., 1997) and stress and stress hormones are also known to trigger neuronal atrophy and memory impairment (Cerqueira, et al., 2005)(Magariños, et al., 1996) that also characterize AD pathology. Despite the above evidence, the significance interplay of all these risk factors on the initiation and/or progression of AD remain unclear and not well understood.

1.2 ALZHEIMER´S DISEASE PATHOLOGY AND ITS PROGRESSION

Over the last decades, huge research efforts have been focused on clarifying the detailed anatomical progression and underlying neurodegenerative mechanisms of AD pathology. Histopathologically, AD brains are characterized by two principal hallmarks: 1) deposits of amyloid β peptide (Aβ), a cleavage product of the transmembrane protein called Amyloid Precursor Protein (APP) and 2) intracellular neurofibrillary tangles (NFT's), formed by the aggregates of abnormally hyperphosphorylated Tau protein (Gendron & Petrucelli, 2009). In addition, AD brains are characterized by neuronal atrophy and loss of synaptic connections that is followed by neuronal loss leading to severe whole brain atrophy at the late stages of the disease (Figure 1a).

Along the disease progression, different brain regions are affected starting from trans-entorhinal region (stage I/II) and hippocampus and later the pathology spreads to forebrain nuclei, thalamus and amygdala (stage III/IV – limbic stages), and in the last stages of the disease, diffusing through neocortical regions (stage V/VI) (Braak & Braak, 1991) (Nagy, et al., 1999).

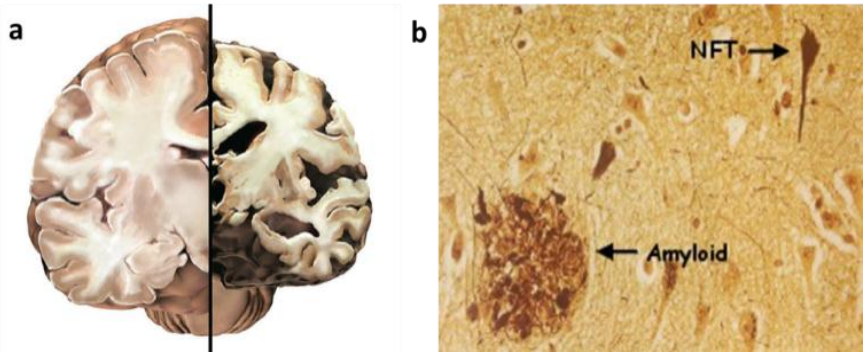

FIGURE 1. Histopathological hallmarks of Alzheimer's disease. (a) Crosswise brain slice where AD brain (right) exhibits severe atrophy in comparison to healthy aged-matched one (left); (b) Histological detection of NFT's and Amyloid plaques in AD brain (adapted from Kelly, 2008 and Vogels, 2009).

1.2.1. AMYLOID PLAQUES AND APP PROCESSING

Amyloid plaques consist of a central extracellular core of aggregated amyloid β-peptide (Aβ), a 39-43 aminoacid peptide, arranged as β-sheet filaments surrounded by dystrophic axons and dendrites. Aβ is a cleavage product of amyloid precursor protein (APP) (Huang & Jiang, 2009) (Figure 2).

APP is a trans-membrane protein with a large extracellular domain. It is synthesized in the endoplasmic reticulum, transported to Golgi network, and then to the cell membrane, via the secretory pathway (Mattson, 2004). It is expressed in many cells and tissue types including endothelial, glial and neuronal cells. APP undergoes post-translational proteolytic cleavage via two different pathways: a) the non-amyloidogenic pathway where APP is sequentially cleavage by α-secretase and γ-secretase (Figure 2a) b) the amyloidogenic pathway where APP is cleaved by BACE-1 (β-secretase) and γ-secretase resulting in the production of Aβ (Golde, et al., 2010) (Figure 2). Cumulative evidence suggest a key role of the amyloidogenic pathway to AD pathology as many cleavage products of this pathway have neurotoxic properties; e.g. the BACE-1 cleavage product, C-terminal fragment (C99) as well as the γ-secretase cleavage product, Aβ and AICD are demonstrated to process cognition-impairing properties damaging many cellular pathways related to neuronal malfunction and survival (Turner, et al., 2003) (Lichtenthaler, et al., 2011).

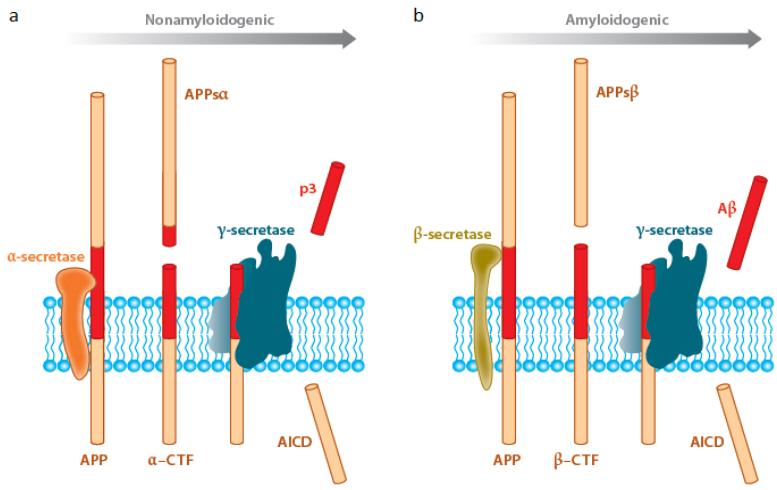

FIGURE 2. Mechanisms of APP processing. (a) Non-amyloidogenic pathway – the consecutive cleavage of APP by α-secretase and γ-secretase generates p3 fragment and AICD at the end; (b) Amyloidogenic pathway – the cleavage of APP by β-secreatase and γ-secretase results to the production of AICD and amyloid **β (Aβ).** (Adapted from O'Brien & Wong, 2011).

Based on the predominant hypothesis for AD, Aβ is the triggering parameter of the disease causing synaptic atrophy and loss, neuronal atrophy and disconnection which results in cognitive deficits, defining Aβ neurotoxic properties. Previous studies have shown that the soluble Aβ, and not the amyloid deposits, appear to exert neurotoxic effects, rapidly blocking long-term potentiation (LTP) in the hippocampus (Walsh, et al., 2002), increase oxidative stress activating Fyn signaling pathways, and stimulating GSK3β-mediated hyperphosphorylation of the cytoskeletal protein, Tau (First described in (Takashims, et al., 1998) (Small & K., 2008). Indeed, many studies demonstrated the neurotoxic actions of Aβ are mediated by Tau protein (Rapoport, et al., 2002) (Roberson, et al., 2007) (Shipton, et al., 2011).

1.2.2 NEUROFIBRILLARY TANGLES AND TAU PROTEIN

Neurofibrillary tangles (NFT's) are the other main hallmark of AD pathology. NFT's are made of highly insoluble paired helical filaments (PHF) that appear as left-handed double helices and straight filaments (SF), consisted of abnormally hyperphosphorylated TAU.

Tau is a soluble microtubule associated protein that modulates the stability and assembly of MTs, inhibiting MT depolymerisation and reducing their dynamic. Thus, Tau has an important role in axonal growth and development, maintaining axonal morphology, cellular trafficking, and transporting cargo from the pre- to the post-synaptic regions, critical for synaptic function (Gendron & Petrucelli, 2009). Tau protein is attached to the MTs by the C-terminal region, and the N-terminal protrudes from the MTs, determining the space between them and playing an important role in the interaction with other cytoskeleton proteins (Gendron & Petrucelli, 2009). The phosphorylation of certain residues within the Mt-binding domain of Tau protein impairs its interaction with MT, leading to the detachment of Tau and its subsequent aggregation into NFTs and MT's destabilization (Deshpande, et al., 2008) (Gendron & Petrucelli, 2009).

The human Tau gene is located in chromosome 17. Alternative mRNA splicing of exons 2, 3 and 10, leads to 6 isoforms of the protein, which are all expressed in the adult brain. The expression of the six isoforms varies during brain development (Ballatore, et al., 2007). The isoform of Tau expressed in fetal human or rodent brain is the smallest one (see Figure 4) which is highly phosphorylated. Following the brain development, Tau expression pattern changes and fetal Tau expression is reduced while all the six isoforms are expressed in adults humans (Billingsley & Kincaid, 1997) but only 4 isoforms are expressed in adult rodent brain (Figure 3) (Ballatore, et al., 2007).

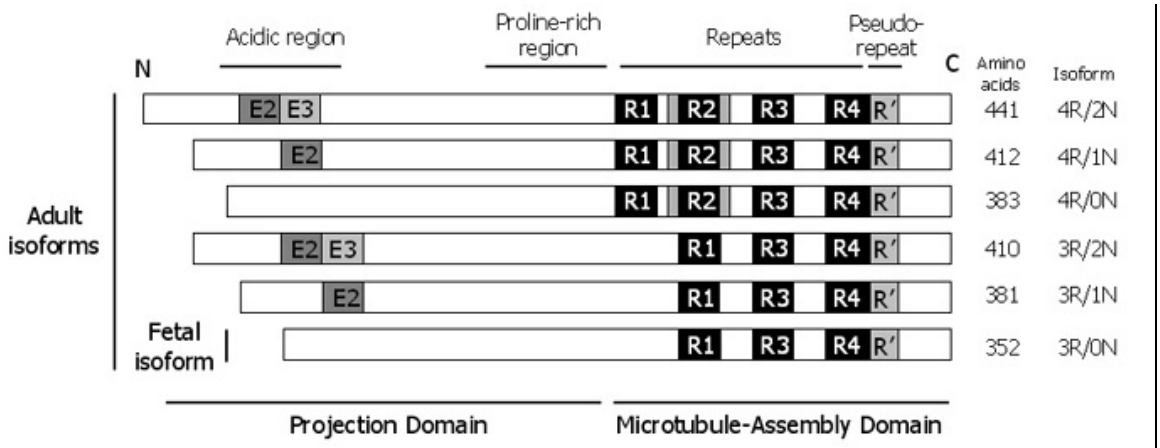

FIGURE 3. TAU isoforms in the fetal and adult brain. Tau isoforms are generated by the alternative splicing of exon 2 and 3 (E2 or E3) and exon 10 (R2) which results in the generation of two main subgroups of isoforms depending of the number of repeats located in the Microtubule-assembly domain (three or four repeats; 3R or 4R) (adapted by Chun & Johnson, 2007).

TAU PHOSPHORYLATION AND ITS ROLE IN AD PATHOLOGY

Tau protein can be phosphorylated at several sites (such as serine, threonine and tyrosine residues) along its structure. Specifically, 80 putative sites of threonine and serine phosphorylation exist in the longest isoform of Tau (441 residues). Kinases are the responsible enzymes that phosphorylate Tau by transferring phosphate groups from high-energy donors, like ATP, to specific substrates by a process known as phosphorylation. Tau kinases are divided in two groups: i) proline-directed kinases, such as GSK-3β, cdk5, MAP kinase and stress-activated kinases and ii) non-proline directed kinases, such as PKA, CaMKII and MARK/PAR-1 (Avila, et al., 2004). In addition to kinases, Tau phosphatases [such as protein phosphatase 1 and 2 A (PP1 and PP2A)] also regulate its phosphorylation pattern as they remove phosphate groups from Tau protein. PP2A is the most activated enzyme in dephosphorylation process of abnormally phosphorylated Tau and its levels are decreased in AD brains (Wang, et al., 2007).

But is phosphorylation itself a detrimental effect for Tau and its function? Indeed, Tau phosphorylation is a natural and dynamic process in cells known to regulate tau function (e.g. binding to MTs). Under pathological conditions e.g. AD pathology, Tau is abnormally hyperphosphorylated in many sites such as within the repeat region which impairs the interaction between Tau and microtubules leading to the detachment of Tau for microtubules (Gendron & Petrucelli, 2009) (Deshpande, et al., 2008). In addition, hyperphosphorylation is suggested to alter conformation of Tau protein which leads to Tau aggregation into oligomers and then, into insoluble paired helical filaments (PHFs) and subsequently to Neurofibrillary tangles (NFTs) (Figure 4). In the "pre-tangle state", where Tau monomers/oligomers aggregate into β-sheets (Ballard, et al., 2011), the hyperphosphorylated Tau is soluble. Then, it gradually aggregates into non-soluble fibrillary inclusions in the dendrites and cell body. These aggregates are resistant to proteasomal or lysosomal degradation resulting in higher accumulation of Tau aggregates, and consequent NFTs formation (Braak & Tredici, 2011). Curiously, NFT's bearing neurons appear to survive for decades (Morsch, et al., 1999) and MT's reduction in AD occurs independently of tau filaments and NFT formation (Cash, et al., 2003). Indeed, tau hyperphosphorylation, without the formation of filaments, can result in neurotoxicity; e.g. phosphorylated Tau at, pT231, pS262 and pS396/404have neurotoxic functions by interfering with MT stability and assembly, comprising defective dendritic plasticity and axonal transport.

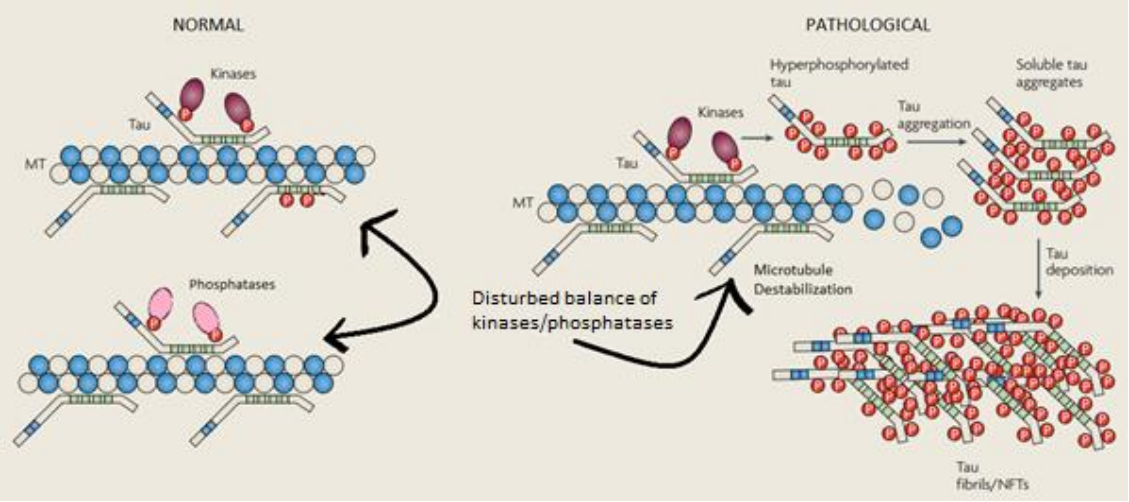

FIGURE 4. Normal and pathological Tau phosphorylation. Under normal conditions, tau phosphorylation is a dynamic procedure for maintenance of Tau normal function trough the balanced action of kinases vs phosphatases that phosphorylated/dephosphorylate Tau. Under pathological conditions, Tau is abnormally hyperphosphorylated leading to its detachment from the microtubules and the formation of soluble aggregates and later insoluble aggregates. (Modified from Götz & Ittner, 2008).

Furthermore, recent evidence aims to distinguish between cellular mechanisms triggered by Tau hyperphosphorylation versus these initiated by Tau aggregates. Kimura et al., (JBC 2010) has shown that animals expressing human non-mutated tau exhibit Tau hyperphosphorylation (but not Tau aggregates), synaptic loss and cognitive deficits by age while animals expressing the human mutated Tau saw tau aggregates and neuronal loss.

1.3 TAU PATHOLOGY – BEYOND ALZHEIMER'S DISEASE

Besides AD, intracellular aggregates of hyperphosphorylated Tau protein are also found in many other neurodegenerative disorders, in the absence of amyloid deposits, that are clinically characterized by dementia and/or motor syndromes, so called "Tauopathies" (Hernández & Avila, 2007). This heterogeneous group of disorders include frontal temporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease, and progressive supranuclearplasy and corticobasal degeneration. Each Tauopathy exhibits a characteristic regional pattern of NFT formation, and degeneration of vulnerable neuronal networks follows a stereotypical pattern (see Table 1). Despite the diverse phenotype and clinical presentation, the progressive accumulation of

NFTs is a common marker to all Tauopathies (Gendron & Petrucelli, 2009). Clinically, most tauopathies present a diagnostic challenge for the clinician and neuropathologists, due to the substantial overlap of pathological features that exist among tauopathies, with many cellular lesions being encountered in more than one disease. (Tolnay & Probst, 2003).

TABLE 1. Tauopathies: Some of characteristic disorders exhibiting tau protein aggregates. (Tolnay & Probst, 1999) (Tolnay & Probst, 2003) (Hernández & Avila, 2007)

Disease	Clinical	Tau inclusions	References
Alzheimer's Disease	Progressive loss of memory and cognitive functions, resulting in a severe dementia.	NFT's, NTh's and dystrophic neurites.	(Braak & Braak, 1991); (Nagy, et al., 1999).
Pick's Disease	Behavior changes, speech difficulty(aphasia), and impaired cognition	Pick bodies, BN's and neuritic inclusions.	(Brion, et al., 1991); (Buée-Scherrer, et al., 1996).
FTDP-17	Behavioral disturbances, cognitive impairment and parkinsonism.	NFT's, NTh's and BN's.	(Spillantini MG, 1998)
PSP	Non-hereditary neurodegenerative disorders clinically characterize by akinesia, rigidity, occulomotor abnormalities and late-onset dementia	NFT's and NTh's.	(Steele, et al., 1964); (Bergeron, et al., 1997).
CBD		Various neuronal inclusions and NTh's.	(Rebeiz, et al., 1967); (Rinne, et al., 1994).

(FTD) Fronto-temporal dementia. (NThs) Neuropil threads. (NFTs) neurofibrillary tangles. (ArGs) argyrophilic grains. (BNs) ballooned cells. (OMMs) oligodendroglial microtubular masses

Tauopathies are linked to different Tau mutations (32 Tau mutations have been so far described) which are missenses, deletions or silent mutations in the coding region of the protein, or intronic mutations located after exon 10 (Goedert, 2005). Tau mutations fall into two large categories: the first affect the protein while the second influences the alternative splicing of Tau pre-mRNA. Several mutations in exon 10 of Tau, such as P301L, K280, N296 and N296H, have effects on both protein and mRNA levels, (e.g. the splicing) and can result in the expression of different isoforms (Goedert, 2005). The most frequent mutation of human Tau gene is the well-characterized P301L that reduces Tau ability to bind to microtubules and enhances its ability to aggregate (Kimura, et al., 2010).

1.4 MORE PARTNERS IN TAU-RELATED NEURODEGENERATIVE MECHANISMS

1.4.1 ABNORMAL CONFORMATION AND TRUNCATION OF TAU

Besides hyperphosphorylation, other Tau posttranslational changes, such as truncation and conformational changes have been found to participate in Tau-triggered neurodegenerative pathways linked to Tau aggregation mechanism(s). Truncation is the process that leads to a protein with less a.a. residues than the normal protein due to mutations and/or mistranslated RNA or due to cleavage by proteases, giving rise to a malfunctioning protein. Tau cleavage (at a highly conserved aspartate residue (Asp421) in the C-terminus of the protein) and caspase activation are both co-localized in AD brain suggesting that activation of caspases and cleavage of tau may proceed to the formation of NFT's (Gamblin, et al., 2003) (Quintanillaa, et al., 2012). Cathepsin D is also known to cleave Tau protein, generating fragments similar to those found in NFT's. Conformational changes lead to alterations in the structure of Tau protein and consequent alteration in its function; e.g. MC1-detected conformational changes of Tau is found in AD brains (Weaver, et al., 2000) related with its malfunction and aggregation in neuronal soma. Both truncation and abnormal conformation changes in tau protein have been linked to AD pathology, raising new questions about the development of tangle pathology. Overall, hyperphosphorylation, truncation and abnormal conformation of Tau are all interconnected with its neurotoxicity as well as its aggregation and tangle formation while the absolute sequence of these events remains a fundamental question in Tau-related neurodegeneration.

1.4.2 DEGRADATION MECHANISMS

A major factor that determines the half-life of a protein is the presence of signals that control its degradation and turn-over. Regarding tau protein, its stability and degradation is shown to be dramatically affected by the aforementioned post-translational modifications that occur in AD brains. The existence of ubiquitin-independent proteasomal degradation of tau protein has been previously reported as Tau protein is degraded by the 20S proteasome in vitro (Feuillette, et al., 2005). However, there is strong evidence of tau degradation by the ubiquitin-proteasome system (UPS) (David, et al., 2002). Accordingly, attachment of ubiquitin marks misfolded or/and

hyperphosphorylated Tau protein for degradation (Kirkin, et al., 2009). Chaperones, such as Heat Shock Protein 70 (Hsp70), Hsp90 and co-chaperone CHIP (carboxyl terminus of the Hsp70-interacting protein) are the main proteins responsible for Tau ubiquitination and degradation (Koren, et al., 2009) while Tau hyperphosphorylation is necessary for the addition of ubiquitin by the E3 ubiquitine ligase known as CHIP. After misfolded Tau is recognized by Hsp40, Hsp70 and CHIP proteins, the addition of Hsp90 with the help of Hop (Hsp70/Hsp90 organizing protein), results to Tau ubiquitination and transportation to the proteasome (Salminen, et al., 2011)(Figure 5).

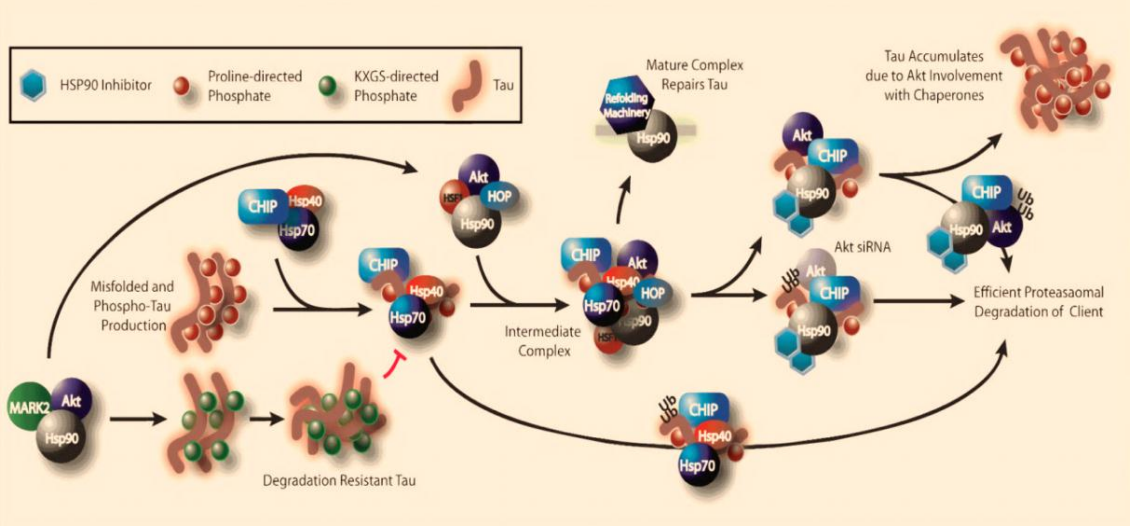

FIGURE 5. Model of Tau protein processing through the chaperone network. Hyperphosphorylated Tau affects its degradation by the molecular chaperones. This aberrant Tau is recognized by Hsp40/Hsp70 complex, and then can be directly degraded or passed on to Hsp90, forming an intermediate complex, together with Akt. After this complex formation, refolding machinery can repair Tau or degradation of the protein can take place, by the inhibition of Hsp90 (adapted from Koren et al, 2009).

Furthermore, small Hsp, like Hsp22 and Hsp27, participating in the chaperone network can also be involved in Tau degradation machinery, as it happens after phosphorylation by stress activated kinases, such as SAPK/P38 MAPK (Stetler, et al., 2009) (Koren, et al., 2009). These proteins can guide abnormal proteins to the proteasome in an ATP-dependent manner.

1.4.3 APOPTOTIC MECHANISMS

Despite the ubiquination of misfiled and/or aggregated Tau, their degradation is very problematic as they are resistant to proteasome clearance and this result to their accumulation in neuronal soma

that may lead to extensive cell damage and consequent cell death. Indeed, neuronal loss is found in entorhinal cortex, hippocampus, frontal, parietal and temporal cortices of AD patients while apoptotic mechanisms have been involved (Engidawork, et al., 2001a) (Engidawork, et al., 2001b).

Apoptosis, also referred to as type I programmed cell death, is morphologically characterized by membrane blebbing, cytoplasmic shrinkage and pyknosis, as well as condensation of the chromatin, and karyorrhexis (fragmentation of the nucleus), ultimately leading to the formation of apoptotic bodies, a prominent morphological feature of apoptotic cell death (Galluzzi, et al., 2012). In AD brains, caspase-3 is shown to be critical initiator of apoptotic pathways (Shimohamaa, et al., 1999) (H. Sua, et al., 2001) in which B cell lymphoma 2 (Bcl-2) family members are also involved. This family of proteins is divided into two groups: pro-apoptotic proteins (e.g. Bax and Bak) and anti-apoptotic proteins (e.g. Bcl-2 and Bcl-xL). The pro-apoptotic members cause the formation of pores in the outer mitochondrial membrane (which is counteracted by the anti-apoptotic members) resulting in the release of cytochrome c into the cytosol that causes the formation of apoptosomes. Once the apoptosomes are formed caspase-9 is activated. Caspase-3 activation is necessary but not enough to execute an apoptotic cell death, and in some cases it may exert unrelated cell death roles by targeting specific substrates. Indeed, fine tuning of caspase-3 activity may avert completion of a full apoptotic program but safeguard physiological activities unrelated to cell death.[reviewed in (D'Amelio, et al., 2012)]. It remains unclear whether apoptotic cell death is part of AD neuronal death, due to accumulation of abnormal tau or if caspase-3 is part of a cellular response for maintaining homeostasis and achieving survival.

1.5. RISK FACTORS OF ALZHEIMER´S DISEASE – CAN CHRONIC STRESS "CROSS-TALK" WITH THEM?

As clinical therapeutic trials against AD pathology failed to provide a promising approach yet, recent research efforts have been focusing on risk factors of the disease towards a prophylactic strategy. Increasing evidence support a causal role of lifetime stressful events in AD suggesting the involvement of chronic stress and glucocorticoids (GC), the primary stress hormones, in the pathogenesis and/or progression of the disease and associating elevated GC levels with hippocampal degeneration and atrophy and cognitive deficits in AD patients (Elgh, et al., 2006) (Hartmann, et al., 1997). But what is stress and how does our organism receive and cope with it?

Stress may be defined as a disruption of homeostasis following the impact of internal or external challenges ('stressors'). Stressors are recognized and perceived by the brain through the release of molecules that target different responses (e.g. dopamine, serotonin, corticosteroids) as an adaptive mechanism to the adverse change (Joëls & Baram, 2009). The principal neuroendocrine system activated upon stressful conditions is the hypothalamo–pituitary–adrenal (HPA) axis, with results in the production of corticosteroids (cortisol in humans adnd corticosterone in rodents) which serve as a feedback inhibitory signal back to hypothalamus and pituitary (Figure 6).

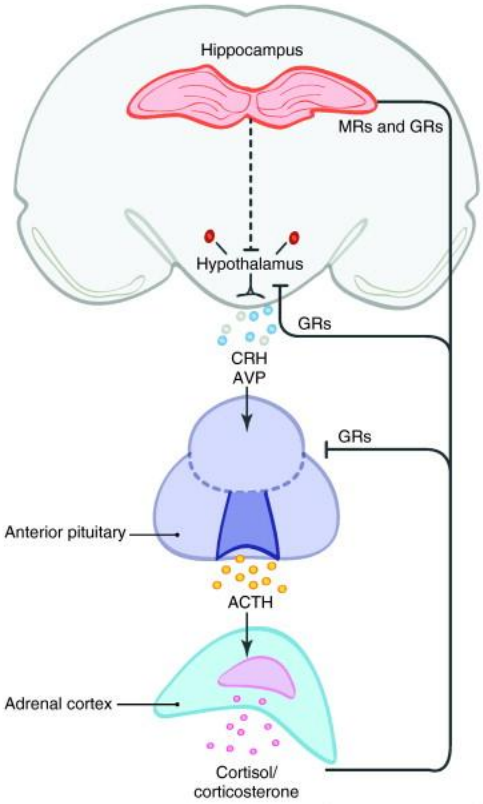

FIGURE 6. HPA axis and its feedback inhibition. Stressful stimuli trigger the hypothalamic release of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) activating the synthesis of adrenocorticotropin hormone (ACTH) at pituitary. ACTH secretes into the bloodstream stimulating corticosteroids secretion from adrenal cortex into the blood which serve as a feedback inhibitory signal to hypothalamus and pituitary (Adapted from Schloesser et al, 2012).

Corticosteroids are divided in two main categories, glucocorticoids (GC) **(gluco**se + **cort**ex + ster**oid)** and mineralocorticoids (MC). In mammalian brain, they primarily act through mineralocorticoid and glucocorticoid receptors (MR and GR, respectively). MR's have higher affinity for corticosteroids and they are the first to be occupied (even at low corticosteroid levels). On the other hand, GR have ten-

fold lower affinity. Accordingly, on basal, non-stressful conditions, GR are partially occupied and become increasingly occupied when corticosteroid levels are elevated (as in stressful conditions) (Kloet, et al., 2008). While stress and stress response are adaptive and important for maintenance of mental and physical health, exposure to stressful conditions for longer periods can become maladaptive resulting in damages in brain and many peripheral tissues affecting their function (Joëls & Baram, 2009). Indeed, elevated GC levels and GR activation are connected with the detrimental effects of chronic stress on neuronal and brain function (Sapolsky, et al., 1990) (Stein-Behrens, et al., 1994)[see above and for review (Sotoripoulos, et al., 2008)], compromising neuronal survival and causing cognitive and emotional deficits through dendritic atrophy and synaptic loss in hippocampus and prefrontal cortex (Cerqueira, et al., 2005) (Cerqueira, et al., 2007) (Rothman & Mattson, 2010). These brain areas are critically affected in AD also exhibiting neuronal and synaptic atrophy/loss (for review, see Sotiropoulos et al, 2008). Indeed, AD patients also exhibit increased circulating GC levels, consistent with a suggested HPA-axis deregulation, possible relating AD with an altered stress-response (Rothman & Mattson, 2010). Indeed, elevated GC levels are associated with hippocampal degeneration, atrophy and cognitive deficits in these patients (Hartmann, et al., 1997). Furthermore, animal studies have also demonstrated that chronic stress and/or GC triggers both AD neurodegenerative mechanisms (Green, et al., 2006) (Catania, et al., 2007) (Sotiropoulos, et al., 2008). Specifically, chronic stress and GC affect APP processing favoring the amyloidogenic pathway which results in increased C99 and Aβ levels affecting enzymes involved in APP misprocessing such as BACE-1, nicastrin and presenilin (Catania, et al., 2007) (Sotiropoulos, et al., 2008). In addition, exposure to chronic stress and/or GC treatment in both cellular and animal models induced Tau hyperphosphorylation followed by decrease degradation and accumulation of Tau in neuronal perikarya (Sotiropoulos, et al., 2008) (Sotiropoulos, et al., 2011).

But does chronic stress damage our brain in the same way? Many studies have demonstated that chronic stress exhibit a different detrimental effect on genders affecting differentially their behaviour with female demonstrating a higher HPA axis response (for review see Dalla et al., 2010; see Figure 10). Interestingly, gender differences also play an important role in the development and progression of the disease as women are more prone to develop AD pathology, associated with a decline in the estrogens production after menopause (Fratiglioni, et al., 1997). In addition, aged individuals have higher risk for AD with the prevalence of dementia doubling approximately every 5

years between the ages of 65 and 95 (Fratiglioni, et al., 1991) while it is also known that aged brain in more vulnerable to detrimental effect of chronic stress (Bloss, et al., 2011).

As a recent study from Sotiropoulos and colleagues (2011) suggested an interactive role of stress and AD mechanisms through Tau, this Master thesis aimed to clarify the interaction of different risk factors of the disease, such as adverse stress, gender and aging, towards the development of AD brain pathology (Figure 7).

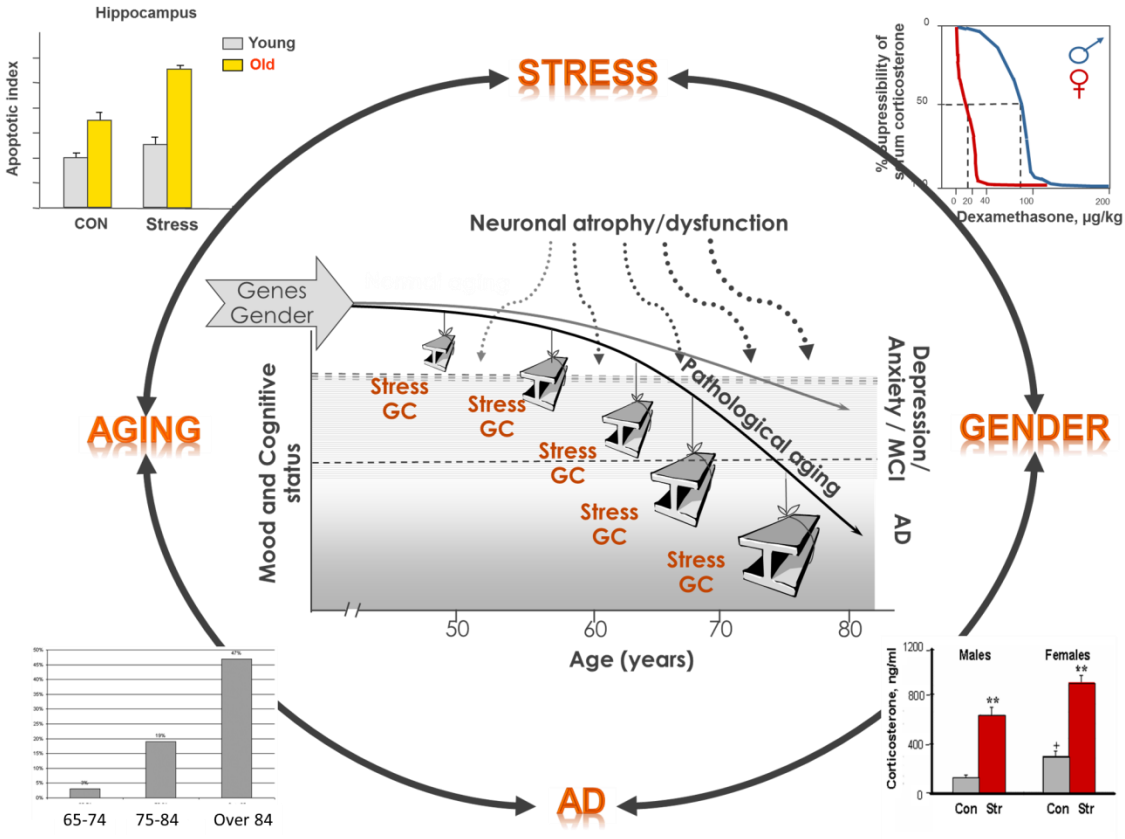

FIGURE 7. Interplay of AD risk factors. Schematic representation of the interconnections among stress, aging, gender and Alzheimer's disease. (Drawn by J. Silva & I. Sotiropoulos).

2. OBJECTIVES

2. OBJECTIVES

Previous studies suggest that Tau protein may lie at the core of stress/GC-induced brain pathology, raising Tau hyperphosphorylation as critical mechanism in stress-triggered neuronal dysfunction and cognitive impairment. While aging, gender and chronic stress are risk factors for Alzheimer´s disease, their inter-significance on the development of Tau pathology is still unclear. Thus, this Master thesis aims to:

I. Understand the behavioral importance of Tau on stress detrimental effects on cognitive and emotional deficits.

II. Obtain molecular evidence about the role of stress and GC on different parameters of Tau pathology.

III. Clarify the interaction of stress and Tau protein on cellular pathways related to neuronal survival.

IV. Identify the significant interplay of gender, stress and aging towards the development of AD pathology.

V. Monitor the influence of stress hormones on Tau subcellular localization.

3. STRESS-GENDER INTERPLAY ON TAU PATHOLOGY

3. STRESS-GENDER INTERPLAY ON TAU PATHOLOGY

This manuscript is submitted to Molecular Psychiatry Journal

Title: Female hippocampus is more vulnerable to stress-triggered Tau neurodegeneration: implication for human Tauopathies

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Running title: Stress-gender interplay on Tau pathology

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3.1. BACKGROUND

Tau pathology has been postulated as a common mechanism of neurodegeneration in many prolong neurological diseases termed Tauopathies, including Alzheimer's disease (AD), frontotemporal dementia (FTD) and frontotemporal dementia with parkinsonism linked to chromosome-17 (FTDP-17) (Binder, et al., 2005) (Gong, et al., 2005). The identification of *Tau* mutations in FTDP-17 patients has established that Tau protein dysfunctions are central to the neurodegenerative process and the resulting dementia (Clarka, et al., 1998) (Spillantinia & Goeder, 1998). Several *Tau* mutations have been expressed in transgenic mice; studies in mice expressing P301L, the most frequent Tau mutation (Nasreddine, et al., 1999), confirm that pathogenic Tau is a direct cause of Tau aggregates and NFT formation associated with AD neurodegeneration and pathology. Indeed, aggregation-prone P301L-Tau promotes its self-assembly forming insoluble, conformational abnormal Tau accumulations that trigger neurodegeneration processes that culminate in neuronal loss (Ballatore, et al., 2007) (Kimura, et al., 2010). Tau aggregates, neuronal loss and region atrophy are found in different AD brain areas such as hippocampus, entorhinal cortex, prefrontal cortex (PFC) and amygdala (Poulin, et al., 2011); such topographic pattern of neuropathology underlies the overall clinical manifestation of the disease, namely memory loss but also non-cognitive neuropsychiatric disturbances such as anxiety (Price & Morris, 1999) (Rosen, et al., 2006) (Bruen, et al., 2008).

Tauopathies, namely AD, are complex multifactorial disorders with multiple precipitating factors such as aging, gender and stressful life events. Importantly, there are several interactions between these precipitating factors (for review, see Sotiropoulos et al. 2008). If aging has the highest impact on the development of AD as the probability of subjects is increased 5% for each year over 65, it is also known that the aged brain is more vulnerable to insults such as chronic stress (Sotiropoulos, et al., 2011). Moreover, epidemiological studies suggest that women are more prone to develop AD (Fratiglioni, et al., 1997) (Stein, 2001) and it has been demonstrated that the detrimental role of chronic stress seems to differ between males and females (Dalla, et al., 2011). Interestingly, elevated GC levels, the primary stress hormones, are associated with hippocampal degeneration and atrophy and cognitive deficits in AD patients (Elgh, et al., 2006) (Hartmann, et al., 1997) (Wilson, et al., 2007)and experimental studies confirm the detrimental effect of stress and/or GC on APP misprocessing and Tau hyperphosphorylation (Catania, et al., 2007) (Green, et al., 2006) (Sotiropoulos, et al., 2011). Despite the fact that many clinical studies highlight the important

contribution of stress, gender and aging on AD, few experimental studies have focused on dissecting their interplay towards the establishment of disease pathology.

While our recent studies demonstrated that the stress/GC trigger Tau hyperphosphorylation and accumulation in brain areas targeted by stress, such as hippocampus and PFC (Sotiropoulos, et al., 2011), the underlying mechanisms through which stress promotes Tau pathology are still unknown. Herein, we aim to provide novel molecular and behavioral evidence on: a) the detrimental role of adverse stress on Tau-triggered neurodegeneration focusing on both cognitive and emotional component of AD pathology and, b) the importance of gender and aging of stress-evoked pathology using middle-age and old male and female animals of a newly-generated transgenic mouse model that expresses the human mutated P301L-Tau (Kimura, et al., 2010).

3.2 MATERIAL AND METHODS

ANIMALS AND TREATMENT

Tg mice used in this study express the P301L mutated human Tau on a C57BL/6J background and were generated as previously described in (Kimura, et al., 2010). Tg mice and their littermate wild-type of both sexes that used in this study were separated at two age groups, 10-14 (middle-aged) and 20-24 months old (old), respectively. Animals were housed 4-5 per cage under standard environmental conditions (temperature 22◦C; relative humidity 70%; 12 h light/12 h dark cycle [lights on at 8 a.m.]; ad libitum access to food and drinking solution). Animals were subjected to prolonged stress over a period of 28 days (one stressor for 45 min/day in a random order; stressors: overcrowding; restraint; placement on a rocking platform; i.p. injection of hypertonic (9%) saline, 1ml/100g). Biometric and biochemical evidence of efficacy of the prolong stress was obtained based on measurements of daytime serum corticosterone levels (measured with a radioimmunoassay kit from ICN, Costa Mesa, CA), body weight changes, and glucocorticoid receptor (GR) levels (measured by Western blot analysis). All stressed animals showed significant elevations in daytime serum CORT levels ($p<0.05$), net loss of body weight ($p<0.05$) reflecting the stress efficacy (see Supplementary Table 1 and Supplementary Figure 1).

BEHAVIOURAL TESTS

Cognitive performance and emotionality were tested by Morris water maze (MWM) and Elevated plus maze (EPM) at the end of the stress period. For spatial reference memory, animals were tested over 9 consecutive days (3 trials/day; 60 s trial period) as previously described (Kimura, et al., 2010)). For emotionality assessment, animals were tested in EPM apparatus for 7 min as previously described (Tanemura, et al., 2002). Briefly, animals were placed in the center of the EPM apparatus and entries as well as time spent in open and closed arm were measured. Data were collected using a CCD camera by the use of NIH Image program (http://rsb.info.nih.gov/nih-image/) and were analyzed using customized software based on Matlab (version 7.2, Mathworks Co Ltd, CA) with image analysis tool box (Mathworks Co Ltd, CA) (Kimura, et al., 2007).

TISSUE COLLECTION

One day after the last behavioral tests, half of animals were killed by decapitation. Brains were excised immediately. Prefrontal cortex, amygdala and hippocampus were dissected (on ice) and immediately stored at –80ºC until they were used for Western blot analysis. The rest of animals were used for immunohistochemical analysis.

WESTERN BLOT

Sarkosyl-insoluble and –soluble fraction of tissue protein extracts was prepared as previously described (Kimura, et al., 2010) (Sahara, et al., 2002). Briefly, after homogenization, hippocampal and PFC lysates were centrifuged and supernatant was collected. Sarkosyl-insoluble, paired helical filament-enriched fractions were prepared from TBS-insoluble pellets which were re-homogenized in salt/sucrose buffer (0.8M NaCl, 10mM Tris/HCl, 1 EGTA, pH=7.4, 10% sucrose solution including protease and phosphatases inhibitors as above mentioned) and centrifuged. One tenth volume of 10% sarkosyl solution was added to the supernatant, and after incubation at 37oC (1h), and centrifugation (150.000g), the resulting pellet was analyzed as the sarkosyl-insoluble fraction. After SDS–PAGE electrophoresis, and semi-dry transfer, all membranes were incubated in different antisera while blots were revealed by enhanced chemiluminescent (ECL, GE Healthcare Bio Science). Quantitation of immunoreactivity was performed with a computer-linked LAS-3000 Bio-Imaging Analyzer System (Fujifilm).

IMMUNOSTAINING

As previously described (Kimura, et al., 2007), deeply anesthetized animals (pentobarbital 50mg/Kg) were transcardially perfused with 10% formalin. Brains were post-fixed for 16hours and embedded in paraffin and sectioned (4 mm) in coronal plane. Deparaffinised sections were treated with various antisera against Tau-C3 (1:300), MC1 (1:100), active-caspase3 (1:300).

STATISTICAL ANALYSIS

Results are expressed as group means ± SEM. Statistical analysis (T-student's Test, One-way ANOVA and Two-way ANOVA) was performed using SPSS, and differences were considered to be significant if $p < 0.05$.

3.3 RESULTS

3.3.1 STRESS ALTERS COGNITIVE AND ANXIETY BEHAVIOR IN P301L-TAU ANIMALS IN A GENDER-SPECIFIC WAY

In light of clinical evidence highlighting the important contribution of stress, gender and aging on AD pathology, we first investigated the effects of prolonged stress on cognitive performance in both male and female P301L-Tau animals in two different ages, middle aged (10-14 months old) and aged (20-24 months old) using the Morris water maze (MWM) test for assessing spatial reference memory. Middle-aged female Tg mice stressed (STR) exhibit a significant delay in learning position of the platform in comparison to non-stressed (CON) animals as assessed by error score curves, using repeated-measured ANOVA test (Figure 8A). This stress effect was even more pronounced in old female Tg animals (Figure 8B) as it is also shown by the probe test analysis (Figure 8C).

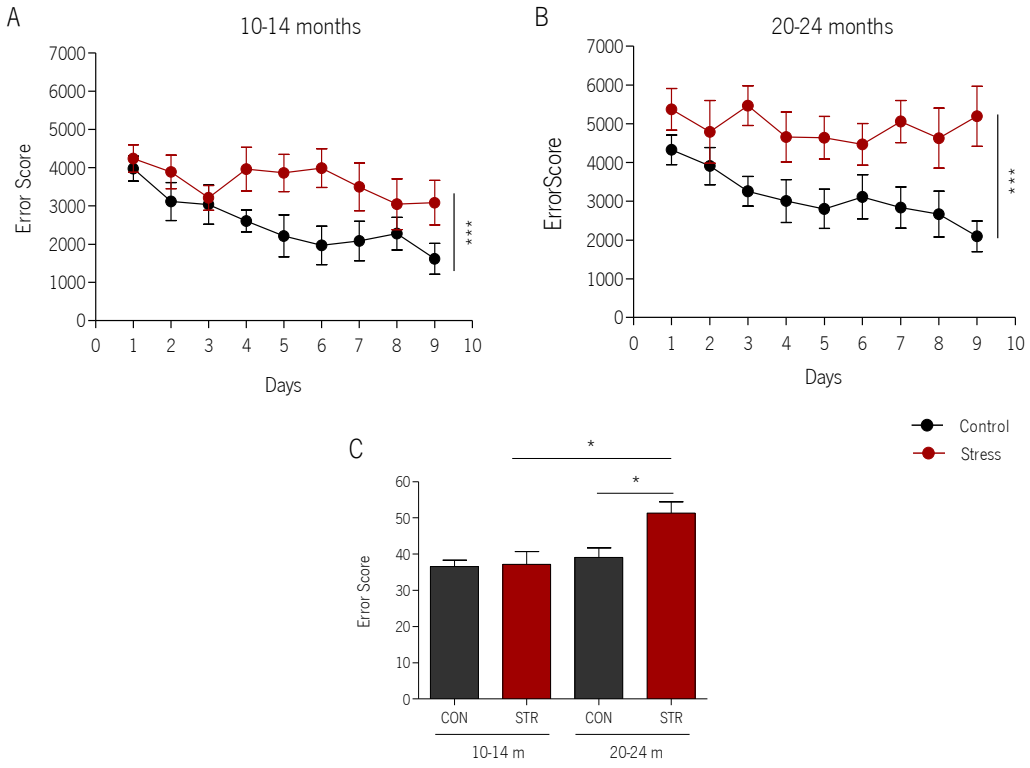


FIGURE 8. Adverse stress induced cognitive impairment in P301L-Tau Tg females. Morris Water Maze test was used to assess spatial reference memory in middle-age (10-14 months old; A) and old (20-24 months old; B) P301L-Tg female animals, represented as error score. Probe test score (C) at the end of learning acquisition period showing that old stressed animals were more affected in comparison to middle-aged stressed ones. All numerical data shown represent mean ± SEM (N=10-12) (* $p<0.05$; *** $p<0.001$).

In contrast, male P301L-Tau Tg animals did not alter their spatial learning ability at both tested ages as revealed by MWM data (Figure 9A and 9B), confirmed by probe test (Figure 9C). Furthermore, no effect of stress was found in non-Tg littermates (Supplementary Figure 2) and no gender, age or strain differences were detected under non-stressed (CON) conditions. These findings reveal that gender (female) and age (older animals) increase the susceptibility to stress-induced cognitive impairments in P301L Tg mice.

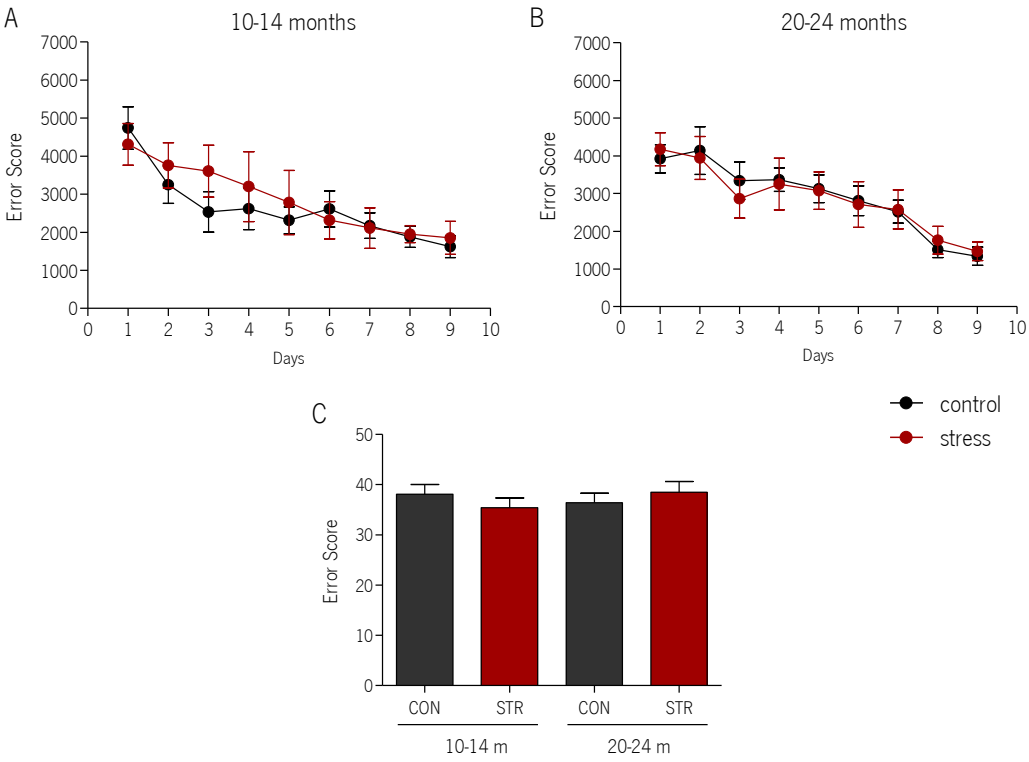


FIGURE 9. Male P301L-Tau Tg animals were not cognitively affected by stress. Morris Water Maze test and Probe test was used to assess spatial reference memory in middle-age (10-14 months old; A) and old (20-24 months old; B) P301L-Tg male animals showing that stress don´t affect male P301L-Tau tg animals. All numerical data shown represent mean ± SEM (N=10-12) (* $p<0.05$).

Anxiety is increasingly recognized in AD patients (Wilson, et al., 2011). Herein, we assessed anxiety levels in the EPM test. As shown at Figure 11, stress significantly increased anxiety behavior in both middle-aged and old female and male P301L-Tau animals as revealed by the time spent on the open arm of the apparatus (Figure 10A and 10C, respectively) and the number of entries (Figure 10B and 10D). As above, exposure of non-Tg littermates to stress did not impact on anxiety-like behavior (Supplementary Figure 3). Note that two-way ANOVA analysis revealed a gender x age

effect given that male animals of both genotypes (P301L Tg and non-Tg) exhibited significantly decreased entries and the time spent in the open arm indicating a clear anxiogenic role of aging independently of the presence of P301L-Tau protein.

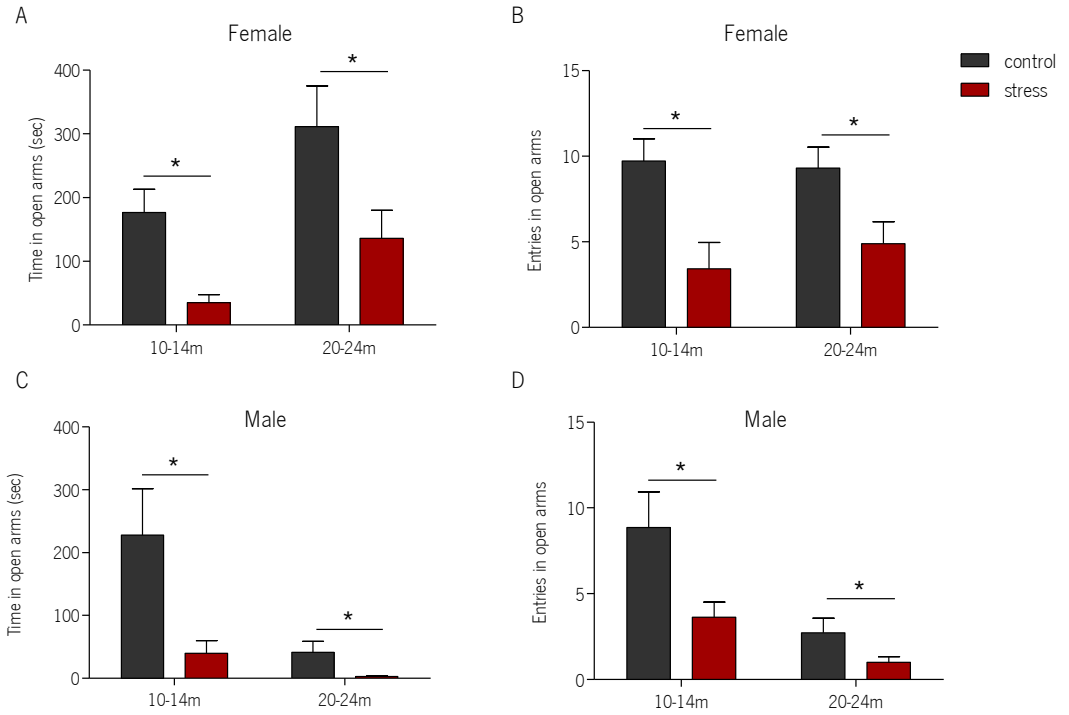


FIGURE 10. Adverse stress induced anxiety levels in both male and female P301L-Tau Tg mice independently of age. Stress increased anxiety levels as assessed by time spent in the open arms (A-C) and number of entries in the open arms (B-D). A-B) Time and entries in the open arms of EPM apparatus in female P301L-Tg mice C-D) Time and entries in the open arms in male P301L-Tau Tg mice. All numerical data shown represent mean ± SEM (N=10-12) (* $p<0.05$).

3.3.2 STRESS TRIGGERS TAU AGGREGATION PATHOLOGY IN FEMALE HIPPOCAMPUS

In the light of experimental reports implicating stress and GC in AD neuropathology (Green, et al., 2006) (Catania, et al., 2007) and in our previous findings that stress triggers Tau hyperphosphorylation and accumulation (Sotiropoulos, et al., 2008) (Sotiropoulos, et al., 2011), here we monitored the effects of stress on different indicators of Tau pathology focusing on hippocampus which is a brain area primarily affected in AD pathology involved in both cognitive and emotional behavior (Braak & Braak, 1991) (Poulin, et al., 2011).

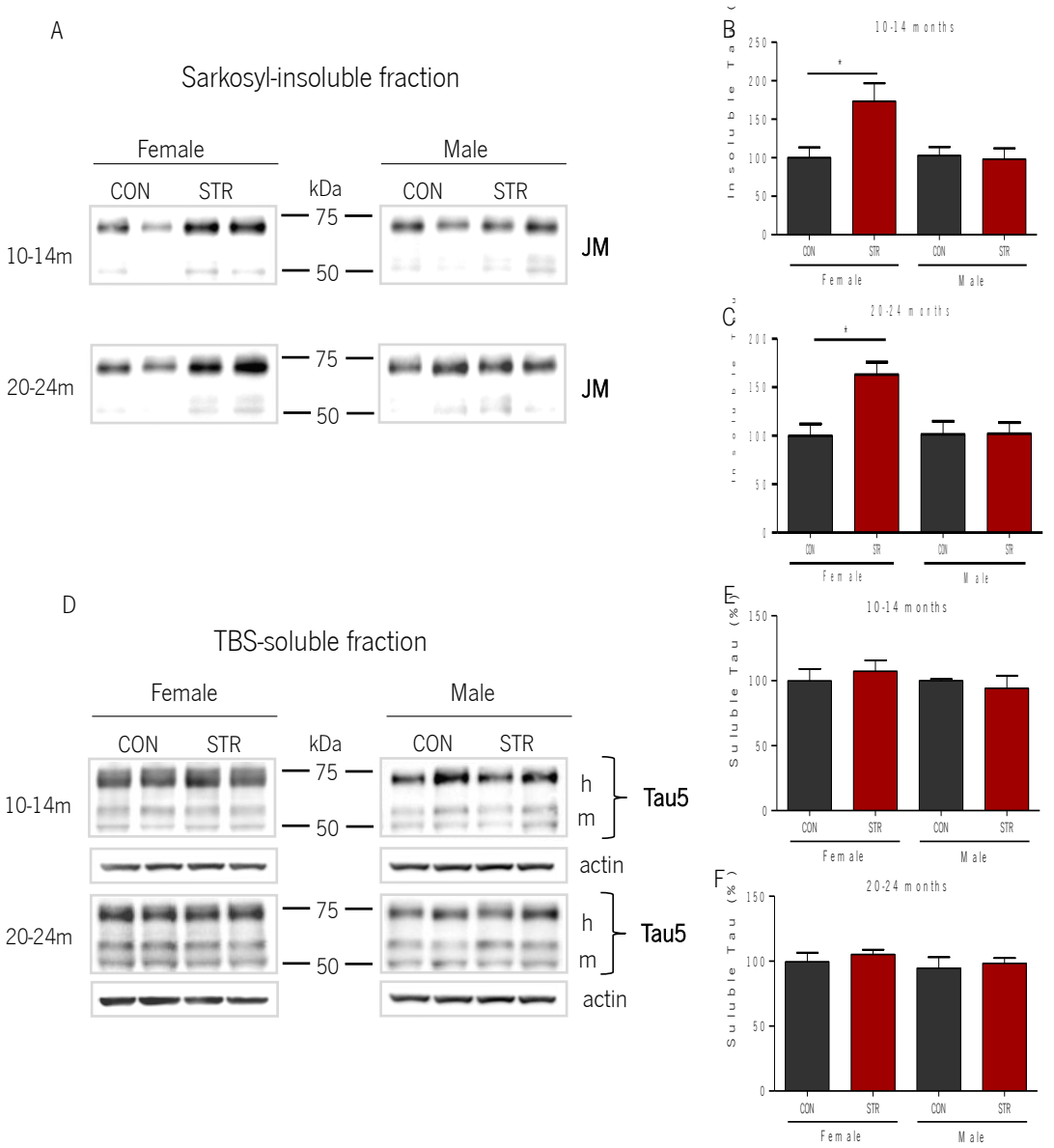


FIGURE 11. Stress increased levels of sarkosyl-insoluble Tau in female but not male hippocampus. A-C. Western-blot analysis of sarkosyl-insoluble fraction of P301L-Tg mice; A. Representative immunoblots of sarkosyl-insoluble fraction, using JM antibody; B. Quantification of insoluble Tau levels in midle-age(10-14 months) P301L-Tg mice; C. Quantification of insoluble Tau levels in old P301L-Tg; D-F. TBS-soluble Tau fraction of P301L-Tau Tg mice hippocampus; D. Representative imunoblots of TBS-soluble fraction, using Tau-5 antibody; E. Quantification of soluble Tau levels in midle-age P301L-Tg; C. Quantification of soluble Tau levels in old(20-24 months) P301L-Tg mice. All numerical data shown represent mean ± SEM values (N = 6-7), depicted with respect to data obtained in control tissues (CON), set at 100%. (* $p<0.05$).

As P301L mutation promotes the self-assembly of mutant Tau into aggregates and filaments, we first monitored the levels of sarkosyl-insoluble level of Tau aggregates by Western blot analysis. In

contrast to their non-Tg littermates, sarkosyl-insoluble Tau (recognized by JM antibody) was extracted from the hippocampi of P301L Tg mice in both sexes (Figure 11). This finding is compatible with the presence of Tau aggregates that is characteristic for AD and other Tauopathies. As shown at Figure 12A-C, stress exposure resulted in a further increase in the levels of sarkosyl-insoluble Tau levels in hippocampus of both adult and aged female P301L-Tau Tg animals. In contrast to females, male P301L Tg animals did not exhibited a significant change of sarkosyl-insoluble Tau levels of hippocampus. In addition, no effect of stress was found on overall TBS-soluble Tau hippocampal levels detected by a pan-Tau antibody at both genders and ages (Figure 11D-F). As Tau pathological changes found in AD include aberrant conformation of the protein, we next monitored the conformational abnormal MC1-positive forms of Tau. As shown at Figure 12, MC1 staining was highly increased after exposure to stress (Figure 12B) in hippocampus of female Tg animals but not in males (data not shown).

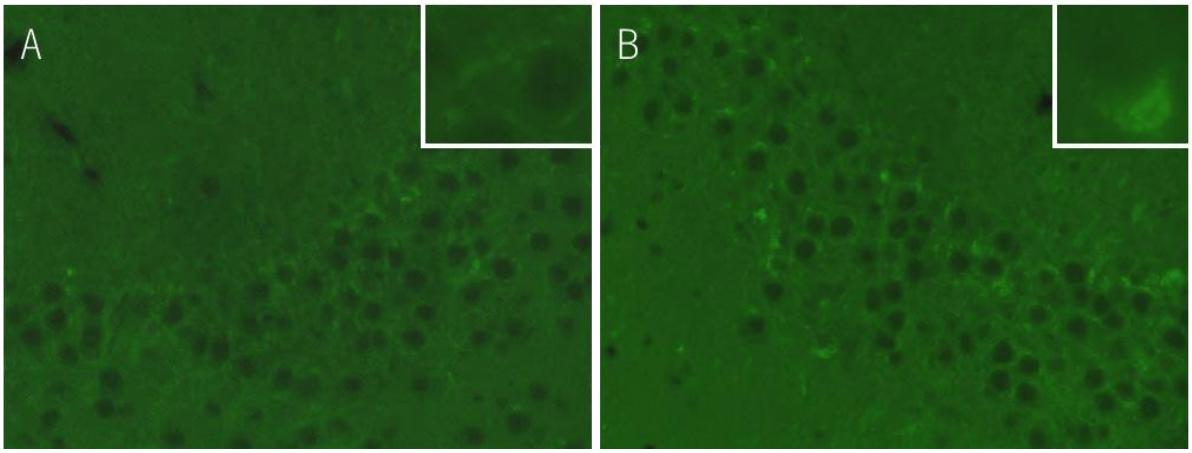


FIGURE 12. Tau conformation changes by adverse stress in P301L-Tg female hippocampus. MC1 staining of control (A) and stressed (B) female hippocampus of P301L-Tau Tg animals.

3.3.3 STRESS INDUCES DYSREGULAYTION OF CHAPERONE-MEDIATED DEGRADATION MACHINERY AND TRIGGERS APOPTOTIC PATHWAYS IN P301L-TAU ANIMALS

Tau degradation pathways are also critically connected with Tau aggregation and pathology. Based on our previous in vitro findings suggesting that stress hormones reduce Tau turnover resulting in its

accumulation (Sotiropoulos, et al., 2008), we analyzed different molecular chaperones, namely Hsp90 and 70 that are involved in Tau aggregation-degradation machinery (Dickey, et al., 2007); e.g. Hsp70 inhibits Tau aggregation by promoting its degradation (Petrucelli, et al., 2004). It is relevant to note that the same molecules are also bind to GC receptors participating in their cell signaling pathway (Conway-Campbell, et al., 2011). We found that stress increased protein levels of Hsp90 but reduced these of Hsp70 and its co-chaperone, Hsp105 while others such as Hsp60, Hsp27 and 40 were not affected (Figure 13A). Furthermore, other co-chaperones and transcription factors involved in the Tau degradation were affected by stress. Specifically, the levels of CHIP protein, a co-chaperone molecule that is involved in protein folding and it is known to direct interact with the above chaperones for facilitating degradation of insoluble tau aggregates (Petrucelli, et al., 2004), were increased by adverse stress (Figure 13A); in contrast, a small decrease in HSF1 levels suggesting an overall deregulation on Tau degradation machinery.

P301L-Tau expression is correlated with cell loss (Kimura, et al., 2010). Similarly, GC triggers apoptotic cell death affecting the ratio of Bax/Bcl-2 (Crochemore, et al., 2005). Thus, we next monitored the possible interaction of stress and P301L-Tau on apoptotic pathways in our Tg animals. Western blot analysis revealed that exposure to adverse stress evoked a significant increase in the protein levels of the pro-apoptotic molecule Bax with no significant changes in the levels of Bcl-xL and Bcl-2; this results in an altered ration of Bax/Bcl2 (Figure 13B and C). Subsequently, we monitored the levels of the active caspase-3, critically involved in Tau pathology as it is connected with the generation of truncated forms of Tau. As shown in Figure 13F, adverse stress increased levels of active-caspase3 while this increase is also accompanied by an elevation in the levels of truncated Tau (Figure 13G).

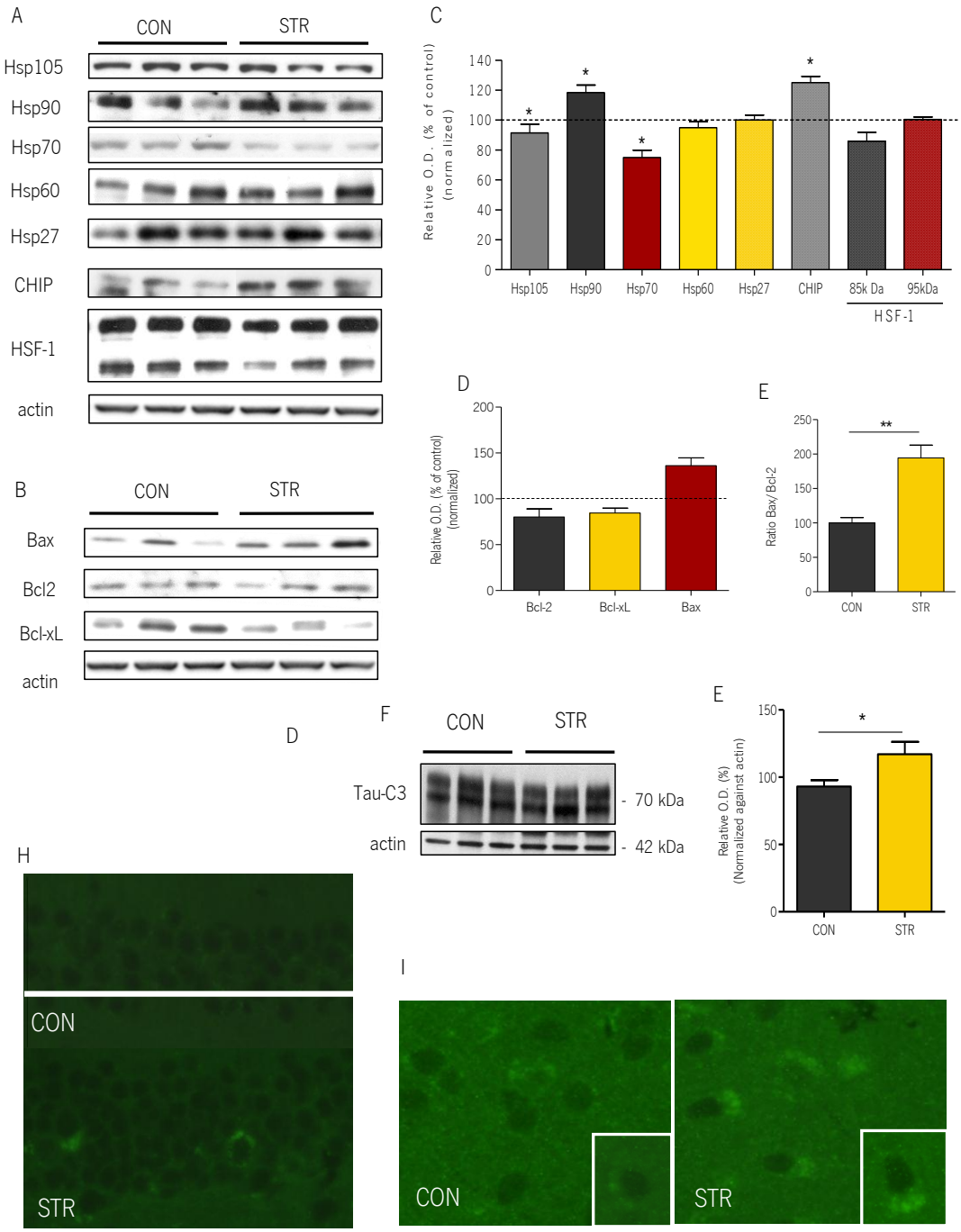


FIGURE 13. Stress-induced changes in molecular chaperones and apoptotic proteins in female P301L-Tau hippocampus. A-B. Representative blots and quantification analysis of different molecular chaperones involved in Tau degradation showing that stress decreased the Hsp70 and Hsp105 protein levels but increased Hsp90 and CHIP ones; C-E. Pro- and anti-apoptotic-related molecules are increased and decreased, respectively, by stress; F-G. Representative blots and quantification analysis of Tau-C3; H-I. Active caspace-3(H) and truncated Tau (I) staining was increased by adverse stress in hippocampus of female P301L-Tau Tg animals. All numerical data shown represent mean ± SEM values (N = 6-7), depicted with respect to data obtained in control tissues (CON), set at 100%. (* $p<0.05$).

We next, counted cell numbers of male and female hippocampus showing that stressed animals exhibit a significant reduction in cell density in CA3 of aged female hippocampus but not in the male hippocampus (Table 2).

TABLE 2. Reduced cell density in CA3 region of Hippocampus in females but no in male P301L-Tau anaimals.

		Female		Male	
		CON	STR	CON	STR
Hippo (CA3)	*10-14m*	7,723 ± 0,341	7,941 ± 0,389	7,849 ± 0,256	7,818 ± 0,148
	20-24m	7,539 ± 0,152	6,796 * ± 0.133	7,562 ± 0,167	7,911 ± 0,279

3.4 DISCUSSION

3.4.1 THE NEURODEGENERATIVE POTENTIAL OF STRESS ON TAU PATHOLOGY

The importance of mechanistic understanding of TAU-mediated neurodegeneration as well as of involvement of risk factors in Alzheimer's disease (AD) and related Tauopathies has been recently recognized towards prevention of Tauopathy-associated dementias. Cumulative evidence from human and animal studies suggests the implication of stress and its primary physiological response, GC, in the onset and/or progression of AD (Wilson, et al., 2007) (Johansson, et al., 2010); for review see Sotiropoulos et al., 2008b). Our recent studies show that stress triggers Tau hyperphosphorylation and accumulation probably by affecting Tau turnover and aggregation (Sotiropoulos, et al., 2008) (Sotiropoulos, et al., 2011). Here, we demonstrate that exposure to prolong stress in the presence of the mutated human Tau protein (P301L) also triggers other parameters of Tau pathology, such as Tau aggregation, abnormal conformation and truncation as stress elevated sarkosyl-insoluble Tau aggregates, conformational abnormal MC1-positive form of Tau and Tau-C3-recognized truncated Tau.

Both MC1-positive and truncated Tau are early pathological change in AD (Gamblin, et al., 2003) (Rissman, et al., 2004) (Weaver, et al., 2000) (de Calignon, et al., 2012), but others like sarkosyl-insoluble Tau aggregates are suggested, by experimental and clinical evidences, to precede NFT formation, and consequently associated with neuronal and cognitive losses, being also considered as better indicators of neurodegenerative process and disease progression (Kimura, et al., 2010) (SantaCruz, et al., 2005) (Berger, et al., 2007). The sequence and relationship of different parameters of Tau-related neurodegeneration are still not well defined. Although we could not understand the clear progression of Tau pathology (conformational changes, truncation and aggregation), our results clearly demonstrate that chronic stress induced increased levels of Sarkosyl-insoluble Tau as well as increased levels of both MC1 and truncated Tau (cleaved by caspase 3 (Gamblin, et al., 2003), supporting relationship between stress and AD, given the induction of AD histopathological hallmarks by chronic stress. As C-terminus truncation of Tau facilitates its aggregation by enhancing the association of N-terminus with the Microtubule binding repeat of the protein, resulting in its aberrant pathogenic conformation of Tau (detected by MC1) (Rissman, et al., 2004) (Gamblin, et al., 2003), Tau misfolding, could be a result of Tau cleavage

by caspase-3 at Asp421; the same applies for oligomerization and formation of pathological (pre-tangle) Tau aggregates. In addition, the Tau fragment produced by proteolytic cleavage clearly augments the nucleation of full-length Tau proteins into insoluble aggregates (Wang, et al., 2007).

Disturbances in degradation of misfolded Tau protein are suggested to determine accumulation of tau aggregates and therefore, play an essential role in pathogenesis of Tau-related diseases. An essential step in Tau degradation is its binding to molecular chaperones such as Hsp70 as their alterations can lead to failure of Tau degradation (Dou, et al., 2003) (Sahara, et al., 2005). Indeed, human data suggested a significant inverse correlation between insoluble Tau and Hsp70 protein in AD brains (Sahara, et al., 2007). Accordingly, our findings support adverse stress demonstrated a decrease in Hsp70 and Hsp105 levels, which ubiquinates Tau protein marking it for further degradation preventing its aggregation. Note that Hsp105 was shown to suppress apoptosis while its reduction could contribute to activation of apoptotic pathways by stress as shown in this study. In parallel, stress increased Hsp90 and CHIP levels and insoluble tau aggregates. Indeed, Hsp90 binds Tau protein and promote its conformational change and aggregation (Tortosa, et al., 2009) while Hsp90 inhibition results in increased tau degradation and reduced subsequent aggregation with high therapeutic potential (Koren, et al., 2009). Note that CHIP correspond directly to Hsp90 levels but not to Hsp70 as is previously shown by Sahara et al., (Sahara, et al., 2005).The fact that the same molecular chaperones bind to GC receptors regulating their cytoplasmic activation (Conway-Campbell, et al., 2011) suggests that this is the mechanistic link for the interplay between stress and Tau aggregation pathology in the P301L mice.

The current study also demonstrated that stress in P301L mice alter the ratio of pro vs anti-apoptotic molecules in favor of the previous; this is associated with reduced neuronal density in several brain regions. In this respect, it is important to note that the expression of P301L-Tau is correlated with cell death (Kimura, et al., 2010) (Wegmann, et al., 2011) and that increased GCs, the stress hormones, increase the ratio of Bax/Bcl-2 and trigger apoptotic cell death (Crochemore, et al., 2005). The demonstration of augmented caspase3 in P301L mice exposed to stress clearly suggests that this neurodegenerative process is triggered by stress in the context of an animal model of Tauopathies while additional, non-apoptotic neurodegenerative mechanism cannot be also excluded (Allen, et al., 2002) (Zehr, et al., 2004).

3.4.2 BEHAVIORAL EFFECTS OF STRESS IN P301L-TAU MICE AND THEIR GENDER-SPECIFIC PROFILE

The presence of Tau pathology in the hippocampus of stressed P301L mice, particularly in females, translated into functional deficits in the spatial reference memory, a hippocampal dependent task. Interesting, however, was the fact the cognitive deficits were confined to Tg females and were accentuated by age. While this fits with the pathological findings of increased Tau pathology in the hippocampal of these mice, it also reveals the existence of important interactions between genetic factors, stress, gender and sex, all of which are recognized to be precipitating factors of Tauopathies, namely AD (Elgh, et al., 2006) (Hartmann, et al., 1997) (Fratiglioni, et al., 1997) (Bloss, et al., 2011). Indeed, the absence of effects in non-transgenic animals clearly demonstrates that the expression of stress effects is potentiated in the presence of human mutated Tau, most likely due to the converging actions on common mediators of the neuropathological processes highlighted above. Noticeably, this phenotype is in good accordance with clinical data (REFS) showing that women exhibits higher risk in AD pathology.

Although not central in dementia symptomatology, anxiety is a very frequent symptom in demented subjects; even though frequently observed in AD it is particularly common in FTD (Levy, et al., 1996) (Porter, et al., 2003). Importantly, increased anxiety, largely determined by the combined activity of the amygdala and mPFC (Kim, et al., 2011), may precede cognitive impairment by several years (Jost & Grossberg, 1996). A few experimental studies suggest that Aβ is a central molecule in manifestation of anxious behavior as intraneuronal Aβ overproduction in amygdala of 3xTg mice (España, et al., 2010) and exogenous Aβ administration (Catania, et al., 2007) (Pamplona, et al., 2010) resulted in symptoms of anxiety. The present findings of stress-induced heightened anxiety in P301L-Tau Tg mice demonstrate, for the first time, a role of Tau protein and its malfunction in the manifestation of anxiety behavior in the context of AD pathology. In contrast to the cognitive deficits, the present results show that stress-induced anxiety was similar among different ages of both male and female animals expressing the aggregation-prone P301L-Tau protein. This phenotypic profile is again in good correlation with pathological findings in the amygdala and PFC, but also with clinical characteristics (Seignourel, et al., 2008).

Epidemiological studies show gender differences in the incidence and prevalence of AD with females being in higher risk, even after the age adjustment (Baum, 2005) (Andersen, et al., 1999) (Fratiglioni, et al., 2000) (Hy & Keller, 2000).Despite the fact that both AD and stress response

have a clear gender profile, there is paucity of research examining sex differences at a neurobiological and mechanistic level (National Institute of Mental Health 2011). Scrutiny of the existing AD Tg models reveal that several Aβ-based Tg mice exhibit a common pattern of higher female Aβ pathology, but not Tau one, under non-stressed conditions (Callahan, et al., 2001) (Wang, et al., 2003) (Schäfer, et al., 2007) (Rosario, et al., 2010) (Hirata-Fukae, et al., 2008). Curiously, there is only one Tg model (Lewis, et al., 2001) displaying higher Tau pathology in female animals as a direct consequence of the higher transgene expression in this gender (Hsiao, et al., 1996) (Sahara, et al., 2002). Our P301L-Tau Tg model demonstrates no gender differences on Tau pathology under non-stress conditions (Kimura, et al., 2010) (Kimura, et al., 2007). However, the present data provide molecular and behavioral evidence that the stress augment in hippocampal Tau pathology is gender specific; in line with the clinical evidence, we also found striker stress-induced deleterious effects in the female P301L. Our experimental findings are in line with a recent study showing that short repeated stress (5 days) triggered Aβ overproduction on the hippocampus of female but not male (5xTg) mice (Devi, et al., 2010). Interestingly, this gender-susceptibility was not observed in other brain regions (e.g. PFC) nor in behaviors in which hippocampus is not typically implicated (e.g. anxiety); these findings are in agreement with our findings where both anxiety and Tau pathology in PFC was equally aggravated by adverse stress in both female and male P301L-Tau animals (data not shown).

But what are the mechanisms underlying the gender susceptibility to stress-induced Tau pathology in the hippocampus of the female P301L mice? An age-dependent decline of estrogen hormones with loss of their neuroprotective effects have been widely suggested to be involved in AD gender susceptibility differences (Andersen, et al., 1999) (Fratiglioni, et al., 2000) (Wang, et al., 2003) (Gandy & Duff, 2000) (Manthey & Behl, 2006). While female rodents do not typically experience menopause and the associated depletion of estrogens, they exhibit after 12-16 months persistent diestrus periods with low estrogen levels (Felicio, et al., 1984) (Nelson, et al., 1982) (Alkayed, et al., 2000) (Jezierski & Sohrabji, 2001) (Nordell, et al., 2003). The reduced estrogenic stimulus can render neurons more vulnerable to stress- and disease-related processes (Wang, et al., 2003) (Gandy & Duff, 2000) (Manthey & Behl, 2006) (McEwen, 2002). This, however, would not explain why the gender differences; while no define explanation can be provided at this moment, it is important to note that estrogen receptors (ERβ) are decreased in hippocampus of 12 and 24 months-old female animals (Yamaguchi-Shima & Yuri, 2007).

Alternatively, changes in the organization role of sex hormones during early brain development (Cosgrovea, et al., 2007) (Mesquita, et al., 2007) may contribute to the described sex differences in both AD risk and stress vulnerability (Dalla, et al., 2011). The supporting evidence for the impact of the programming role of sex hormones in the vulnerability to AD neurodegenerative mechanisms comes from a recent study where demasculinization of neonatal male 3xTg mice that led to increasing hippocampal Aβ levels to approximately that of females and diminished the differences of adulthood hippocampal Aβ pathology (male << female) of male hippocampus (Rosario, et al., 2010). Together with our current findings, the above evidence further supports the emerging idea that "feminized" hippocampus is inherently more vulnerable to stress-triggered pathology. An interesting, and not mutually exclusive, hypothesis stems from studies on the structure and function of the locus coeruleus (LC) that were shown to exacerbate AD pathology (Heneka, et al., 2006); importantly, this brain region exhibits increased sensitivity to stress in females (Curtis, et al., 2006) (Bangasser, et al., 2011) (Bangasser, et al., 2012)

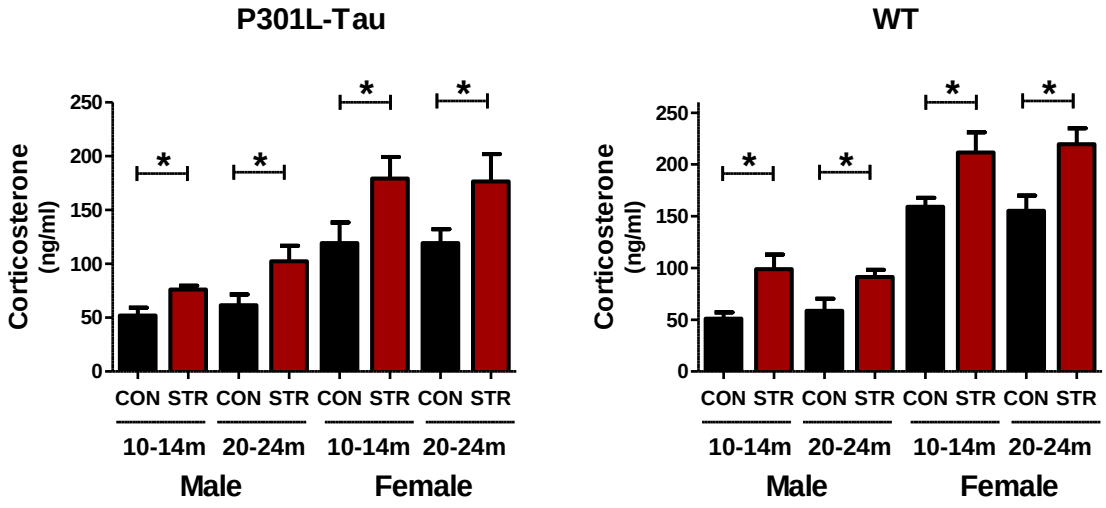
3.5 CONCLUSION

As previously suggested (Carroll, et al., 2011), our findings clearly demonstrate that prolong stress exhibit a gender-specific detrimental effect on Tau pathology and associated cognitive component of AD pathology mirroring the clinical and epidemiological profile of the disease in humans. Furthermore, in contrast to their WT littermates, P301L-Tau mice were vulnerable to prolong stress suggesting that the presence of mutated Tau predisposes animals to detrimental stress effect, a fact that is supported by reports of an enhanced neurotoxic response to stress or stress hormones (Roberson, et al., 2007), in presence of Tau protein (Sotiropoulos, et al., 2008) (Rothman, et al., 2012). Indeed, this study provides important evidence on association of genetic causes and risk factors such as adverse stress of Tau pathology as well as their interaction with gender, towards the optimization of treatment modalities in clinical use.

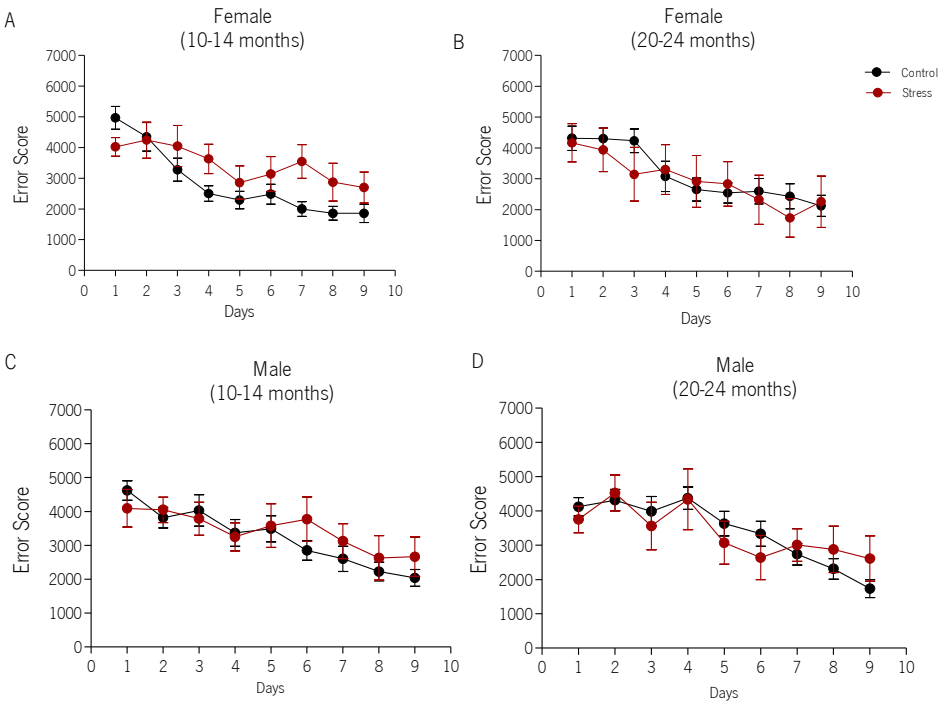
3.6 SUPPLEMENTARY DATA

Supplementary Table I. Biometric data of control and stressed animals. Effects of stress on body weight and blood corticosterone levels of both P301L-Tg and WT animals at sacrifice.

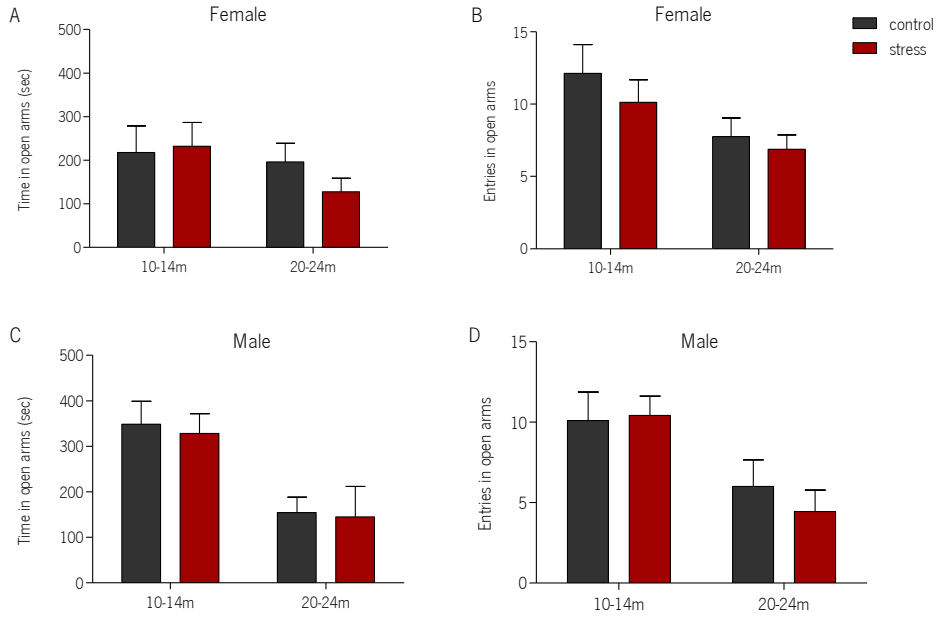
			Body weight changes (%)	
	Gender	Age	CON	STR
P301L-Tau	♂	10-14m	+4.42	-7.32
		20-24m	-1.04	-10.68
	♀	10-14m	+4.5	-5.95
		20-24m	+2.07	-6.28
WT	♂	10-14m	+3.86	-2.35
		20-24m	-0.76	-10.28
	♀	10-14m	+2.54	-7.13
		20-24m	+1.76	-8.67



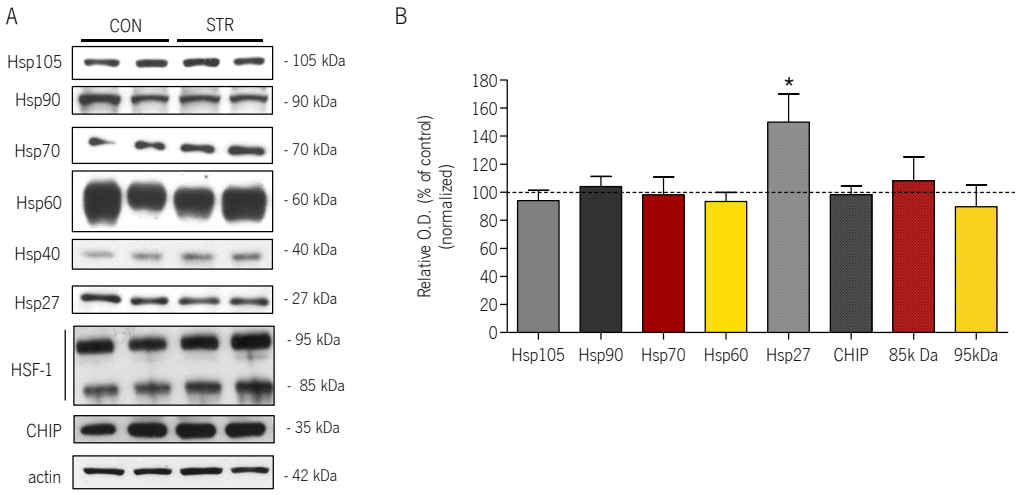
SUPPLEMENTARY FIGURE 1. Corticosterone levels. Corticosterone circulating levels in both P301L-Tau and WT animals. All data shown represent mean ± SEM values (* $p<0.05$).



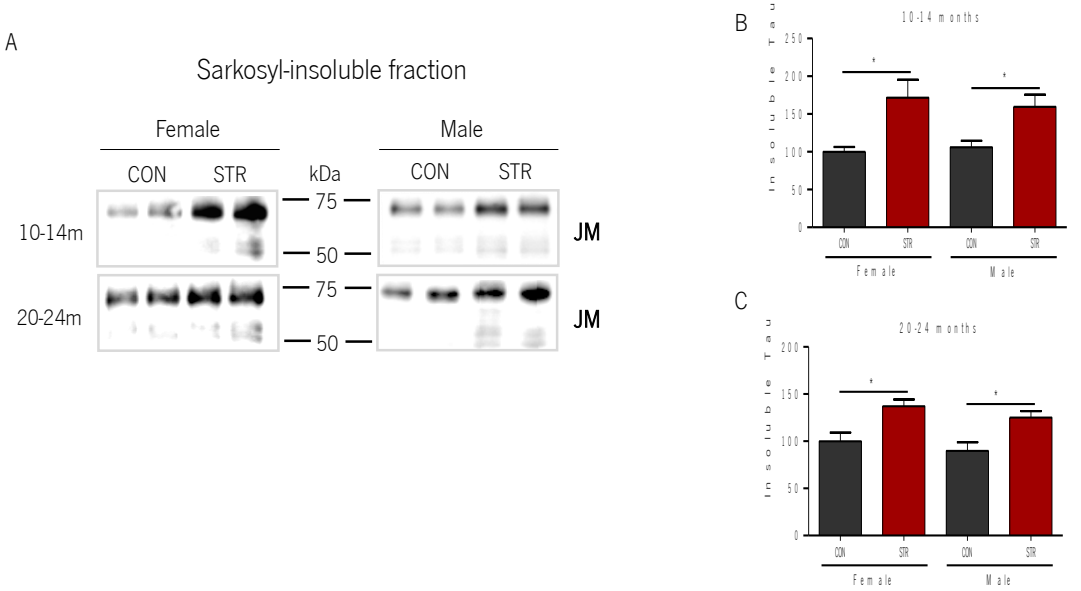
SUPPLEMENTARY FIGURE 2. Adverse stress has no impact on cognitive performance of WT animals. Morris Water Maze test used to assess spatial reference memory in middle-age (10-14 months old; A and C. old (20-24 months old; B and D. WT female and male animals, showing that stress don´t affect male P301L-Tau tg animals. All numerical data shown represent mean ± SEM values (N = 10-12).



SUPPLEMENTARY FIGURE 3. No stress-evoked changes in anxiety levels of WT animals. Stress had no effect on anxiety levels as assessed by time spent in the open arms (A-C) and number of entries in the open arms (B-D). A-B) Time and entries in the open arms of EPM apparatus in female WT mice; C-D) Time and entries in the open arms in male WT Tg mice. All numerical data shown represent mean ± SEM values (N = 10-12) (* p≤0.05).



SUPPLEMENTARY FIGURE 4. No changes induced by stress in molecular chaperones female WT hippocampus. A-B. Representative blots and quantification analysis of different molecular chaperones involved in Tau degradation showing that stress as no effect on molecular chaperones, increasing only Hsp27.



ADDITIONAL FIGURE 1. Adverse stress increases sarkosyl-insoluble Tau levels in both female and male, regarding age. A-C. Western-blot analysis of sarkosyl-insoluble fraction of P301L-Tg mice; A. Representative blots of sarkosyl-insoluble fraction, using JM antibody; B. Quantification of insoluble Tau levels in mild-age P301L-Tg; C. Quantification of insoluble Tau levels in old P301L-Tg. All numerical data shown represent mean ± SEM values (N = 6-7), depicted with respect to data obtained in control tissues (CON), set at 100%. (* $p<0.05$).

4. STRESS HORMONES INFLUENCE ON TAU LOCALIZATION

4. STRESS HORMONES INFLUENCE ON TAU LOCALIZATION

4.1 BACKGROUND

Microtubule-associated protein Tau (Tau) is postulated as a common crucial protein in mechanism of neurodegeneration in many chronic neurological diseases termed Tauopathies, including AD raising this protein as strong candidate for therapeutic intervention. While the mechanism(s) by which Tau mediates neurodegeneration are not completely understood, many studies have established that Tau dysfunction are central to the neurodegenerative process and the resulting dementia (Binder, et al., 2005) (Iqbal, et al., 2005) highlighting its hyperphosphorylation and mislocalization as initial steps of neuronal dysfunction. So, where is Tau protein localized and is Tau phosphorylation profile related to its distribution? The current view suggests TAU as a cytoskeletal, mainly axonal, protein with a major role in stabilizing microtubules (MT). Indeed, it is well known that Tau facilitates tubulin assembly by binding to MT, thus stabilizing polymerized MT, nucleating and orienting MT. In addition, Tau inhibits the rate of MT depolymeraziation e.g. reducing their dynamic instability (Avila, et al., 2004). During brain development, Tau and other microtubule-associated proteins are initially distributed ubiquitously throughout neurons but during differentiation processes, Tau becomes sorted mainly into axons. Although most of the studies state that Tau is an axonal microtubule associated protein, several observations suggest that Tau is also distributed in the cell body and dendrites in adult rat brain (Papasozomenos & Binder, 1987) (Tashiro, et al., 1997) while Tau mRNA was also detected in the synaptosomal fractions of rat brain (Crispino, et al., 2001). Furthermore, as Tau is known to interact with other cytoskeletal proteins such as spectrin, F-actin and Fyn, very recent evidence suggest a novel role of Tau at neuronal dendrites connecting it with early stages of neurodegenerative mechanism involving Aβ toxicity and/or Tau hyperphosphorylation (Hoover, et al., 2010) (Ittner, et al., 2010). As chronic stress and GC hormones are shown to trigger Tau hyperphosphorylation (Sotiropoulos, et al., 2008) (Sotiropoulos, et al., 2011) and in parallel, induce dendritic and synaptic atrophy/loss (Sousa, et al., 1999), this study aims to provide detailed in situ demonstration of TAU topology using electron microscopy and monitor the influence of stress hormones, GC, on Tau subcellular localization in neurons of hippocampus; a well-known target area for both stress and AD pathology.

4.2 MATERIAL AND METHODS

ANIMALS

Animals used in this study were divided into two groups: a) animals i.p. injected with the synthetic glucocorticoid Dexamethasone (DEX; 300μg/mL/kg BW; 1:10 Fortecortin TM, Merck, Darmstadt, Germany) for 15 days and control animals (vehicle-injected). Animals were group housed per cage under standard environmental conditions (temperature 22°C, relative humidity 70%, 12h light/dark cycle, ad libitum access to food and drinking solution). Control, vehicle-injected and DEX-injected animals exhibited a 29.73%, 3.11% and -7.47% body weight changes, respectively.

TISSUE AND SLICES PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY

At the end of DEX treatment, animals were sacrifice by decapitation and brain was removed. Immediately, hippocampi were fixed in 4%PFA for 3 days(4°C). Tissue was transferred to 0,8% gluteraldehyde in 0.1M of PB, pH=7.4. After PB washing, tissue was embedded in Epon resin and ultrathin sections (500 Å) were cut onto nickel grids.

TAU IMMUNOGOLD STAINING

Slices were treated with heated Citrate buffer (1x; Thermo Scientific, CA, USA) for 30min and left to cool down for 5-10min. After 20mM phosphate buffer (PB) wash (5min), non-specific binding blocked by 5% BSA(extra pure, EM specified, SIGMA-ALDRICH, ST Louis, MO, USA) at R.T. (10min). After PB wash, grids were incubated with primary antibody diluted in 1%BSA in PB (Tau-5, 1:50; p202Tau, 1:50; p231Tau, 1:150; Abcam) for 14h at R.T. followed by appropriate secondary gold antibody (anti-rabbit-gold or anti-mouse-gold, 1:15; Abcam). Negative control grids were incubated without primary antibody. Grids were stained with uranyl acetate, followed by lead citrate and observed in a Zeiss 902A electron microscope.

4.3 RESULTS AND DISCUSSION

In line with the well-known axonal role of microtubule-associated Tau protein, we found that Tau expression was enriched at axonal compartment exhibiting the high dot signal forming the characteristic parallel arrays that are continuous over long distances (Figure 15A). These electron microscope findings support the main function and regulating role of Tau protein on axonal growth and structure (Mandelkow, et al., 2003). In addition, Tau was detected at dendrites but at significantly lower levels of expression in comparison to the axons (Figure 15A). Indeed, previous immunohistochemical studies in rat brain and neuronal cultures suggested the presence of Tau protein at the somatodendritic compartment (Tashiro, et al., 1997) (Ballatore, et al., 2007). Most importantly, Tau was also found to be present at dendritic spines and synapses, specifically at postsynaptic part including postsynaptic density (Figure 15B-D). This finding further supports a very recent suggestion for synaptic Tau role where it binds to NMDA receptors through Fyn (Ittner, et al., 2010). Surprisingly, Tau seems to be also detected pre-synaptically providing a completely new insight about novel Tau role and function. Further experimental evidence is required in order to confirm and further investigate a potential presynaptic role for Tau protein.

In addition, endoplasmatic reticulum and Golgi apparatus (GA) exhibit Tau immunogold staining (Figure 16A, B) highlighting the fact that Tau is subject to several types of post-translational modifications such as glycosylation and phosphorylation that may occur when Tau is transferred to this cellular compartment. Previous studies have immunoisolated Tau with the Golgi membranes and immunodetected Tau at GA of motor neurons by electron microscope immunocytochemistry suggesting that TAU may regulate of GA structure by mediating their association with microtubules (Farah, et al., 2006). In agreement with previous studies describing association of Tau with mitochondria (Jung, et al., 1993), we found mitochondrial distribution of Tau (Figure 16C).

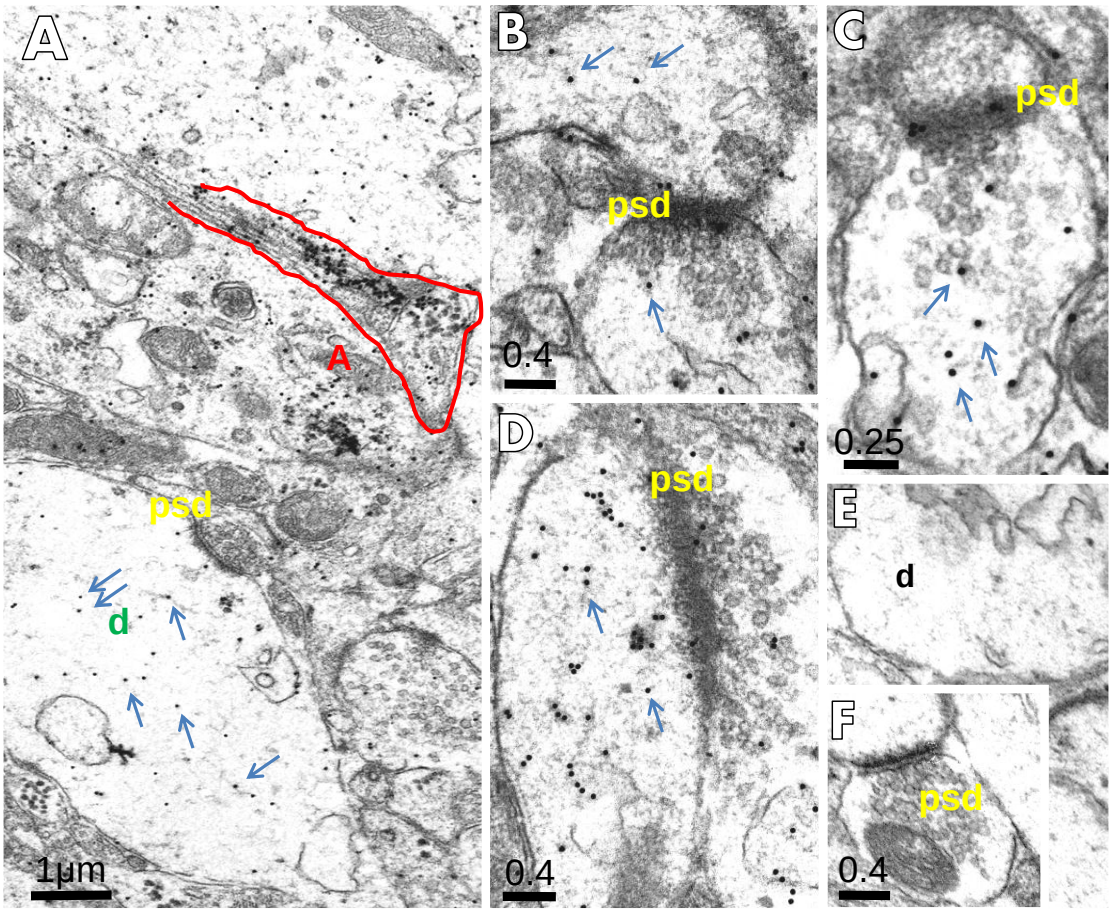


FIGURE 15. Electron microscope subcellular localization of Tau protein. Tau localization was primarily found at axons (red; A) and dendrites (green; d), but also at synapses (yellow; B-D). E-F: negative control.

Supporting the fundamental involvement of Tau protein at cargo cellular mechanism at axon but also at soma (Stamer, et al., 2002) (Mandelkow, et al., 2003). In addition, Tau was detected on cellular membrane providing further support of the interaction of Tau with membrane as shown by Brandt et al., (Figure 16D). Accordingly, further support of Tau role in transport of membranous organelles was based on Tau overexpression in neuronal but also non-neuronal cells which resulted in accumulation of organelles such as mitochondria, Golgi membranes, peroxisomes in the region around the nucleus (Drewes, et al., 1998) (Stamer, et al., 2002).

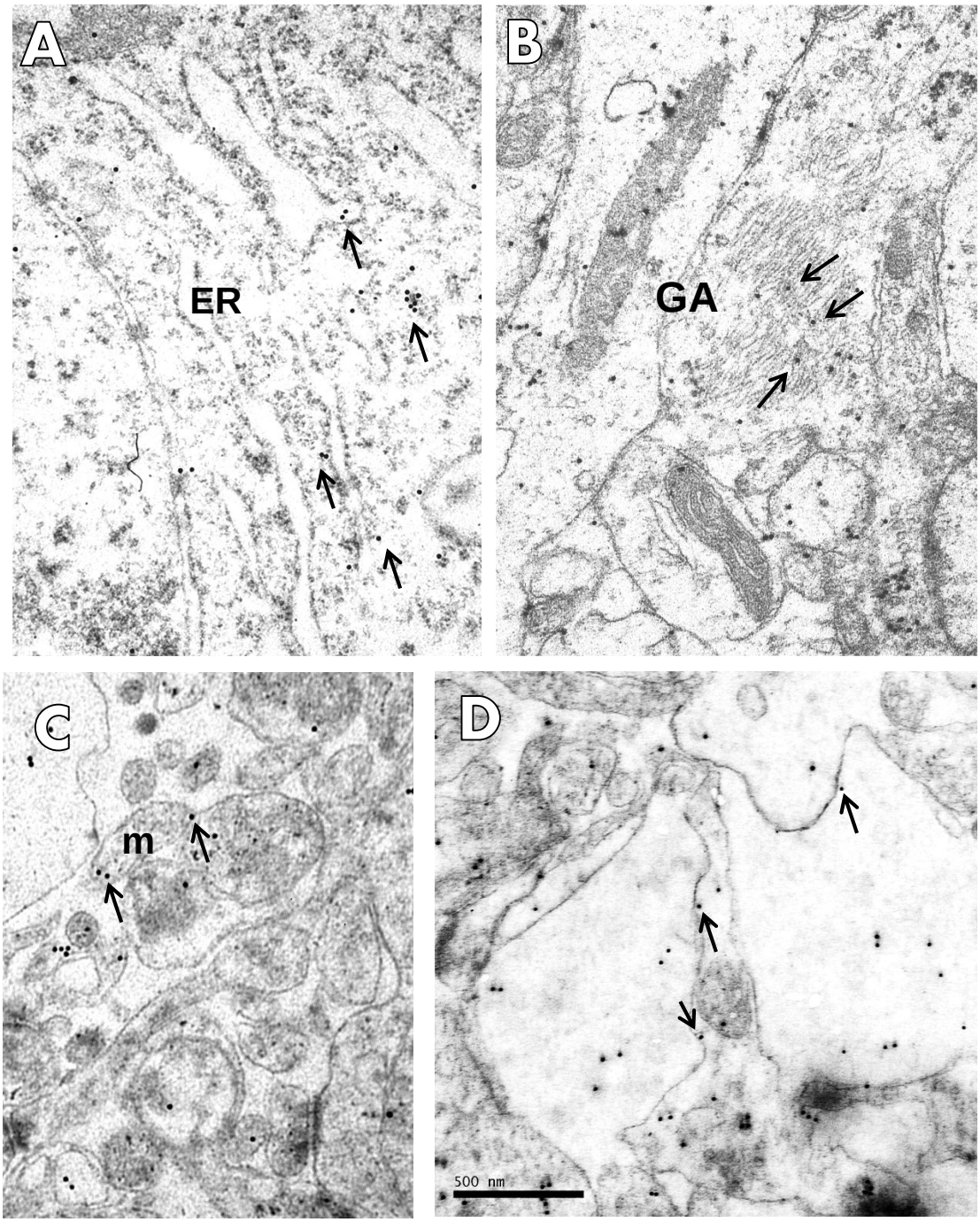


FIGURE 16. Tau localization at membranes of cellular organels. Micrographs of imunogold staining for Tau (Tau-5) showing that Tau protein is present at Endoplasmatic reticulum (ER), Golgi apparatus (GA), mitochondria outer membrane (M) and plasma membrane in hippocampus tissue.

Phosphorylation of Tau protein is shown to regulate its function as well as its localization in different neuronal compartments. This is also true for pathological conditions such as Alzheimer´s disease (AD) pathology where tau is hyperphosphorylated and accumulated in the neuronal soma where

synaptic loss occurs. Indeed, previous studies have shown that chronic stress and stress hormones, glucocorticoids (GC), trigger Tau hyperphosphorylation and accumulation (Sotiropoulos et al., 2008; 2011) followed by dendritic atrophy and synaptic loss. Based on the above findings, we aim to *in vivo* monitor the effect of prolong exposure to elevated GC levels on Tau subcellular localization as part of stress/GC-evoked synaptic pathology using animals that were i.p. injected with the synthetic glucocorticoid, dexamethasone (DEX) for 15 days. As electron microscope-based immunogold staining revealed (Figure 17), DEX treatment resulted in reduction of total Tau staining in dendrites and synapses in hippocampal tissue.

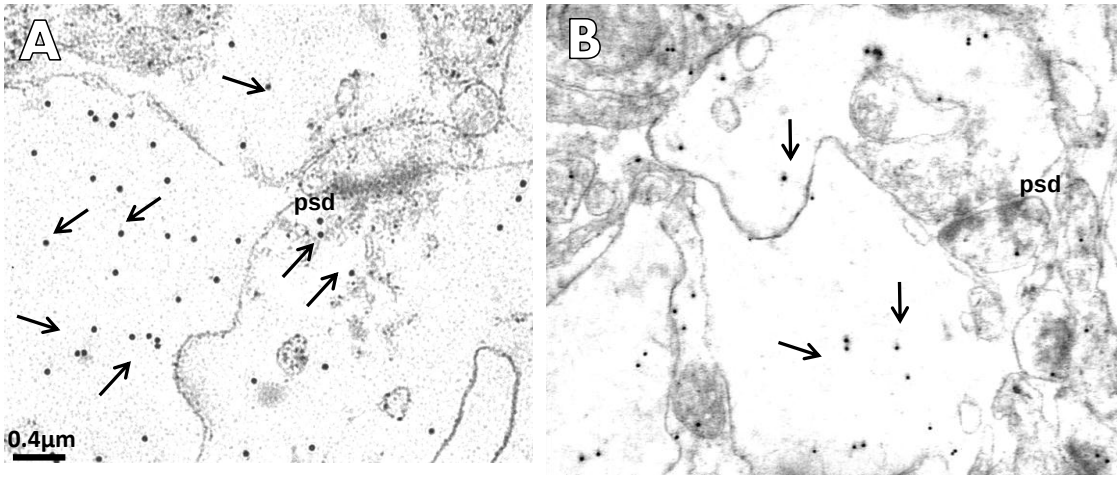


FIGURE 17. DEX reduces dendritic and synaptic imunogold staining of Tau. Micrographs of imunogold staining for total Tau (Tau 5) showing a decreased staining of Tau in the hippocampal dendrites and synapses in DEX-treated animals (B) when compared to vehicle-treated ones (A).

At the next step, we monitored the presence and localization of phosphorylated Tau isoforms in hippocampus of DEX-treated animals focusing on Thr231-phosphoTau as this epitope phosphorylation is strongly implicated in the neuropathology of AD associated with the appearance of tau aggregates and tangles as well as the severity of AD memory impairments of AD patients (Augustinack, et al., 2002) (Ewers, et al., 2007). We noticed an increase in staining of phosphorylated Tau at Thr231 epitope (Figure 18A and B), a primed site for GSK3β (Goedert, et al., 1994) a Tau kinase that is known to be activated by stress and GC in both in vitro and in vivo studies (Sotiropoulos, et al., 2008) (Sotiropoulos, et al., 2011). The phosphorylation at Thr231 diminishes the ability of Tau to microtubules and it is mainly found at soluble fractions (Cho &

Johnson, 2003) (Hamdane, et al., 2003), pointing for the role of stress in the initiation of Tau pathology through soluble abnormal Tau, supported by our previous studies (Sotiropoulos, et al., 2011). Furthermore, this notion is in agreement with the recently suggested accumulation of hyperphosphorylated species of Tau at dendritic spines as one of the earlier parts of synaptic pathology characterizing AD (Hoover, et al., 2010).

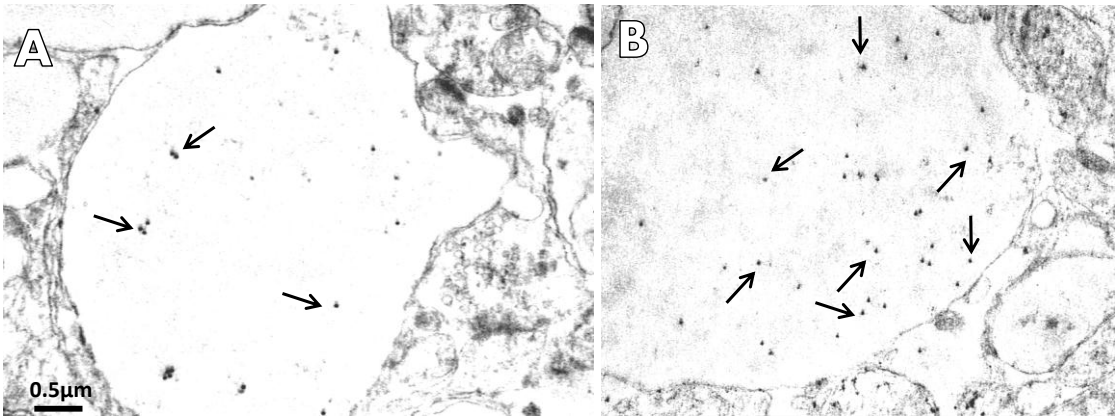


FIGURE 18. Increase phosphorylated Tau in dendrites of DEX-treated animals. Micrographs of immunogold staining of phosphorylated Tau at Thr231 epitope is increased in dendrites of DEX –treated animals (B) in comparison to vehicle-treated ones (A).

4.4 CONCLUSION

This ultrastructure study provides a direct, *in situ* evidence for TAU distribution in different neuronal compartments underscoring the dendritic and synaptic distribution of TAU protein under normal and stressful conditions (Hoover, et al., 2010) (Ittner, et al., 2010). Specifically, these results highlight a subcellular mislocalization of Tau protein as a critical underlying mechanism of neuronal damage and possible atrophy due to exposure to high GC levels. As Tau is recently shown to structurally interact with synaptic receptors, our findings points towards novel mechanistic involvement and role of TAU at neuronal synaptic plasticity and signalling and ultimately in functionality of neuronal networks that mediate cognitive functions and provide a substrate for stress-triggered pathology.

5. GENERAL DISCUSSION

5. GENERAL DISCUSSION

Epidemiological studies show that AD is the leading cause of dementia in the population over 65 years presently affecting more than 27 million people worldwide while this number will be increased to 106 million by 2050 (1 in 85 persons) raising the number of caregivers at 216 million (Brookmeyer, et al., 2007). Although the understanding of the pathophysiology and neurobiology of neurodegenerative disorders has considerably increased over the last decades, AD remains a complex multifactorial disease with many risk/predisposing factors, such as aging, gender and psychological stress, to be involved in its etiopathogenesis. In the light of lack of proper treatment for AD patients, better identification of mechanistic interactions of these risk factors is of great importance towards the exploration of potential biological targets for preventative and curative intervention which is urgently needed.

Mounting evidence from human and animal studies suggests the implication of stress and its primary physiological response, GC, in the onset and/or progression of AD (see introduction; for review, see Sotiropoulos et al., 2008). Indeed, a role for high glucocorticoid levels in AD had been hypothesized since 1968 (McEwen, et al., 1968) (Sapolsky, et al., 1985) (Wolkowitz, et al., 1990) (Lupien, et al., 1998). Many AD patients show signs of GC hypersecretion and HPA axis deregulation (Hartmann, et al., 1997) (Elgh, et al., 2006) (Johansson, et al., 2010) while cortisol increase seems to be a very sensitive marker of cognitive decline in AD patients (Weiner, et al., 1997). Recent studies from our group have shown that stress triggers Tau hyperphosphorylation and accumulation affecting Tau turnover and degradation (Sotiropoulos, et al., 2008) (Sotiropoulos, et al., 2011). These Master studies provide further evidence on stress role of other parameters of Tau pathology such as tau aggregates, pathological conformation changes as well as truncation of Tau (Figure 19). Indeed the last was connected to the increased activity of caspace-3 resulting in activation of apoptotic cascades and decreased cell density. Side by side, all these findings supports the neurodegenerative potential of chronic stress towards the development of AD pathology. Furthermore, we saw that adverse stress affect some critical molecular chaperones involved in misfolded and/or aggregated Tau degradation. Specifically, protein levels of inducible Hsp70 and Hsp105 were decreased by stress pointing towards the reduced Tau degradation in agreement of human data suggested a significant inverse correlation between insoluble Tau and

Hsp70 protein in AD brains (Sahara, et al., 2007). On the opposite, Hsp90 were increased by stress which could explain the inducible reduction of Hsp70 through a transcriptional effect of Hsp90 on HSF1 that triggers the production of inducible chaperones (e.g. Hsp70). Indeed, Hsp90 inhibitor has been suggested as a therapeutic tool for enhanced degradation of misfolded/aggregated proteins in many neurodegenerative diseases (e.g. Huntington, Machado-Joseph disease) (Koren, et al., 2009).

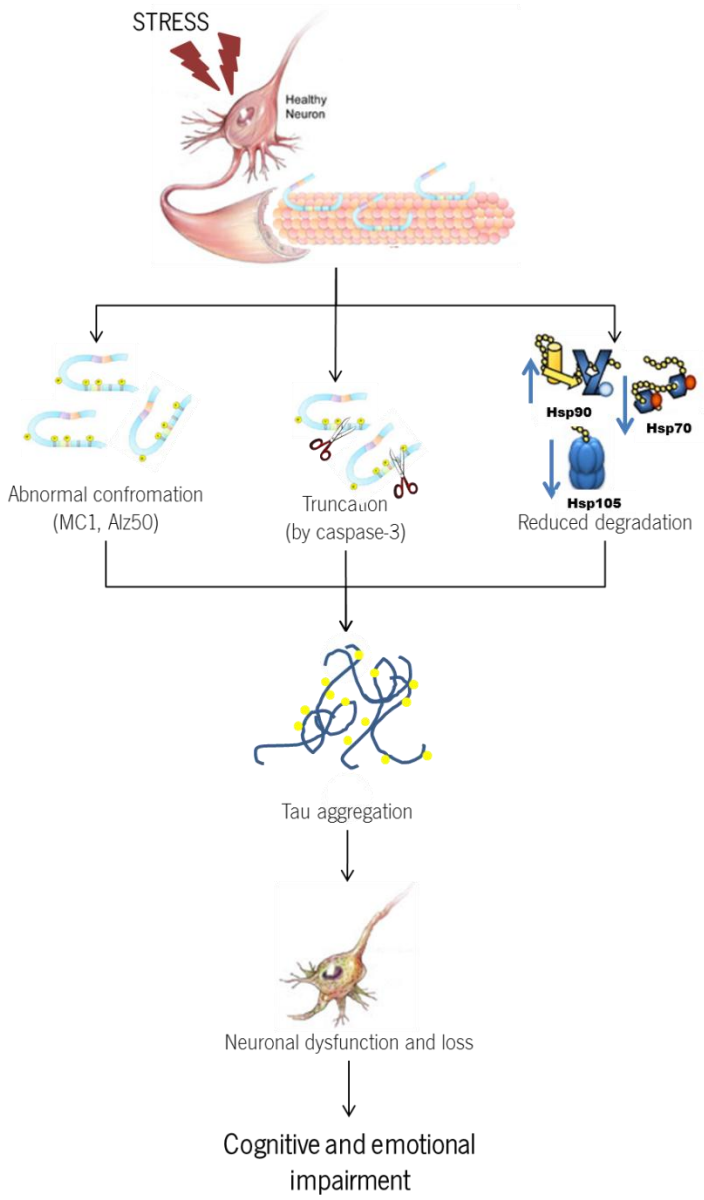


FIGURE 19. Stress-trigged Tau aggregation pathology. Hypothetical model describing the detrimental role of adverse stress on different parameters of Tau pathology resulting in neuronal dysfunction and loss as well as in cognitive and emotional deficits. Specifically, stress increases tau aggregation by diminishing its chaperone-mediated degradation and in parallel triggers apoptotic mechanisms that result in neuronal dysfunction and loss affecting both cognitive and emotional performance.

Another interesting finding of this thesis focuses on the increase female hippocampal vulnerability to stress-triggered Tau pathology and the associated cognitive impairment. Despite the fact that both AD and stress response have a clear gender profile, there is paucity of research examining sex differences at a neurobiological and mechanistic level (National Institute of Mental Health 2011). Thus, our study is among the few ones that provides further evidence on gender differences on stress-trigged AD pathology that is in line with clinical evidence on gender differences in incidence and prevalence to AD (Gandy & Duff, 2000) (Wang, et al., 2003). In contrast to cognition, emotionality was similarly affected by stress between male and female animals. Indeed, clinical studies also demonstrated that there no gender differences in anxious behavior among AD patients suggesting that other brain areas don´t exhibit this gender-dependent vulnerable to AD pathology. This notion is in line with our findings that PFC is similarly affected between male and females (findings of current study and Devi et al, 2010). While we cannot provide a mechanistic evidence for this gender difference, the increased stress susceptibility of locus coeruleus (LC) in females (Bangasser, et al., 2011) (Bangasser, et al., 2012), could explain the higher stress damage of female hippocampus as LC damage is shown to exacerbate hippocampal AD pathology (Heneka, et al., 2006)(Figure 20).

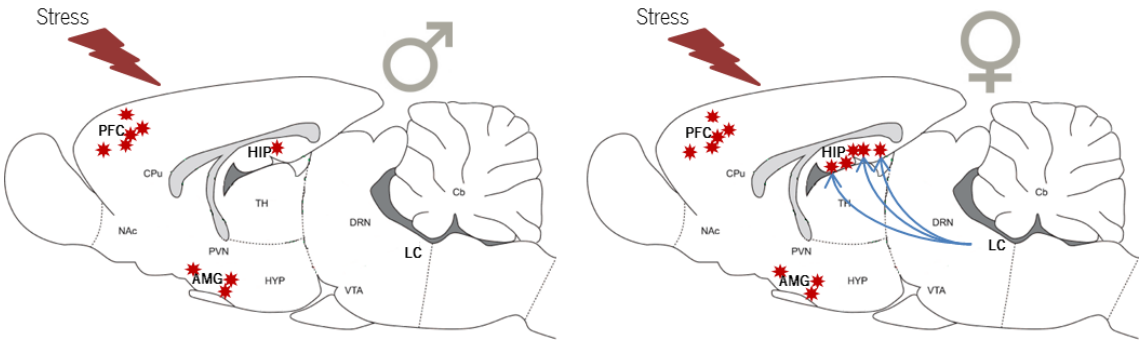


FIGURE 20. Working model of gender differences in stress-triggered Tau pathology. Schematic representation of the areas affected by stress in male and female P301L-Tau animals suggesting the LC key contribution to this gender-specific vulnerability. In contrast to other brain areas, female hippocampus exhibits increased stress vulnerability to Tau pathology through LC damage (✸ Tau pathology; drawn by J. Silva & I. Sotiropoulos).

Our findings provide experimental evidence on stress-gender interaction on AD pathology highlighting the importance of use of both genders in experimental studies for clarifying

mechanisms of disease pathology. We demonstrate for the first time that prolong stress exhibit a gender-specific detrimental effect on Tau pathology and associated cognitive and emotional component of AD pathology mirroring the clinical and epidemiological profile of the disease in humans. Furthermore, in contrast to their WT littermates, P301L-Tau mice were vulnerable to prolong stress suggesting that the presence of mutated Tau predisposes animals to detrimental stress effect; a notion that is in line with previous studies that demonstrated an enhanced neurotoxic response to stress or stress hormones, in presence of Tau protein (Sotiropoulos, et al., 2008) (Rothman & Mattson, 2010).

These studies provide novel mechanistic knowledge about the detrimental role of chronic stress towards the development of brain pathologies, highlighting Tau and its malfunction as a potential drug target for preventing or blocking neuronal degeneration and death.

6. MAIN CONCLUSIONS

6. MAIN CONCLUSIONS

Accumulating evidence suggest a detrimental role of chronic stress of the initiation and progression of AD pathology. Many clinical studies demonstrate that AD patients exhibit higher GC levels and deregulation of HPA axis while previous animal studies saw that stress and/or GC trigger APP misprocessing and tau hyperphosphorylation. The current Master thesis provides further evidence of stress detrimental role on other parameters of Tau pathology showing that adverse stress increase levels of truncated and conformational altered Tau followed by elevated Tau aggregates, probably through failure of chaperone machinery. This results in activation of apoptotic pathways that leads to neuronal loss. Importantly, this stress effect exhibit a gender- and area-specific profile with female hippocampus being more vulnerable to stress-triggered Tau neurodegeneration. This study provides important evidence on association of genetic causes and risk factors such as adverse stress of Tau pathology as well as their interaction with gender, towards the optimization of treatment modalities in clinical use.

7. FUTURE PRESPECTIVES

7. FUTURE PRESPECTIVES

This Master thesis clearly demonstrated that adverse stress increased neurotoxic Tau aggregates resulting in activation of apoptotic pathway affecting protein levels of Bax, Bcl-2 and Bcl-XL. As these molecules are also implicated in autophagy (Zhou & Da Xing, 2011) which is a cellular mechanism involved in many neurodegenerative diseases (Schaeffer & Goedert, 2012) (Cheung & Ip, 2011) (Caballero & Coto-Montes, 2012), a next step analysis could include the influence of stress on autophagic pathways in our P301L-tau animals.

Furthermore, based on our current findings unrevealing a clear interplay on stress and gender on Tau-related neurodegeneration, future studies could focus on detailed clarification of physiological and cellular mechanisms underlying the increased vulnerability of female hippocampus. Specifically, as LC lesions are suggested to trigger hippocampal neurodegeneration in AD Tg models (Heneka, et al., 2006) and LC is known to be more affected by stress in female animals (Bangasser, et al., 2011), future studies could analyzed the role of stress on LC of female and male P301L-Tau animals. In addition, it would be interesting to monitor whether this female vulnerability is based on age-related estrogen decline by supplementation of 17beta-estradiol in the female P301L-Tau animals before their exposure to adverse stress.

8. REFERENCES

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