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Tau (mal)function in brain plasticity and pathology – a gateway beyond Alzheimer's Disease

(Dis)função da proteína Tau na plasticidade e patologia do cérebro – para além da Doença de Alzheimer

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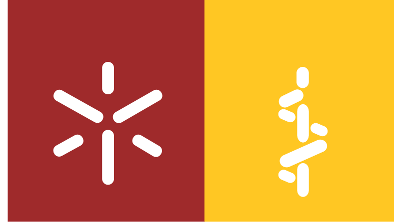

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Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação do
Doutor Ioannis Sotiropoulos
e do
Professor Doutor Nuno Jorge Carvalho de Sousa

fevereiro de 2017

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Domínio de Neurociências

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“A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales. “

Marie Curie

ABSTRACT

TAU (MAL)FUNCTION IN BRAIN PLASTICITY AND PATHOLOGY – A GATEWAY BEYOND ALZHEIMER'S DISEASE

Despite that research efforts have been increasingly focused on Alzheimer's disease (AD) over the last decades, it was only recently that Tau protein was suggested as an essential regulator of neuronal plasticity as well as pathology triggered by different intrinsic and extrinsic factors. Consistent with suggestions that lifetime stress may be a clinically-relevant precipitant of AD pathology, previous experimental studies showed that Tau is at the core of chronic stress-induced pathological brain aging, raising Tau malfunction as a critical mechanism through which stress and glucocorticoids (GC) exert their neuro-remodeling and neurodegenerative effects upon the substrates of cognition and emotion. While experimental evidence, including some previous work from our group, showed that chronic stress and GC trigger Tau hyperphosphorylation, accumulation and aggregation, the molecular mechanisms by which Tau contributes to stress-driven brain malfunction and pathology are poorly understood.

In this thesis, we investigated the cellular mechanisms through which chronic stress and/or GC trigger Tau malfunction and pathology leading to cognitive and mood deficits. Our findings demonstrate that prolonged exposure to high GC levels triggers two different Tau-related cellular cascades in dendrites and dendritic spines, respectively: a) Tau hyperphosphorylation and somatodendritic accumulation of different phosphorylated Tau isoforms accompanied by reduced microtubule stability and dendritic remodeling and, b) synaptic missorting of specific hyperphosphorylated epitopes of Tau and overactivation of GluN2B receptor leading to synaptic atrophy/loss. Furthermore, this thesis aimed to clarify the molecular mechanisms of stress/GC-driven neurotoxic accumulation and aggregation of Tau. We showed, using both *in vitro* and *in vivo* studies, that chronic stress and GC evoke an mTOR-dependent blockage of autophagic clearance machinery with parallel induction of histone deacetylase 6 (HDAC6) and formation of stress granules (SGs). These findings implicate induction of HDAC6 and SGs as well as the inhibition of autophagy in stress-driven Tau aggregation identifying novel mechanisms through which chronic stress precipitates brain pathology, which may contribute for future improved therapeutic strategies.

While abnormal hyperphosphorylation and aggregation of Tau are well-established key events in AD neuropathology, the impact of the loss of normal Tau in neuronal function in adult brain is still under intense debate; e.g. while *in vitro* evidence supports an essential role of Tau for

microtubule stabilization, axonal maintenance and transport, adult animals with constitutive deletion of *MAPT* (and thus, Tau protein) fail to display obvious behavioral, neurostructural or functional deficits highlighting a significance gap of knowledge about the actual role of Tau in neuronal function. In order to avoid the developmental compensation mechanisms suggested to be present in the currently available (constitutive) Tau knock-out (KO) mouse lines, these studies present and analyze a novel mouse model of conditional deletion of Tau in adult brain, based on a tamoxifen-inducible LoxP/Cre system, that offers temporal and brain-area specificity in knocking-out *MAPT* gene. After confirming that the generation of this new mouse model didn't affect the developmental, neurological and behavioral profile of the animal, we show for the first time that CaMKII-driven conditional Tau deletion in the adult forebrain doesn't affect cognitive performance, but it triggers the induction of anxious and depressive behavior in adult mice. Importantly, these behavioral deficits were accompanied by neuronal atrophy and synaptic alterations in prefrontal cortex and amygdala, two essential brain areas for the manifestation of mood deficits. These findings provide novel evidence about the essential role of Tau on neuronal and brain homeostasis.

Overall, the findings of this thesis provide novel evidence about the involvement of Tau in different parameters of brain plasticity as well as brain pathology.

RESUMO

(DIS)FUNÇÃO DA PROTEÍNA TAU NA PLASTICIDADE E PATOLOGIA – PARA ALÉM DA DOENÇA DE ALZHEIMER

Ao longo das últimas décadas a investigação tem-se focado com mais intensidade na Doença de Alzheimer, mas apenas recentemente foi sugerido a proteína Tau como um regulador essencial da plasticidade neuronal e da patologia, despoletada por diferentes fatores intrínsecos e extrínsecos (ex.: beta-amilóide, excitotoxicidade, epilepsia). De acordo com a hipótese de que o stress crónico pode ser um fator clinicamente relevante na patologia da Doença de Alzheimer, estudos experimentais sugerem que a proteína Tau pode ter um papel nuclear no envelhecimento patológico induzido pelo stress crónico, relevando o mau funcionamento desta proteína como um mecanismo crítico através do qual o stress e os glucocorticoides (GC) exercem efeitos neuro-remodeladores e degenerativos, levando a alterações na cognição e emoção. Apesar das provas experimentais, incluindo alguns trabalhos anteriores do nosso grupo, mostrarem que o stress crónico e os GC despoletam a hiperfosforilação, acumulação e agregação da Tau, os mecanismos precisos através dos quais esta proteína contribui para um mau funcionamento do cérebro e patologia induzidos pelo stress são pouco claros.

Nesta tese foram monitorizados os mecanismos celulares através dos quais o stress crónico e/ou os GC despoletam o mau funcionamento e patologia da Tau que resultam em défices cognitivos e de humor. Os dados atuais demonstram que uma exposição prolongada a níveis altos de GC ativam duas cascatas celulares que estão relacionadas com a localização da proteína Tau nas dendrites e espinhas dendríticas: a) hiperfosforilação e acumulação somatodendrítica de diferentes epítopos fosforilados da Tau, acompanhadas de uma redução na estabilidade dos microtúbulos e remodelação das dendrites e, b) localização anormal nas sinapses de isoformas hiperfosforiladas da Tau e sobreativação do recetor GluN2B, que leva a atrofia/perda sináptica. Para além disto, esta tese teve como objetivo esclarecer os mecanismos moleculares induzidos pelo stress/GC envolvidos na agregação e acumulação neurotóxica da Tau. Os estudos *in vitro* e *in vivo* realizados demonstraram que o stress crónico e os GC conduzem a um bloqueio da maquinaria de limpeza autofágica, dependente da ativação da cascata de sinalização do mTOR, paralelamente, com a indução da histona desacetilase 6 (HDAC6) e formação de grânulos de stress; os últimos estando causalmente relacionados com a agregação da tau. Estas descobertas implicam a indução da HDAC6 e dos SGs assim como a inibição da autofagia numa agregação da Tau induzida pelo stress,

identificando assim novos mecanismos através dos quais o stress crónico precipita a patologia cerebral, podendo no futuro contribuir para o desenvolvimento de estratégias terapêuticas mais eficientes.

Apesar da hiperfosporilação e agregação anormais da Tau serem mecanismos-chave da neuropatologia da Doença de Alzheimer, o impacto da perda da função normal da Tau no funcionamento/mau-funcionamento neuronal no cérebro adulto está ainda debaixo de um intenso debate: por exemplo, enquanto provas *in vitro* suportam o papel essencial da Tau na estabilização dos microtúbulos, transporte e manutenção dos axónios, a deleção constitutiva da proteína não leva a nenhuma alteração comportamental, neuronal ou funcional no animal adulto, demonstrando que existe uma falha significativa no nosso conhecimento sobre o papel da Tau na função neuronal. Desta forma, evitando mecanismos de compensação que estão descritos nos animais com deleção constitutiva da Tau (KO), nestes estudos de doutoramento foi desenvolvido um novo modelo de ratinho com uma deleção condicional da Tau no cérebro adulto, baseado no sistema LoxP-Cre (indutível por tamoxifeno), que oferece flexibilidade temporal e de área cerebral na deleção do gene *mapt*. Após se ter observado que esta nova linha de ratinhos não apresentava o perfil de desenvolvimento neurológico e comportamental alterado, demostramos pela primeira vez que a deleção condicional da Tau, induzida pelo promotor CaMKII no cérebro adulto, não afeta a performance cognitiva dos animais, mas despoleta a indução de comportamento ansioso e depressivo em ratinhos adultos. É de notar que estes défices comportamentais foram acompanhados por atrofia neuronal e alterações sinápticas no córtex pré-frontal e na amígdala, duas áreas do cérebro essenciais na manifestação de défices de humor. Estas descobertas providenciam novas provas acerca do papel essencial da Tau na homeostasia neuronal e cerebral.

Em suma, as descobertas desta tese de doutoramento fornecem novas provas acerca do envolvimento da Tau em diferentes parâmetros da plasticidade cerebral e também na patologia do cérebro, com a monitorização das cascatas envolvidas na função, disfunção e patologia da proteína Tau.

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ABBREVIATIONS

μm – micrometers

3-MA – 3-methyladenine

3R – 3-microtubule binding repeat

4R – 4-microtubule binding repeat

Aβ - Amyloid-β

ABL – Abelson murine leukemia oncogene

ACTH – Adrenocorticotropin hormone

AD – Alzheimer's Disease

AIS – axon initial segment

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AND – Anogenital Distance

ANOVA – Analysis of Variance

AnxA2 – Annexin A2

AOSs – Antisense oligonucleotides

ApoE – Apolipo protein E

APP – Amyloid precursor protein

ARG – Abelson-related gene

Asp – aspartate amino acid

Atg – Autophagy related gene

ATP – Adenosine triphosphate

BACE-1 - β-secretase

BAG1 – Bcl-2 associated athanogene

BLA – Basolateral amygdala

BSA – Bovine serum albumin

CA1 – cornu ammonis

CAMKII – Calmodium dependent protein kinase II

CAMKIIa – Calcium/Calmodium-dependent protein kinase type II alpha chain

CBD – Corticobasal degeneration

Cdk5 – Cyclin-dependent kinase 5

CeA – Central amygdala

CFC – Contextual Fear Conditioning

cm – centimeters

CMV – Cytomegalovirus

CREB – cAMP response element-binding

CRH – Corticotropin-releasing hormone

DAB – 3,3'-diaminobenzidine

DEX – dexamethasone

DG – Dentate gyrus

DMEM – Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic acid

EGFP – Enhanced Green Fluorescent Protein

EGTA – Ethylene glycol bis(2-aminoethyl ether)tetraacetic acid

ELISA – enzyme-linked immunosorbent assay

EPM – Elevated Plus Maze

ER – Estrogen Receptor

F-actin – Filamentous actin

FBS – Fetal bovine serum

FKBP – FK506 binding protein

FST – Forced-Swim Test

FTD – Frontotemporal dementia

FTDP-17 – Frontotemporal dementia with parkinsonism linked to chromosome 17

FUS – RNA-binding protein fused sarcoma

G3BP1 – GTPase-activating protein-binding protein 1

GC – Glucocorticoid

GFP – Green Fluorescent Protein

GlcNAc – N-acetylglucosamine

GR – Glucocorticoid receptor

Grb2 – Growth factor receptor-bound protein 2

GSK3β - Glycogen synthetase 3β

H13 – Minor histocompatibility antigen

HDAC6 – Histone deacetylase 6

HOP – Hsp70-Hsp90 organizing protein

HPA – Hipothalamus-Pituitary-Adrenal

Hsc70 – heat shock cognate

Hsp – Heat shock protein

i.p. – intraperitoneal

KO – Knock-out

LC3 –Light chain 3

LCK – Lymphocyte-specific protein tyrosine kinase

LDB – Light-dark Box

LTD – Long-term depression

LTP – Long-term Potentiation

Lys – lysine amino acid

mA – milliamp

MAP – Microtubule associated protein

MAPK – mitogen-activated protein kinase

MAPT – Microtubule Associated Protein Tau

MAPT – Tau gene

MARKs – Microtubule affinity-regulating kinase

mg – milligrams

mGluR – Metabotropic glutamate receptor

Min – minutes

miRNA – micro RNA

mM – millimolar

MR – Mineralocorticoid receptor

mRNA – messenger

mRNP – messenger ribonucleoprotein

MT – Microtubule

MTOC – Microtubule organizing center

mTOR – mechanistic target of rapamycin

MWM – Morris Water Maze

NFT – Neurofibrillary Tangle

NMDA - N-methyl-D-aspartate

NSF – Novelty Supressed Feeding

NThs – Neuropil Threads

OF – Open Field

P62/SQSTM1 – sequestosome-1

PBS – Phosphate buffered saline

PCR – polymerase chain reaction

PFA – paraformaldehyde

PFC – prefrontal cortex

PFC – prefrontal cortex

PGK – Phosphoglycerate Kinase

PHFs – Paired-helical filaments

PI3K – Phosphatidylinositol-4,5-bisphosphate 3-kinase

PiD – Pick's Disease

PKA – AMP-dependent protein kinase

PND – post-natal day

PP – Protein phosphatase

Pro – Proline amino acid

PS1 – Presenilin 1

PS2 – Presenilin 2

PSD – Post-synaptic Density

PSP – Progressive superuclearpalsy

PVN – paraventricular nucleus

RBP – RNA binding protein

RIPA – Radioimunopercipitation Assay Buffer

RNA – Ribonucleic acid

RT – room temperature

SAGE – Serial analysis of gene expression

SDS-PAGE – SDS-Polyacrylamide gel electrophoresis

SDT – Sweet-drive test

Ser – Serine amino acid

SF – Straight filaments

SGs – Stress Granules

SHANK - SH3 and multiple ankyrin repeat domain

SIRT1 – Sirtuin 1

SPT – Sucrose Preference Test

SPT – Sucrose preference test

SYK – Spleen tyrosine kinase

Tau – Tubulin associated unit

TBS – Tris buffered Saline

Tg – Transgenic

Thr – Thrionine amino acid

TIA-1 – T-cell intracellular antigen-1

TM – Tamoxifen

TORC1 – mTOR complex

TST – Tail Suspension Test

TTP – Tristetrapolin

Tyr – Tyrosine amino acid

Ub – Ubiquitin

ULK – Serine/threonine protein kinase

VPT – Vertical Pole Test

WB – Western Blot

WT – Wild Type

YM – Y-maze

THESIS PLANNING

The present thesis is divided into 6 chapters. Chapter 1 consists of a general introduction while Chapters 2 to 4 describe the work developed towards the understanding of the role of Tau and its malfunction in brain plasticity and pathology. In Chapter 6, a general discussion of the work is presented.

In **Chapter 1,** a brief overview on the currently available evidence related to Tau protein and its biological structure and function followed by cellular mechanisms involved in Tau pathology found in Alzheimer's disease (AD) and other Tauopathies. In addition, emphasis is also given to risk factors for the disease such as environmental chronic stress and its impact of Tau protein towards the precipitation of AD neuropathology.

Chapter 2, the first chapter of the result section, describes the work recently published in the paper entitle "Tau mislocation in Glucocorticoid-triggered Hippocampal Pathology". Hereby, we described for the first time the role of Tau hyperphosphorylation and its synaptic missorting in mechanisms of GC-driven neuronal atrophy and synaptic damage extending Tau-related neuroplastic mechanisms beyond AD.

In **Chapter 3**, the described work clarifies the molecular underpinnings of the deleterious impact of chronic stress and GC on Tau aggregation. We show for the first time that stress leads to a blockage of the autophagy clearance mechanism and induction of Stress granules towards the generation of the neurotoxic Tau aggregates, cell death and cognitive and mood deficits in P310L-Tau transgenic (Tg) mice. This work adds to our mechanistic understanding of how chronic stress and elevated GC trigger Tau pathology.

The generation of a novel conditional *mapt* knock-out (KO) mouse model is described in **Chapter 4** which includes an extensive characterization of a novel Tau-lox mouse line, the creation of the first conditional model for Tau protein, Tau-lox/CaMK followed by the optimized protocol of tamoxifen-driven deletion of *MAPT* using CAMK2a -Cre expression. This novel mouse line offers a temporal and brain-area specificity in knocking-out *Tau* gene avoiding the developmental compensation mechanisms that are suggested to mask the actual role of Tau in the adult brain.

In **Chapter 5**, using the above conditional Tau-KO, we demonstrate that Tau deletion in the adult brain leads to anxious and depressive behavior correlated with dendritic atrophy and synaptic deficits in prefrontal cortex and amygdala. These findings provide solid evidence for the loss/reduction of *MAPT* gene and Tau protein impact on neuronal and brain structure and function.

A general discussion of the thesis is presented in **Chapter 6**. This chapter summarizes and critically discussed the main questions answered by this thesis work and its contribution to increase our knowledge about Tau function, malfunction and pathology. In addition, different drawbacks and limitations of the work, the analytical platforms as well as the models used are also included in this chapter addressing future questions that should be followed in next studies.

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

One of the most adaptive organs in mammalian organism is the brain which exhibits an astonishing variety of responses to internal and environmental stimuli leading to behavioral, molecular and biochemical changes. We now understand that this ability to receive, analyze and cope with new internal or external situations is attributed to its plasticity, e.g. neuronal and synaptic plasticity, which underlies basic functions of the brain such as learning and memory (Sousa and Almeida 2012). Moreover, the observed decline in brain functions during aging or under pathological conditions is causally related with damaged neuronal plasticity. Thus, a better understanding of the mechanisms that regulate neuroplasticity will contribute to our knowledge about brain function and pathology. As it will be discussed in this PhD thesis, accumulating evidence suggests that the cytoskeletal protein Tau and its interaction with several binding partners could serve as an essential parameter of neuronal plasticity as well as brain pathology (Mondragón-Rodríguez et al. 2012; Frandemiche et al. 2014; Liao, Miller, and Teravskis 2014). Thus, we focused on the analysis of the role of Tau protein in brain function, malfunction and pathology.

1.1 TAU PROTEIN AND BRAIN PATHOLOGY – FROM PAST TO PRESENT

Tau protein was discovered in 1975 (Weingarten et al. 1975) and its original name was given by Marc Kirschner as a "unit" that is "associated" with tubulin promoting their self-assembly into microtubules [**T**ubulin **a**ssociated **u**nit; Tau]. Indeed, Tau was one of the first microtubule associated proteins (MAPs) to be characterized and its discovery started a line of research centered around its role as a microtubule stabilizer in neurons, having an important role in cell differentiation and polarization. Along the 70s and 80s, molecular and biochemical characterization of Tau protein, as well as its gene was achieved (Cleveland, Hwo, and Kirschner 1977a; Cleveland, Hwo, and Kirschner 1977b; Drubin and Kirschner 1986; Goedert et al. 1989; Himmler 1989; Trojanowski et al. 1989) followed by an increase knowledge and identification of Tau presence in neurons (mainly in axons) (Binder, Frankfurter, and Rebhun 1985; Trojanowski et al. 1989) (**Figure 1**). In parallel, and despite the essential cellular functions described for Tau, the generation of the first constitutive Tau knock-out (KO) mouse line in the 90s, created an ongoing debate about the real function of Tau protein as Tau-KO mice exhibit no obvious phenotypic, behavioral or neurostructural anomalies (Harada et al. 1994). While this paradox was suggested to be attributed to compensation mechanisms driven by

other MAPs during brain development, the *in vivo* function of Tau in adult brain is not yet completely understood. Thus, the current thesis uses a novel conditional Tau-KO mouse model providing the first evidence about loss of Tau in the adult brain avoiding developmental compensatory mechanisms that may mask the real function of Tau.

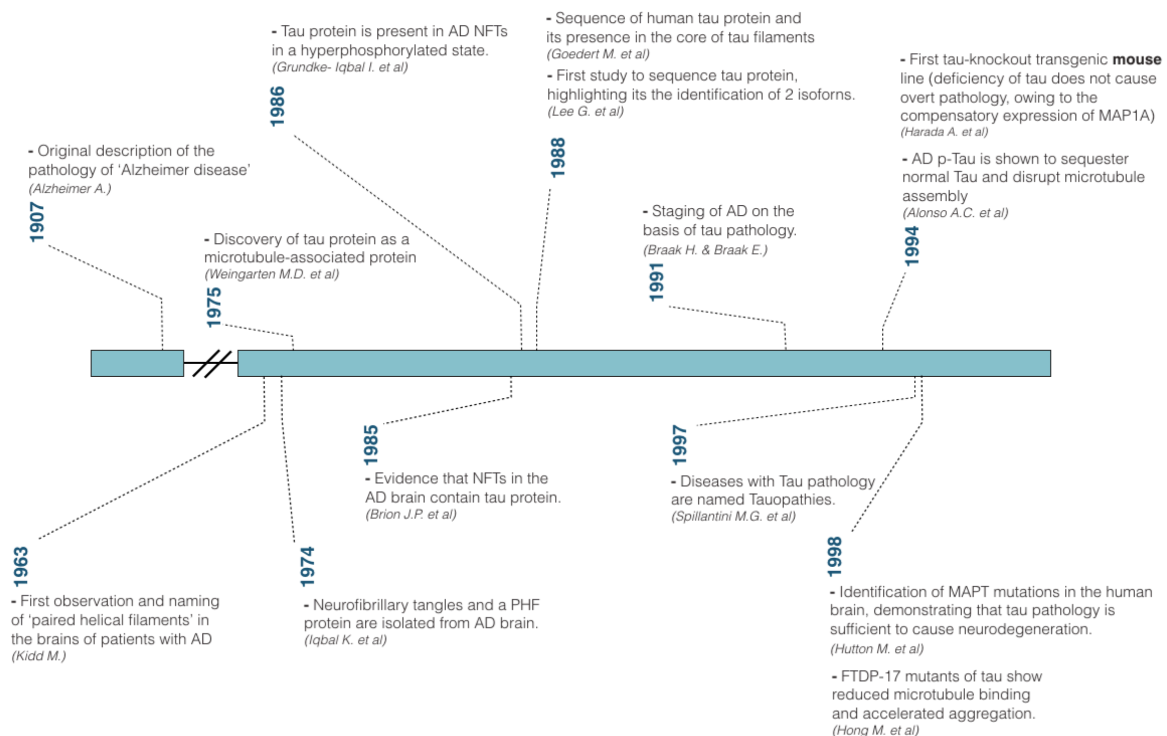

Figure 1. Historical overview of key findings about Tau and its role in Alzheimer's disease (AD) pathology during last century. Since Alois Alzheimer described the AD in the beginning of 1900s, nothing was known about until its discovery in 1975. After that, many research studies provide novel evidence about the Tau biochemical nature and its modifications in AD brain pathology. The creation of the first Tau-KO mouse line was achieved, although these mice didn't exhibit any obvious behavioral or structural phenotype raising uncertainty about the real function of Tau in the brain (drawn by Silva JM and Sotiropoulos I).

Another focus on Tau research was established after the identification of Tau as a major component of abnormal protein deposits in the brain of patients suffering from Alzheimer's disease (AD), a neurodegenerative disorder characterized by brain atrophy and memory loss. Indeed, Tau was the first protein to be identified as the core of neurofibrillary tangles (NFTs), one of the main histopathological hallmarks of AD, (Brion et al. 1985; Grundke-Iqbal et al. 1986); while one year later, amyloid beta (Aβ) was found to be deposited in extracellular amyloid plaques, the other histopathological characteristic of AD brain (Kang et al. 1987). Along the past decades, different pathological Tau modifications (e.g. Tau aberrant hyperphosphorylation, truncation, aggregation) were also identified in AD brain as well as in other neurodegenerative disorders, which today are

called Tauopathies. In addition, a lot of research efforts have been focusing on elucidating the pathological properties of Tau, analyzing the mechanisms that may underlie the neurotoxicity caused by aberrant Tau hyperphosphorylation and accumulation as well as the generation of insoluble Tau aggregates (**Figure 1 & 2**).

Recently, Tau malfunction has been implicated in neuropathologies beyond AD, such as epilepsy and environmental stress; this thesis provides novel data about the implication of Tau in the mechanisms through which chronic exposure to environmental stress or stress hormones precipitate brain pathology. More recently, Tau hyperphosphorylation and neuronal atrophy have been implicated in reversible conditions of brain malfunction such as hypothermia, hypometabolism, hibernation and anesthetics use, raising questions about a potential "threshold" between Tau-related neuroplasticity and neuropathology.

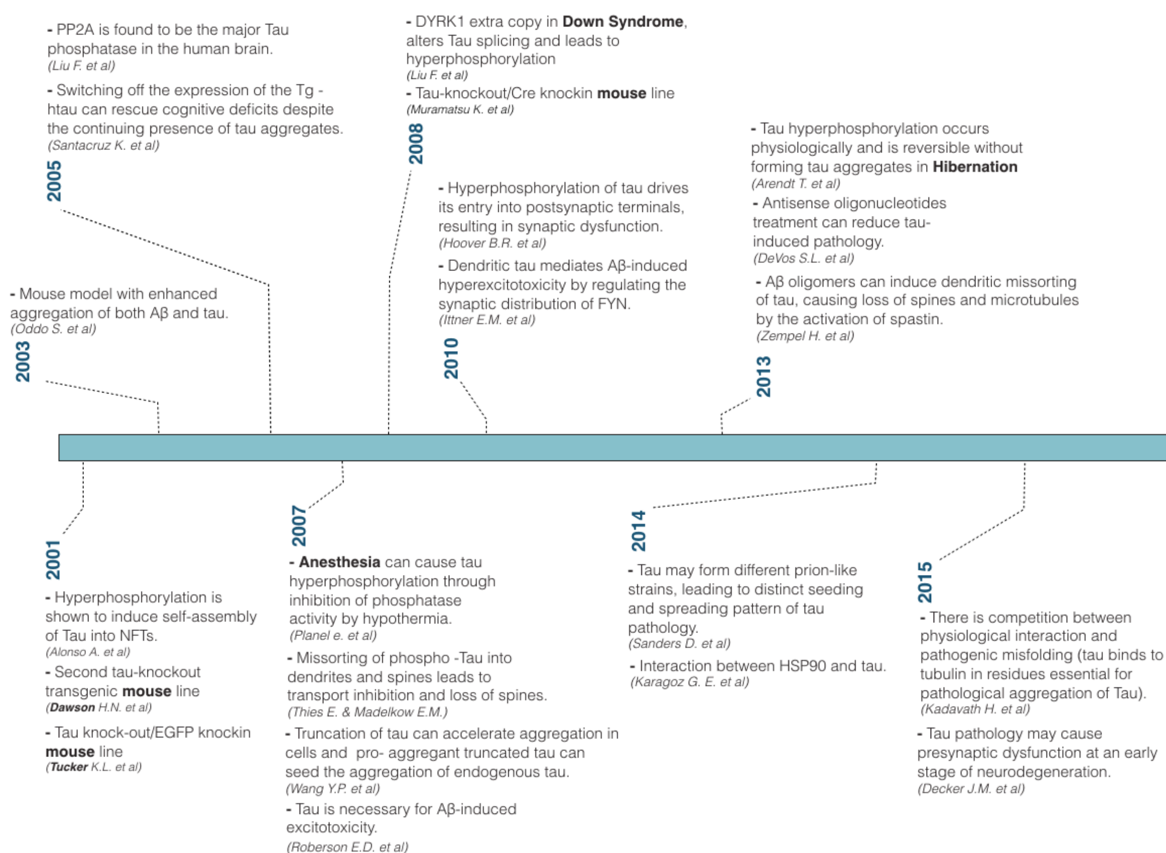

Figure 2. Tau role in and beyond AD during the last 16 years. During the 20th century, considerable progress in understanding AD neurodegeneration has been made providing further evidence about the connection between Aβ neurotoxicity and Tau-driven neuronal mechanisms related to dendritic atrophy and synaptic malfunction. Moreover, Tau and its hyperphosphorylation have recently implicated in brain pathologies beyond AD, such as epilepsy, excitotoxicity and prolong exposure to chronic stress or glucocorticoids as well as reversible conditions of brain/neuronal malfunction e.g. hypothermia, anesthesia (drawn by Silva JM and Sotiropoulos I).

1.2 TAU STRUCTURE, PARTNERS AND NEURONAL FUNCTION

1.2.1 Tau gene

In humans, Tau protein is encoded by the *microtubule-associated protein Tau* gene, *MAPT*, which is in chromosome 17q21 and comprises 16 exons, where exon 1(E1), E4, E5, E7, E9, E11, E12 and E13 are constitutive, and the others are subjected to alternative splicing. E0 and E1 encode for 5' untranslated *MAPT* mRNA sequences, where E0 is part of the promoter, which is transcribed but not translated (Andreadis, Brown, and Kosik 1992; Andreadis 2005). Alternative mRNA splicing of exons E2, E3 and E10, leads to the expression of 6 isoforms of the protein in the adult human brain. These isoforms differ on the number of 29 residue near-amino-terminal inserts, which are encoded by E2 and E3 isoforms, containing 0, 1 or 2 inserts and known as 0N, 1N or 2N, respectively. Isoforms can also be categorized depending on whether they contain 3 or 4 carboxyl-terminal repeats (3R and 4R, respectively), where in the 4R-Tau, but not in 3R-Tau, isoforms, E10 is encoded (Lee, Cowan, and Kirschner 1988) (**Figure 3**).

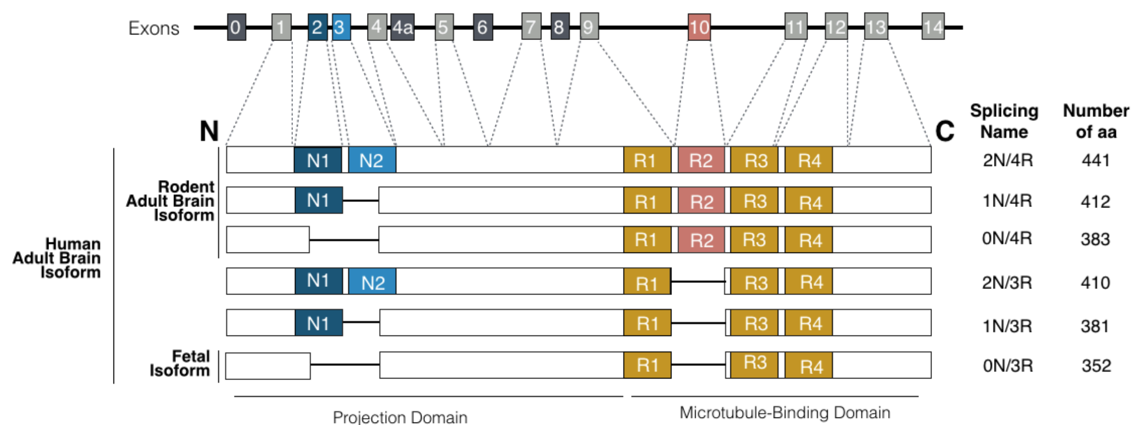

Figure 3. *MAPT* gene and the splice isoforms of Tau. *MAPT* gene contains 16 exons, generating 6 isoforms present in the human brain (4 isoforms in rodent brain). These isoforms differ in the presence of 0,1 or 2 (N0, N1 and N2, respectively) near-amino-terminal inserts, and in the presence of the R2, which leads to the formation of 3R or 4R Tau isoforms. During development, the fetal Tau isoform (0N3R; 352 a.a) is expressed, whereas the adult human brain express all six isoforms with 3R-Tau and 4R-Tau being equally expressed. In rodents, while 3R-Tau expression is high (low 4R-Tau expression) during brain development, the adult brain exhibits a shift towards the expression of 4R (very low or no expression of 3R-Tau). Tau protein includes two major domains: the carboxyl-terminal and the projection domain. (adapted from (Y. Wang and Mandelkow 2015)).

1.2.2 Tau isoforms in health and disease

The expression of the six Tau isoforms is regulated during development (Ballatore, Lee, and Trojanowski 2007). The isoform of Tau expressed in fetal (human or rodent) brain is the smallest one (0N3R; 352 a.a.) which is highly phosphorylated (**Figure 3**). Along brain development, Tau expression pattern changes and fetal Tau expression is reduced. All six Tau isoforms are expressed in adult human brain (Billingsley and Kincaid 1997) but only 4 Tau isoforms are expressed in adult rodent brain (Hanes et al. 2009) (**Figure 3**). Furthermore, in the adult human brain, the levels of 3R and 4R are roughly equal while the 2N isoform is underrepresented compared with the others. In contrast, the adult rodent brain expresses mainly 4R-Tau isoform (Goedert and Jakes 1990; Bullmann et al. 2009; Hanes et al. 2009).

MAPT gene splicing is tightly regulated by several different mechanisms. The E10 splicing is the most studied. RNA-binding protein fused in sarcoma (FUS) may promote splicing of E3 and E10, where FUS knockdown increases the expression of 2N and 4R Tau isoforms (Orozco et al. 2012). Small non-coding RNAs (miRNAs) can also influence Tau splicing; for example, miR-132 reduces 4R expression in mouse neuroblastoma cells (Smith et al. 2011), and miR219 represses Tau synthesis by binding to the 3' untranslated region of Tau messenger RNA (mRNA) (Santa-Maria et al. 2007; Santa-Maria et al. 2015). Another mechanism that could be linked to the regulation of Tau isoform expression is the formation of ribonucleoprotein granules, that results in a shift towards the expression of larger tau isoforms. The expression Tau mRNA-binding proteins (e.g. RAS GTPase-activating protein-binding protein 1 (G3BP1), minor histocompatibility antigen H13 or IMP1), promote the formation of this granules, leading to shift in the expression of larger Tau isoforms, changing *MAPT* expression pattern, towards more active forms and thus, controlling axonal sprouting (Moschner et al. 2014). Furthermore, Tau can bind itself to RNA, maintaining RNA integrity while this interaction may also induce Tau aggregation, contributing to neurodegeneration (Kampers et al. 1996; Violet et al. 2014).

Regarding regional expression in the brain, Tau expression shows considerable regional variation. The mRNA and protein levels in the neocortex are ~2fold higher than those in the white matter and cerebellum (Trabzuni et al. 2012). The splicing of *MAPT* gene also presents regional differences; for example, fetal (0N3R) Tau is lower in the cerebellum than in other regions (Boutajangout et al. 2004; Trabzuni et al. 2012). These variations may contribute to the different vulnerability of the different brain regions to Tau pathology while specific disturbances of the 4R/3R

ratio (1:1 in healthy brain) are associated with distinct Tauopathies (Dickson et al. 2011) (see section 1.1.2).

1.2.3 Protein domains and structure of Tau

Tau is a highly water-soluble protein, due to its unusual hydrophilic nature, and does not adopt the folded structure that is typical of most cytosolic proteins. It is a "natively unfolded" and "disorganized" protein, with a highly flexible and mobile polypeptidic chain, and a range of different negative and positive residues over its structure, creating an asymmetry of charges, that is important for: i) interactions with MT and other partners (**Table 1** & **Figure 4**), ii) internal folding and iii) aggregation under pathological conditions. Tau protein can be divided into two major domains, based on its interaction with MTs: the C-terminal assembly domain and the N-terminal projection domain (**Figure 4**).

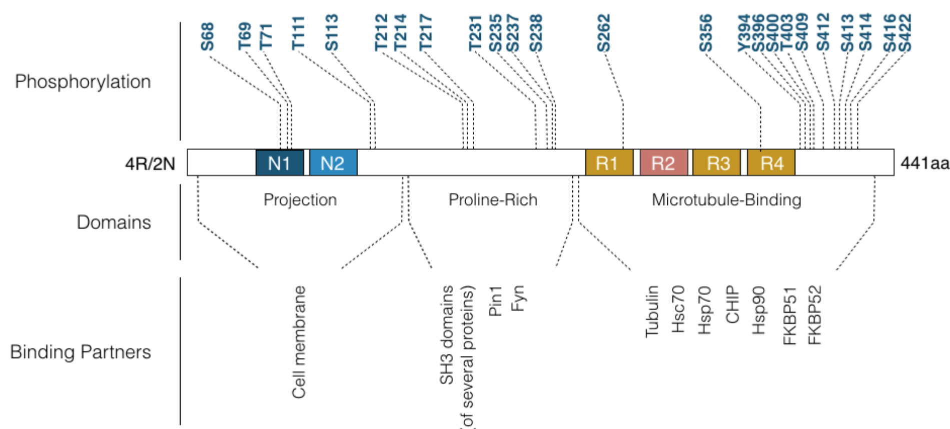

Figure 4. Domains, phosphorylation sites and binding partners of Tau protein. Tau protein includes two major domains: the carboxyl-terminal section that includes the repeat domain and the flanking regions (responsible for MT binding and aggregation); and the projection domain, the amino-terminal section that projects away from microtubules. In the middle region of the protein, there is a Proline-rich domain that contains multiple Thr-Pro and Ser-Pro residues. Tau protein is phosphorylated in several epitopes along its structure, and these post-translational modifications are crucial to regulate Tau protein interaction with its binding partners. (drawn by Silva JM)

Despite its unfolded character, Tau protein shows a preference for changing its global conformation to form "paperclip"-like shape, in which the C-terminal, N-terminal and repeat domains all approach each other (Jeganathan et al. 2006). The formation of this structure might protect Tau from aggregation, has the truncation of Tau prevents the formation of this structure and promotes Tau aggregation. In between these two major domains, there is a Proline rich region, which serves

as a binding site for signaling proteins, such as Fyn (Lee et al. 1998; Lee 2005; Ittner et al. 2010). In the repeat domains, there is a conserved consensus motif KXGS (Drewes et al. 1995; Ozer and Halpain 2000), which can be phosphorylated at serine; this phosphorylation is shown to decrease Tau binding to MT and consequently to lead to cytoskeleton destabilization.

Table 1. Tau binding partners. Tau protein has several binding partners, from signaling molecules, cytoskeleton elements and lipids, supporting its multifunctional role as a protein. Tau can regulate signaling pathways, activate and inhibit enzymes, and interacts with chaperones and co-chaperones.

Tau Binding Partner	Interaction function	References
Beta Tubulin	Cytoskeleton	(Kar et al. 2003)
cSrc	Src-family kinase; facilitates cSrc-mediated actin rearrangements	(Lee et al. 1998; Sharma et al. 2007; Reynolds et al. 2008)
F-actin	Cytoskeleton; Tau connects microtubules and actin filament network	(Fulga et al. 2007)
Fyn	Src-family kinase; traffic of Fan into postsynaptic sites in dendrites	(Lee et al. 1998; Reynolds et al. 2008; Ittner et al. 2010)
Growth Factor Receptor-bound protein 2 (Grb2)	Adaptor protein for growth factor signaling; mediates interaction of Tau with growth factor receptors, facilitating their signaling	(Rouzier et al. 2005; Reynolds et al. 2008; Souter and Lee 2009)
HDAC6	Enzyme inhibitor, regulating microtubule stability	Flanagan et al., 1997
Hsp90	Regulating Tau ubiquitylation and degradation	(Dickey et al. 2007; Tortosa et al. 2009)
CHIP	Co-regulates, with Hsp90, abnormal Tau ubiquitylation and degradation	(Dickey et al. 2008)
FKBP51/FKBP52	Regulation of Tau protein turnover, inhibiting or enhancing Tau interaction with Hsp90/CHIP complex	(Tortosa et al. 2009; Chambraud et al. 2010)
Hsp70	Stabilization of binding of Tau with microtubules; promoting Tau degradation with CHIP	(Petrucelli et al. 2004; O'Leary, et al. 2010; Jinwal, Koren, et al. 2010)
Hsc70	Promotes Tau-mediated microtubule stabilization; Facilitates MC1 Tau conformation as a protective mechanism	(Koren, et al. 2010)
Pin1	Its binding to Tau stimulates dephosphorylation of cdk5 phosphorylation sites; modulates Tau phosphorylation in response to A(beta)	(Kimura et al. 2013; Hamdane et al. 2006; Ma et al. 2012)
TIA-1	The interaction regulates stress granule formation as well as misfolding and aggregation of Tau	(Vanderweyde et al. 2016)

1.2.4 Posttranslational modifications in Tau function and malfunction

Tau is subjected to a complex array of posttranslational modifications, with Tau phosphorylation being one of the major modifications studied. Like its splicing, Tau phosphorylation is also developmentally regulated, as fetal Tau is highly phosphorylated in comparison to adult Tau (Kanemaru et al. 1992; Köpke et al. 1993; Yu et al. 2009). There are numerous phosphorylation epitopes in the longest Tau isoform (2N4R). All these phosphorylation sites cluster in the flanking regions while among them, there are epitopes/motifs that are abnormally hyperphosphorylated in AD and other Tauopathies. Phosphorylation in the microtubule-binding domain (residues 244-368) of Tau is believed to be crucial in regulating MT stabilization, as phosphorylation in the microtubule-binding repeat region (e.g. S262 and S356) leads to Tau detachment from the MT (Buée et al. 2000). Phosphorylation outside this area, like in the Proline-rich domain, is also involved in regulation of cytoskeletal stability, since S214 and T231 also results in reduced Tau ability to bind to MT (Cheng et al. 2008) (**Figure 4**).

Furthermore, different kinases are responsible for phosphorylation of distinct epitopes of Tau protein. These kinases are divided into 2 groups: i) the non-proline kinases such as microtubule affinity-regulating kinases (MARKs), cyclic AMP-dependent protein kinase (PKA) and Ca^{2+} or calmodium dependent protein kinase II (CAMKII) (Hanger, Anderton, and Noble 2009); and ii) the proline-directed kinases, such as glycogen synthetase 3β (GSK3β), cyclin-dependent kinase 5 (cdk5) and the mitogen-activated protein kinase (MAPK), which have received special attention as they are highly expressed in the brain and are causally associated with aberrant Tau hyperphosphorylation and overall Tau pathology in AD brains (Pei et al. 1999; Patrick et al. 1999). Indeed, the contribution of Tau hyperphosphorylation to its aggregation is controversial, since some studies suggest that Tau hyperphosphorylation contributes to Tau aggregation process (Augustinack et al. 2002; Noble et al. 2003) while others also demonstrated that Tau aggregation does not depend on Tau phosphorylation levels (Schneider et al. 1999; Wang et al. 2007). Besides phosphorylation in serine/threonine-proline reach motifs, Tau is also phosphorylated by tyrosine kinases, such as the SRC family members (LCK, SYK and FYN) at Tyr18, and the ABL family members (ARG and ABL1) at Tyr394 (**Figure 4**). These phosphorylations of Tau are also present in the Paired Helical Filaments (PHFs) in AD brain. In addition, Tau phosphorylation negatively affects the binding of the protein to FYN, although there is controversial evidence about it (Bhaskar, Yen, and Lee 2005; Reynolds et al. 2008).

As Tau function and intracellular localization are dependent on a tight regulation of its phosphorylation levels, phosphatases also play an important role. Protein phosphatase 1 (PP1), PP2A, PP2B, PP2c and PP5 have all been implicated in dephosphorylation of Tau (Gong 2000). Among them, PP2A seems to have an essential role as it accounts for 70% of the total human brain Tau phosphatase activity, and its activity has been shown to be reduced in AD brains (Gong et al. 1995; Gong 2000), providing an extra explanation for aberrant Tau hyperphosphorylation in AD brain. Furthermore, as phosphatases are more sensitive to intracellular biochemical changes than kinases, it may explain why tau hyperphosphorylation occurs during animal hibernation and anesthesia-induced hypothermia (Planel et al. 2007; Härtig et al. 2007; Xiao et al. 2013).

Despite research in Tau have mainly been focusing on phosphorylation over the last decades, recent studies monitor the importance of other Tau posttranslational modifications such as glycosylation, isomerization, glycation, nitration and methylation. For example, *N*-Glycosylation[1] is only present in AD brains, and it's suggested to maintain and stabilize PHFs formation as well as facilitate Tau phosphorylation, through some changes in Tau conformation (Wang, Grundke-Iqbal, and Iqbal 1996; Liu et al. 2002a; Liu et al. 2002b). On the other hand, O-GlcNaAcetylation[2] is known to protect Tau from phosphorylation and suppress Tau aggregation. Deamination[3] is detected in PHF-tau but not in normal Tau, indicating that this modification may facilitate Tau aggregation (Ledesma, Bonay, and Avila 1995; Watanabe et al. 2004). The same is observed in glycation[4], which reduces Tau binding to microtubules. Furthermore, nitration[5] is present in normal Tau (Tyr197) and may contribute to important physiological functions, but nitration in Tyr18, Tyr29 and Tyr394 is detected only in AD or other Tauopathies. Depending on the nitration sites, they can promote or inhibit aggregation (Reyes et al. 2008). Methylation[6] of lysine residues in Tau occurs in normal human brains and its thought to suppress aggregation (McCaddon and Hudson 2007; Funk et al. 2014).

Ubiquitylation is another posttranslational modification found in Tau protein as part of its degradation process. Indeed, Tau ubiquitylation by the molecular co-chaperone, CHIP (carboxyl

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[1] *N*-Glycosylation – glycosylation is an enzymatic process that attaches glycans to a nitrogen of asparagine or arginine side-chains from a protein, lipid or another organic molecule. It requires participation of a special lipid called dolichol phosphate.Does not occur in the cytoplasm or nucleus, rather in cytoplasmic organelles.

[2] O-GlcNaAcetylation - *O*-linked glycans attached to the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine, or hydroxyproline side-chains, or to oxygens on lipids such as ceramide. It occurs in the cytoplasm and nucleus and has a faster rate, as it can be added or removed multiple times along the life of a polypeptide, more similar to phosphorylation. It is one of the most abundant post-translational modification within the nucleocytoplasmic compartment of all metazoans.

[3] Deamination – removal of an amine group from a molecule.

[4] Glycation – is the result of a covalent bonding of a sugar molecule, like glucose or fructose, to a protein or lipid, without the action of an enzyme. It is known to impair functioning of biomolecules.

[5] Nitration – introduction of a nitro group into an organic molecule. Reactive oxygen species mediate tyrosine nitration, which is an indicator of cell damage.

[6] Methylation – addition of a methyl group on a substrate by enzymes, and can be involved in the regulation of gene expression, protein expression and RNA processing. Tau protein can be methylated in Lysine residues.

terminus of the Hsp70- interacting protein), targets the protein to proteasome degradation (Shimura et al. 2004; Petrucelli et al. 2004). As ubiquitin is a stress protein implicated in ATP-dependent degradation of short-lived proteins or removal of abnormal or damaged proteins, it has an important function in maintaining a balance between normal Tau and degrade abnormal Tau. On the other hand, deubiquitinating enzymes also play and important role in this process, attenuating the output of Ub signaling (Hutter et al. 2008). This process is not only important for proteasome degradation, but also for autophagy degradation, both mechanisms implicated in AD pathology and Tau impaired degradation.

1.2.5 Intracellular sorting of Tau

Tau is mainly located in the human brain, specifically in neurons. It is ubiquitous in immature neurons, where evenly distributes in cell body and neurites, but becomes axonal during neuronal maturation along the emergence of neuronal polarization. This intracellular sorting of Tau is accompanied by a shift towards high-molecular-weight isoforms (4R-Tau isoforms) and reduced phosphorylation (Drubin, Caput, and Kirschner 1984; Drubin and Kirschner 1986; Papasozomenos and Binder 1987; Sultan et al. 2011). After maturation, low levels of Tau can be found in the nucleus and dendrites as well as in other brain cells, such as oligodendrocytes (**Figure 5**).

The sorting pathway that the neuronal cells use is incompletely understood with evidence supporting it could occur at both mRNA and protein level. One of the suggested mechanisms of Tau sorting is based on the selective Tau transport in axons or selective degradation in dendrites (Hirokawa et al. 1996), while an alternative notion suggests that Tau as higher affinity in axons than that to dendrites (Hirokawa et al. 1996), this could also explain the increased presence of Tau in axonal compartment. More recently, Li and colleagues support the hypothesis that the axon initial segment (AIS) operates as a barrier against retrograde diffusion of Tau into the dendrites, and that Tau phosphorylation and its interaction with microtubules is essential for this barrier to be maintained (Li et al. 2011). Interestingly, the sorting of Tau seems to be isoform dependent, since different isoforms appear in different compartments (Liu and Götz 2013). Axonal sorting of Tau seems to be critical for neuronal function as missorting of Tau into the somatodendritic compartment is recognized as one of the earliest signs of neurodegeneration in AD (Cuchillo-Ibanez et al. 2008; Hoover et al. 2010).

Given the differential distribution of Tau and its isoforms in different cell compartments, it is

possible that Tau serves different function in different subcellular compartments, and disturbances in this sorting could trigger neuronal malfunction and neurodegeneration.

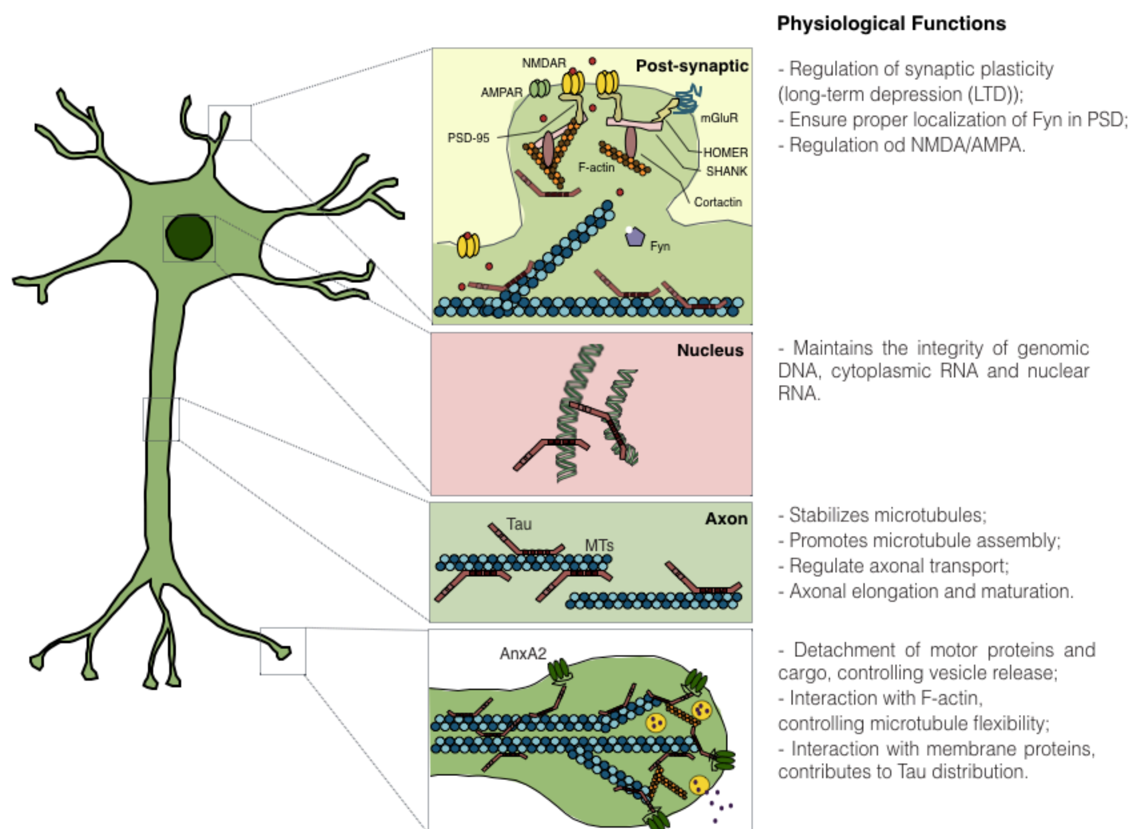

Figure 5. Physiological functions of Tau. The several functions of Tau so far described in healthy neurons are depend on its localization in the neuron. Tau mainly localizes in the axon, where it plays an important role in stabilizing microtubules, promoting their polymerization and playing a role in axonal transport. In the nucleus, a small amount of Tau is also detected, where Tau might play a role in DNA integrity maintenance. Only a small amount of Tau can be detected in dendrites, however its function is still debatable; Tau has been shown to be involved in synaptic plasticity. In the axon terminals, Tau interacts with membrane proteins, F-actin and motor proteins, controlling microtubule flexibility and vesicle release (Drawn by Silva JM and Sotiropoulos I).

1.3 THE MULTIPLE ROLES OF TAU IN NEURONS: BEYOND THE CLASSICAL VIEW.

The initial identification of Tau protein was related to its ability to modulate the stability and assembly of microtubules (MTs) (Weingarten et al. 1975; Cleveland, Hwo, and Kirschner 1977a), promoting MT nucleation, growth and bundling (Brandt and Lee 1993; Drewes et al. 1995), as well as reduce microtubule dynamic and instability (Drubin and Kirschner 1986; Drechsel et al. 1992). But, more recently, several novel functions of Tau have been revealed.

1.3.1 Axonal Tau – microtubules and cargo transport

As Tau interacts with microtubules in a dynamic way, it regulates the dynamic reorganization of the cytoskeleton promoting neurite outgrowth and stabilization (Feinstein and Wilson 2005; Mandelkow and Mandelkow 2011). Direct evidence came from the analysis of cultured neurons in which the expression of Tau protein was suppressed by treatment with antisense oligonucleotides (ASOs). It was observed that distribution of Tau is correlated with the morphological development of the axon, and when treated with ASOs, neurons failed to extend axon-like processes whereas they can extend minor processes (Ferreira, Busciglio, and Cáceres 1989; Caceres and Kosik 1990). On the other hand, acute inactivation of Tau by antibody microinjection had no effect on axonal elongation or microtubule dynamic (Tint et al. 1998). However, *in vivo* studies using animals that lack Tau (Tau-KO) have shown that absence of Tau doesn't affect axonal elongation (Harada et al. 1994), while others demonstrated that Tau-KO animals exhibited significant delay in axonal and dendritic extension (Dawson et al. 2001). The above discrepancy and gap between *in vitro* and *in vivo* data about the role of Tau was bridged by the suggestion of development compensative interaction among MAPS in Tau-KO animals which may hide the real function of Tau during brain development; in fact, there is an increased expression of MAP1A in the first Tau-KO animal model (Harada et al. 1994). Further support of the interdependency and synergic action of different MAPs is based on other studies demonstrating the importance of both MAP1B and Tau during transition from minor process stage to the axonal stage (Takei et al. 2000; González-Billault et al. 2002; Tortosa et al. 2013). However, a conditional deletion of Tau will provide a clear answer about the real function of Tau.

Furthermore, the axonal presence of Tau is also different between distal and proximal end of the axon as Tau is most associated with microtubules at the distal end of the axon, close to the growth cone (Black et al. 1996; Hinrichs et al. 2012). Indeed, Tau phosphorylation is suggested to be involved in this intra-axonal sorting of Tau since the phosphorylation of Tau was also found to differ along the length of the growing axon. A phosphorylation gradient is evident, with a gradual change from phosphorylated to dephosphorylated Tau as we move from soma towards the growth cone (Mandell and Banker 1996). As the microtubules are more dynamic in the distal regions of growing axons, and dephosphorylation of Tau increases its affinity with microtubules, these data strongly suggest that Tau in the growing axon has functions other than increasing microtubule stability. Furthermore, Tau projection domain also exhibits other functions through its interaction with membrane complexes and cytoplasmatic components. It is proposed that Tau interaction with

annexin A2, through the non-microtubule domain (Gauthier-Kemper et al. 2011), contributing for the axon specific distribution of Tau, and this interaction is modulated by phosphorylation (Maas, Eidenmüller, and Brandt 2000), as mutated Tau leads to an abnormal interaction, and possibly lead to the redistribution of Tau away from the axons to the somatodendritic compartment (Gauthier-Kemper et al. 2011).

Tau also plays an important role in influencing the motor function of dynein and kinesin, which transport cargoes towards the minus ends (towards the cell body) and plus ends of microtubules (towards axonal terminus), respectively (Stamer et al. 2002). Different Tau-dependent mechanisms have been described/suggested for the regulation of cargo transport: i) Tau seems to compete with the motor proteins for the binding to MTs, reducing the binding frequency, and thereby slowing down both anterograde and retrograde transport (Stamer et al. 2002; Dixit et al. 2008); the inhibitory effects on kinesin and dynein leads to the accumulation of cargoes (e.g. mitochondria), in the soma of cells overexpressing Tau (Vershinin et al. 2007); ii) Tau reduces the number of motor proteins engaged with cargoes; iii) Tau as a cargo competes with other cargoes for available kinesin, inhibiting axonal transport of other cargoes (Utton et al. 2005; Konzack et al. 2007); and iv) Tau may regulate the release of cargo vesicle from kinesin chains by activation of PP1 and GSK3β (Kanaan et al. 2011). Again, the deletion of Tau in mice has little or no influence on axonal transport (Yuan et al. 2008), implying that there is still a lot to understand about the mechanisms that can counteract the impact of Tau in axonal transport. More recently, it was proposed by Brandt and colleagues that Tau interaction with microtubules is fast and dynamic, in a kiss-and-hop mechanism (Janning et al. 2014; Igaev et al. 2015). The dwell time of interaction between Tau and MTs is of 40ms, much shorter than previously described (Konzack et al. 2007), but still capable of regulating MT dynamics. However, this dwell is not enough to affect kinesin transport along the axon and affect axonal transport. Moreover, mutation in the pseudorepeat-regions or disease-like Tau hyperphosphorylation alter the dynamic between Tau and MTs (Niewidok et al. 2016). These findings shed new light about the role of Tau interactions with MTs and their significance in neuronal function.

1.3.2 Dendritic and synaptic function of Tau

In contrast to axons, a small amount of Tau is present in dendrites and dendritic spines under normal/physiological conditions, but its function has not been well characterized (Mondragón-Rodríguez et al. 2012; Tai et al. 2012). It is suggested that Tau may regulate synaptic plasticity, as

pharmacological synaptic activation induces translocation of endogenous Tau from dendritic shaft to excitatory postsynaptic compartments in cultures mouse neurons and in acute hippocampal slices (Frandemiche et al. 2014). Through its interaction with several cellular partners such as tubulin, F-actin, and Src family kinases (**Figure 4**, **Table 1**), Tau may play an important role in mediating alterations in the cytoskeletal structure of dendrites and spines (Morris et al. 2011). In fact, previous work from our group have shown that Tau-KO mice present alteration in long-term depression (LTD) mechanisms (Kimura et al. 2014) while the impact of Tau loss on long-term potentiation (LTP) is either little or inexistent (Lopes et al. 2016). Nevertheless, the above findings indicate that Tau has a role in regulating synaptic plasticity in the hippocampus.

Localization of Tau at the synapse has been the focus of several recent reports aiming to determine whether (and why) Tau is located at presynaptic, postsynaptic, or both compartments (Tai et al. 2012). We now know that Tau interacts directly with filamentous F actin (Fulga et al. 2007), which are localized both in presynaptic boutons and in the head and neck of dendritic spines (Dillon and Goda 2005). Furthermore, using synaptoneurossomes derived from healthy and AD brains, recent studies demonstrated that Tau is present in both pre and postsynaptic compartments (Tai et al. 2012), although phospho-Tau (detected by PHF1 antibody) was found in greater amounts in the postsynaptic sites. Furthermore, using a mouse model of Tau pathology expressing mutated human Tau (P301L-Tau), PHF-Tau is found in both pre and postsynaptic compartments suggesting that Tau distribution changes in the disease context (Harris et al. 2012).

There are several potential mechanisms by which Tau could affect synaptic function and neuronal excitability. Tau may directly influence synaptic function since, as described above, it has been shown to be localized within both pre- and post-synaptic compartments, possible due to its interaction with important synaptic proteins. Further analysis has shown that Tau is also phosphorylated through the action of NMDA receptor activation (Mondragón-Rodríguez et al. 2012). Together with phosphorylated Tau, there is also non-phosphorylated Tau in this compartments, suggesting that Tau is likely to oscillate between phosphorylated and non-phosphorylated states in the synapses (Mondragón-Rodríguez et al. 2012). This data strongly suggests that in dendritic compartments, phosphorylation of Tau meets its primarily physiological function in synapses. This dendritic localization is much more studied in the context of pathology, where phosphorylated Tau protein is missorted into dendrites and dendritic spines, causing synaptic dysfunction, by suppressing AMPA receptors-mediated synaptic response, through a disruption of postsynaptic targeting and anchoring of glutamate receptors (Hoover et al. 2010).

Furthermore, it was shown that Tau associates with PSD complex (Kornau et al. 1995), and has a role in targeting Fyn kinase to postsynaptic compartments and its involved in coupling NMDARs to PSD95 (Lee et al. 1998; Reynolds et al. 2008; Ittner et al. 2010). The interaction of Tau with Fyn appears to be essential for: i) targeting Fyn to the PSD, where it regulates NMDA receptor (GluN2b) function through phosphorylation (Trepanier, Jackson, and MacDonald 2012), and ii) for interaction of Fyn with membrane associate proteins of the plasma membrane (Usardi et al. 2011; Pooler et al. 2012). This interaction of Tau with Fyn is regulated by phosphorylation of Tau, and therefore can be disrupted in disease, when Tau phosphorylation pattern is altered (Bhaskar, Yen, and Lee 2005; Reynolds et al. 2008; Usardi et al. 2011). Consequently, Tau may play an important role in regulating synaptic function by acting as a postsynaptic scaffolding protein and/or through regulating the presence/absence of neurotransmitter receptors to the synapse.

1.4 TAU PATHOLOGY IN BRAIN DISORDERS

1.4.1 Alzheimer's disease pathology, the first described Tauopathy

In 1906, Dr Alois Alzheimer, a German physician 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, aphasia and agraphia, accompanied by disorientation, auditory hallucinations, paranoia, profound agitation and marked psychosocial impairment (Graeber and Mehraein 1999). Auguste D died four years after the first symptoms appeared, while Dr Alzheimer presented her clinical case in the scientific community; later, the disorder was named after his name by the famous psychiatrist Kraepelin. Today, AD is considered a progressive age-related neurodegenerative 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. Among AD patients, 5-7% of them develop an early onset of the disease (after 40 years old) named as familial AD, usually due to one or more mutations in genes related to the disease; while more than 95% of AD cases (sporadic AD) are affected later in life (>65 years old), without clarified genetic mutations. Currently, more than 46.8 million people worldwide suffer from dementia, while this number is expected to rise to 131.5 million by 2050 due to the increased longevity, raising dementia and AD one of the major health problems for modern societies (Prince et al. 2016). Thus, the importance of an early diagnosis and treatment are of extreme relevance for public health (Wehling and Groth 2011).

AD pathology is characterized by two principal hallmarks: deposits of amyloid β peptide (Aβ), a cleavage product of the transmembrane protein called Amyloid Precursor Protein (APP) (**Figure 6b**); and intracellular neurofibrillary tangles (NFT's), formed by the aggregates of abnormally hyperphosphorylated Tau protein (Alonso, Grundke-Iqbal, and Iqbal 1996; Gendron and Petrucelli 2009) (**Figure 6c**). Furthermore, these alterations lead to neuronal atrophy and loss of synaptic connections that is followed by neuronal loss leading to severe whole brain atrophy at the later stages of the disease (**Figure 6a**).

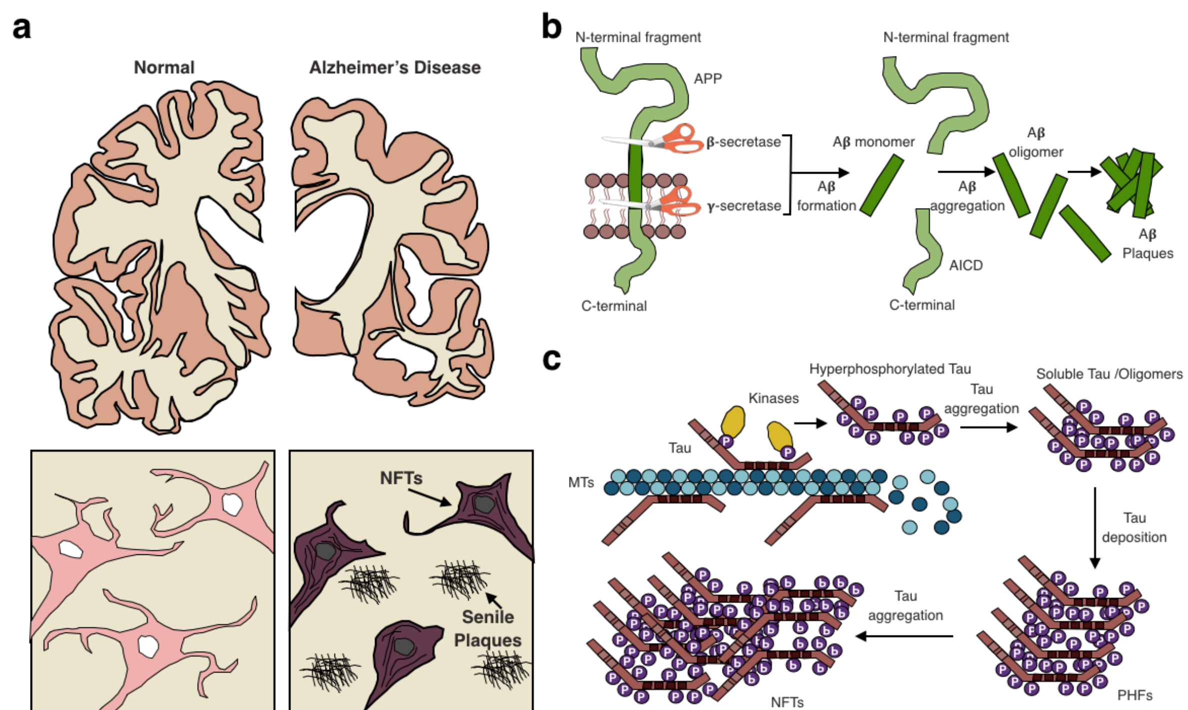

Figure 6. Alzheimer's Disease pathology. **(a)** AD brain is characterized by severe atrophy of several areas, and in terms of brain histopathology, the extracellular senile plaques and intracellular aggregates of neurofibrillary tangles (NFTs) are unique for AD brain. **(b)** Senile plaques are deposits of Aβ, a peptide that is formed as a cleavage product of APP processing by β-secretase and γ-secretase **(c)** NFTS are aggregates of hyperphosphorylated forms of Tau protein which is detached from microtubules and aggregates giving rise to Tau fibrils and insoluble aggregates forming tangles (drawn by Silva JM).

Senile plaques and APP misprocessing

Amyloid plaques consist of a central core of extracellular aggregates of amyloid β-peptide (Aβ), a 39-43 amino acid peptide, arranged as β-sheet filaments surrounded by dystrophic axons and dendrites. Aβ is a cleavage product of amyloid precursor protein (APP) (Huang and Jiang 2009) (**Figure 6b**). APP is a trans-membrane protein ubiquitously expressed, 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; Choy, Cheng, and Schekman 2012). The protein undergoes post-translational proteolytic cleavage via two different pathways: the non-amyloidogenic pathway, where APP is sequentially cleavage by α- secretase and γ-secretase; and the amyloidogenic pathway, where APP is cleaved by BACE- 1 (β-secretase) and γ-secretase resulting in the production of Aβ (Golde, Petrucelli, and Lewis 2010).

Both clinical and animal studies suggest a key role of the amyloidogenic pathway in AD pathology, based on the predominant hypothesis that 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 Tau (Takashima et al. 1998; Small and Duff 2008; Liao, Miller, and Teravskis 2014;). Indeed, some studies demonstrated the neurotoxic actions of Aβ are mediated by Tau protein (Rapoport et al. 2002; Roberson et al. 2007; Shipton et al. 2011).

Neurofibrillary Tangles

Neurofibrillary tangles (NFT's) are the other main histopathological hallmark of AD. NFT's are made of highly insoluble paired helical filaments (PHF) that appear as left-handed double helices and straight filaments (SF), consisting of abnormally hyperphosphorylated Tau. 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 and Braak 1991; Nagy et al. 1999) (**Figure 7**).

In AD brain, Tau is abnormally hyperphosphorylated in many sites (some of them within the repeat region) which impairs its MT-binding leading to the detachment of Tau for MTs (Gendron and Petrucelli 2009; Deshpande, Win, and Busciglio 2008). In addition, hyperphosphorylation is suggested to alter conformation of Tau protein which leads to Tau aggregation into oligomers and then, into insoluble PHFs and subsequently to NFTs (**Figure 6c**). In the "pre-tangle state", the hyperphosphorylated Tau is soluble. Then, it gradually aggregates into insoluble fibrillary inclusions in the dendrites and cell body. These aggregates are resistant to proteasomal or lysosomal degradation resulting in high accumulation of Tau aggregates, and consequent NFTs formation

(Heiko Braak and Del Tredici 2011). Curiously, NFTs bearing neurons appear to survive for decades (Morsch, Simon, and Coleman 1999) and MTs 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 Thr231, Ser262 and Ser396/404 have neurotoxic functions by interfering with MT stability and assembly leading to defective dendritic plasticity and axonal transport (Cuchillo-Ibanez et al. 2008; Rodríguez-Martín et al. 2013). Furthermore, using an inducible model of Tau pathology expressing human P301L-Tau, Santa-Cruz and colleagues demonstrated that neuronal atrophy and cognitive decline was reversed by switching-off the production of pathogenic P301L-Tau in the brain of this mouse model even though Tau aggregation in NFTs was still increasing after the P301L-Tau gene switching-off. Altogether, the above info supports the notion that Tau hyperphosphorylation and Tau oligomers, but not NFTs, are species that are responsible for the toxic effects of abnormal Tau leading to neurodegeneration

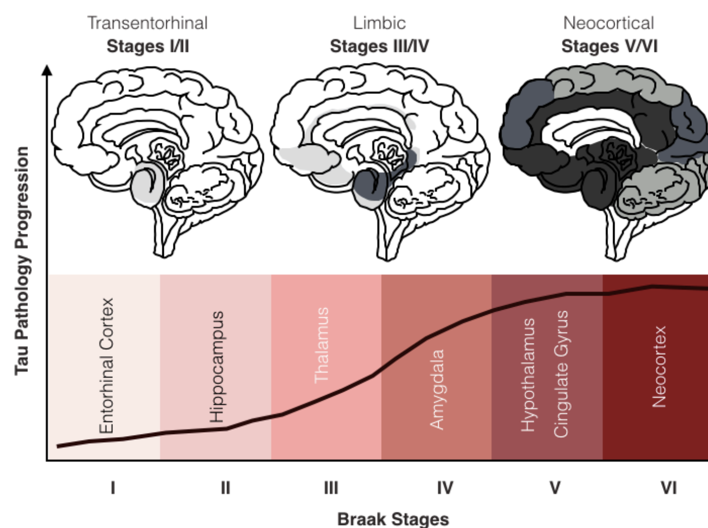

Figure 7. Temporal and spatial development of Tau pathology during AD progression. According to Braak and Braak (1991), affected brain areas are classified into six stages. In the first stage the pyramidal neurons of the entorhinal cortex are affected, and mild changes are observed in the CA1 regions of the hippocampus. During stage III/IV, the pathology spreads to the large cortical projection neurons in the limbic system and association cortices. While sensory areas are relatively spared in stage V, subcortical nuclei shows pronounced changes, and hippocampus becomes highly affected during the stage V and VI, known as neocortical stages. In stage VI, primary sensory areas become markedly affected (drawn by Silva JM).

1.4.2 Tau Pathology – Beyond Alzheimer's Disease

Besides AD, intracellular aggregates of hyperphosphorylated Tau protein are also found in other neurodegenerative disorders, in the absence of amyloid deposits. These disorders are clinically characterized by dementia and/or motor syndromes and are termed "Tauopathies" (Hernández and Avila 2007). This heterogeneous group of disorders include frontal temporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease (PiD), progressive supranuclearplasy (PSP) and corticobasal degeneration (CBD) etc. (see **Table 2**). Tauopathies exhibit distinct clinical features as well as distinct, but overlapping, distributions of Tau pathology within the human brain. Despite the diverse phenotype and clinical presentation, the progressive accumulation of NFTs is a common marker to all Tauopathies (Brandt, Hundelt, and Shahani 2005; Serrano-Pozo et al. 2011). However, all of them present distinct patterns of Tau phosphorylation and isoforms in both soluble and insoluble Tau in the brain (**Figure 8**).

Progressive Supranuclear Palsy (PSP), Pick's Disease (PiD) and Corticbasal Degeneration (CBD) are Tauopathies belonging to a group of diseases known as frontotemporal dementia (FTD) and part of the sporadic group of Tauopathies. PSP is clinically characterized by supranuclear gaze palsy as well as postural instability, driven by an atrophy of the basal ganglia, subthalamus and brainstem, and corresponding neuronal loss and gliosis, with these regions presenting high density of fibrillary Tau pathology, with neuropil threads (NThs) and NFTs (Steele 1994; Tolosa, Valldeoriola, and Marti 1994; Steele, Richardson, and Olszewski 2014). CBD is a progressive neurodegenerative disorder that affects cerebellar cortex, deep cerebellar nuclei and substancia nigra, presenting neuronal loss with spongiosis, gliosis, and prominent glial and neuronal intracytoplasmic filamentous tau pathology (Iwatsubo, Hasegawa, and Ihara 1994; Mori et al. 1994), presenting NThs throughout gray and white matter. PSP and CBD present a similar biochemical profile in terms of Tau protein (**Figure 8**), and are also both associated with A0 allele of Tau gene and the H1 haplotype, with overlapping clinical (Hauw et al. 1994) and pathological features (Feany, Mattiace, and Dickson 1996), indicating that they may be different phenotypic manifestations of the same underlying disease process. PiD s defined by the presence of Tau-imunoreactive Pick bodies (Feany, Mattiace, and Dickson 1996; Constantinidis, Richard, and Tissot 2008) and characterized by frontotemporal lobar and limbic atrophy associated with neuronal loss, spongiosis and gliosis (Dickson 1998). In terms of WB pattern, it differs from AD, PSP and CBD, because the major Tau bands appear to be 3R-tau exclusively (Sergeant et al. 1997; Mailliot et al. 1998) (**Figure 8**).

There is a group of familial Tauopathies which is a group of syndromes known as FTDP-17. They present a diverse, but overlapping, clinical and neuropathological features (Foster et al. 1997) (**Table 2)**. They all present abundant filamentous Tau pathology in neurons and some in glial cells, and the burden of Tau pathology and degeneration reflect the different syndromes and subserves specific cognitive, executive and motor functions (Murrell et al. 1999; Lippa et al. 2000; Rizzini et al. 2000). As Tau gene in located in chromosome 17q21-22 and several groups have identified mutations in the Tau gene that segregated with FTDP-17 (**Figure 9**) which between them change the WB profile of Tau protein (**Figure 8**).

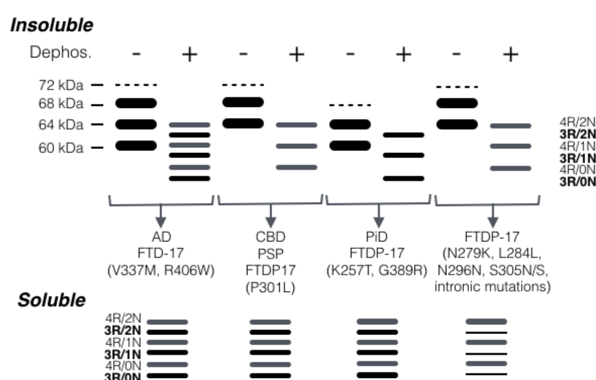

Figure 8. Schematic representation of immunoblotting distribution of soluble and insoluble Tau in different Tauopathies. The figure presents the typical banding pattern of non-dephosphorylated (-) and dephosphorylated (+) insoluble (top) and soluble (bottom) from brains of patients with Tauopathies as indicated (adapted from (Lee, VMY, Goedert, and Trojanowski 2001).

An important finding which changed the overall scientific view about Tau-related neurotoxicity was the discovery of Tau mutations that led to neuronal atrophy and death as well as dementia. To date, more than 80 mutations have been identified so far in the human *MAPT* gene, which are found in the coding region of the protein, or intronic mutations located after exon 10 (Goedert 2005). These mutations are linked to several Tauopathies (but not in AD) (Kara et al. 2012; Kouri et al. 2014) and they can be classified as: i) missense mutations which change Tau sequence and lead to change in alternative splicing, ii) splicing mutations, which alter the relative ratio of the different Tau isoforms, but doesn't result in mutant Tau protein. Many of the missense mutations cluster in the microtubule-binding domain, in both repeats and/or flanking regions (**Figure 9**) (such as P301L, K280, N296K and V337M). These mutations led reduced Tau affinity for MTs and an increase tendency for aggregation (Hong et al. 1998; Barghorn et al. 2000). Some mutations, that are not in the repeat-domain, can also lead to alterations in Tau interaction with microtubules and affect general axonal

transport (Bunker et al. 2006; Magnani et al. 2007). Most splicing mutations are within or near the intron 10, and they usually lead to an increase in the inclusion of E10 and increase the 4R/3R ratio. Nevertheless, there are others that can inhibit the inclusion of E10 and lead to a decrease 4R/3R ratio (K280, L266V and G272V).

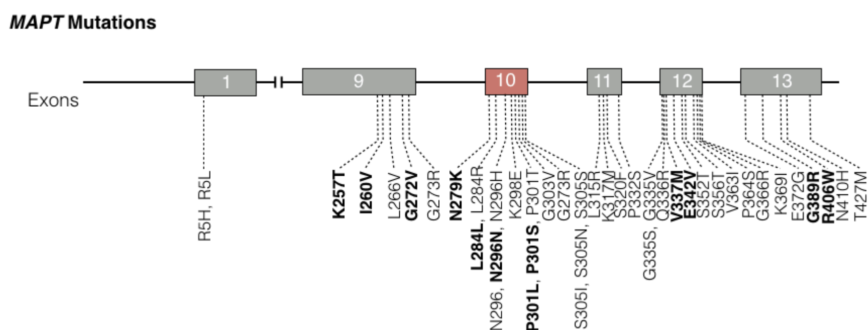

Figure 9. Human *MAPT* gene mutations. Mutations in the *MAPT* gene are mainly located in the exons that transcribe microtubule binding region of Tau protein, and many have been found to cause FTDP-17. Mutations located in or near the exon 10 tend to affect splicing, altering 3R/4R ratio, which can cause pathogenic changes. Furthermore, several of these mutations (e.g. P301L or P301S) are used to study Tau pathology in transgenic animals (drawn by Silva JM).

Table 2. Tauopathies. Clinical characteristics and pathology in some of neurodegenerative disorders that exhibit tau protein abnormalities. (Lee, VMY, Goedert, and Trojanowski 2001; Goedert and Jakes 2005; Hernández and Avila 2007)

Disease		**Clinical Characteristics**	**Tau Pathology**	**References**
Sporadic	**Alzheimer's Disease**	Progressive loss of memory and cognitive functions, resulting in a severe dementia.	NFTs and NTh pathology are found in conjunction with the deposition of amyloid fibrils in the extracellular space.	(Kidd 1963; Brion et al. 1985; Grundke-Iqbal et al. 1986; Goedert et al. 1988; Kosik et al. 1988; Kond et al. 1988; Wischik et al. 1988; Lee, Brian J. Balin, Laszlo Otvos 1991; Braak and Braak 1991; Nagy et al. 1999)
	Pick's Disease	Behavior changes, speech difficulty (aphasia), and impaired cognition	Frontotemporal lobar and limbic atrophy. Pick bodies, Balloned neurons and neuritic inclusions, with marked neuronal loss, spongiosis, and gliosis.	(Pollock et al. 1986; Iwatsubo, Hasegawa, and Ihara 1994; Feany, Mattiace, and Dickson 1996; Probst e al. 1996; Buée-Scherrer et al. 1996; Dickson 1998; Constantinidis, Richard, and Tissot 2008)
	Progressive Supranuclear Palsy	Atrophy of the basal ganglia, subthalamus, and brainstem	High density of fibrillary tau pathology, including NTh's, and NFT's that are typically round or globose. Glial fibrillary tangles in both astrocytes (tufted astrocytes) and oligodendrocytes (coiled bodies) are also often present.	(Hauw et al. 1990; Yamada, McGeer, and McGeer 1992; Hauw et al. 1994; Steele 1994; Litvan et al. 1996; Komori 1999)
	Corticobasal Degeneration	Affects cerebral cortex, deep cerebellar nuclei, and substantia nigra, in association with prominent neuronal achromasia depigmentation of the substantia nigra, as well as an asymmetric frontoparietal atrophy.	Various neuronal inclusions and NTh's throughout gray and white matter. Neuronal loss with spongiosis, gliosis, and prominent glial and neuronal intracytoplasmic filamentous tau pathology.	(Rebeiz, Kolodny, and Richardson 1968; Iwatsubo, Hasegawa, and Ihara 1994; Feany and Dickson 1995; Feany, Mattiace, and Dickson 1996; Komori 1999)
Familial	**FTDP-17**	Autosomal-dominantly inherited neurodegenerative diseases with diverse, but overlapping, clinical and neuropathological features; they are characterized primarily by FTD and parkinsonism, but the different FTDP-17 syndromes appear to reflect the burden of tau pathology and degeneration in brain regions known to subserve specific cognitive, executive, or motor functions	Phenotypic heterogeneity, the neuropathology of FTDP-17 is characterized by marked neuronal loss in affected brain regions, with extensive neuronal or neuronal and glial fibrillar pathology composed of hyperphosphorylated tau protein, with NTh's and NFT's.	(Rizzini et al. 2000; Foster et al. 1997; Ghetti 1998; Murrell et al. 1999; Spillantini et al. 2000; Lippa et al. 2000)

1.4.3 Tau-related Neurodegenerative Mechanisms

Hyperphosphorylation, truncation and abnormal conformation of Tau

Over the last decades, enormous research efforts have been focused on clarifying the underlying neurodegenerative mechanisms of Tau pathology and its specific anatomical spreading in brain. Besides the cytoskeletal disturbances through MT instability, hyperphosphorylated Tau may also trigger neuronal malfunction and neurodegeneration through other cellular mechanisms. For example, it is well-known that, in AD brain, Tau hyperphosphorylation is accompanied by Tau missorting from axon to the somatodendritic compartment. Synaptic missorting of Tau is shown to cause synaptic malfunction by Fyn-driven hyperactivation of NMDA receptors and downstream excitotoxic signaling (Hoover et al. 2010; Zempel et al. 2010; Ittner et al. 2010). Interestingly, hyperphosphorylation of Tau seems to be necessary for missorting of Tau at synapses as only pseudophosphorylated Tau (which mimics hyperphosphorylated Tau), but not phosphorylation-deficient Tau, is mislocalized and accumulated in dendritic spines (Hoover et al. 2010). In addition, hyperphosphorylation of Tau may alter its degradation (through the proteasome or through autophagy), since Tau phosphorylated at Ser262 or Ser356 cannot be recognized by the C terminus of molecular chaperone complex (CHIP–Hsp90) and is spared from proteasomal degradation and thus, accumulated in Tau aggregates (Shimura et al. 2004; Petrucelli et al. 2004). Indeed, Tau aggregation is often considered to be enhanced by Tau hyperphosphorylation (Noble et al. 2003) while Tau truncation and abnormal conformation is also shown to participate in Tau aggregation and consequent neurodegeneration.

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; Filipcik et al. 2009; Quintanilla et al. 2009; Quintanilla et al. 2012). Cathepsin D is also known to cleave Tau protein, generating fragments like those found in NFT's. It is suggested that truncated Tau may exhibit higher tendency for aggregation, probably through the disruption of the "paperclip" structure (Corsetti et al. 2014) as thus, it is believed to contribute to Tau aggregation process.

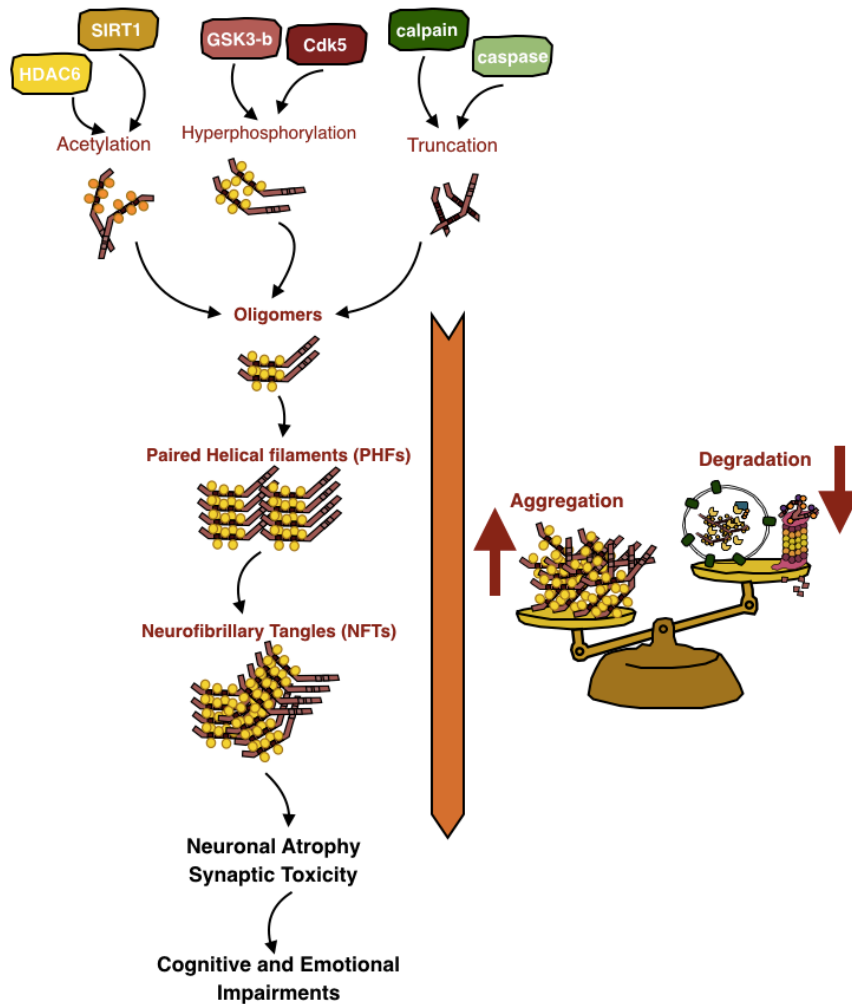

Figure 10. The interplay among different partners of Tau pathology. While the interconnection between different parameters of Tau pathology are still debatable, Tau post-translational modifications of Tau such as hyperphosphorylation and acetylation, followed by truncation and abnormal conformation of Tau are suggested to be involved in the oligomerization, and ultimately aggregation, that eventually result in neuronal atrophy and synaptic loss leading to cognitive and emotional impairments. However, the pathogenic deficits that occur in the regulation of the intracellular balance between Tau aggregation and Tau degradation in the AD brain are poorly understood (drawn by Silva JM and Sotiropoulos I).

Aberrant conformational changes, e.g. detected by MC1 conformational-dependent antibody of Tau, are also found in AD human brain as well as in different mice models of Tau pathology supporting the notion that abnormal conformation of Tau is also involved in Tau aggregation process (Weaver et al. 2000; Garcia-Sierra, Mondragon-Rodriguez, and Basurto-Islas 2008). Recently, acetylation was identified as a novel Tau post-translational modification involved in Tau pathology. Acetylation of Tau can occur by P300 acetyltransferase or by CREB-binding protein at several Lys residues, in the flanking region or in the repeat domain, and deacetylation can occur by sirtuin 1 (SIRT1) and histone deacetylase 6 (HDAC6), respectively (Cohen et al. 2011; Noack, Leyk, and

Richter-Landsberg 2014). Note that both HDAC6 and SIRT1 appear to be essential epigenetic components of AD pathology involved in Tau pathology (Lu et al. 2015) as it will be discussed later in this thesis. Depending on the sites, acetylation of Tau can inhibit or facilitate Tau degradation and suppress or promote its aggregation. For example, Tau acetylation in Lys174 retards the protein turnover and is critical for Tau-induced toxicity (Min et al. 2015) while elevated levels of HDAC6 is shown to alter both Tau acetylation, through its interaction with p300, and Tau phosphorylation pattern, increasing phosphorylation of Tau in Ser262/356 (Noack, Leyk, and Richter-Landsberg 2014).

Even though hyperphosphorylation, truncation, abnormal conformation and, recently, acetylation of Tau are all interconnected and linked to Tau aggregation and neurotoxicity, the absolute sequence of these events and their significance on Tau pathology remain fundamental question(s) in Tau-related research field (**Figure 10**).

The (im)balance of Tau degradation vs. aggregation

Tau protein stability and degradation is shown to be dramatically affected by the above-mentioned post-translational modifications of Tau that occur in AD brains suggesting that potential imbalance between Tau degradation and Tau accumulation/aggregation. Below, we will focus on potential mechanisms that maybe involved in damaged Tau degradation favoring its pathological accumulation and aggregation.

Ubiquitin-Proteasome System

When protein function and abnormal conformation can't be restored from misfolded and aggregated states, molecular chaperones help redirect nonnative clients, single proteins or aggregates, toward degradation by proteasome (Finley 2009) or lysosome (Yang and Klionsky 2010), respectively. Proteasomes are large multi-subunit complexes that consist of a 19S regulatory cap and a 20S proteolytic core (Coux, Tanaka, and Goldberg 1996). The 19S regulatory particle recognizes ubiquitylated substrates, removes ubiquitin chains, and unfolds the client to allow entry into the 20S core, where it is rapidly degraded into peptides (Kisselev et al. 1999; Finley 2009). This degradation process is initiated by the addition of several ubiquitin molecules (Finley 2009; Shang and Taylor 2011) (**Figure 11**). However, ubiquitylation of client proteins is not exclusive from proteasome; K48-linked ubiquitin chains are directed to proteasome (Chau et al. 1989). Ubiquitylation of Tau has been proposed a long time ago, when ubiquitin was found to be a component of NFTs (Mori, Kondo, and Ihara 1987; Perry et al. 1987; Shaw and Chau 1988), and

that insoluble PHFs isolated from AD brains showed ubiquitylation of Tau at several residues (Morishima-Kawashima et al. 1993; Cripps et al. 2006). More interesting is that all the ubiquitylation sites demonstrated are localized in the microtubule-binding repeat region. It is known that phosphorylation or even acetylation of these sites affects Tau-MT interaction, and thus ubiquitination can also play a role in regulating this interaction. Indeed, it is described that poly-ubiquitylated Tau fails to interact with microtubules in cell lines (Babu, Geetha, and Wooten 2005).

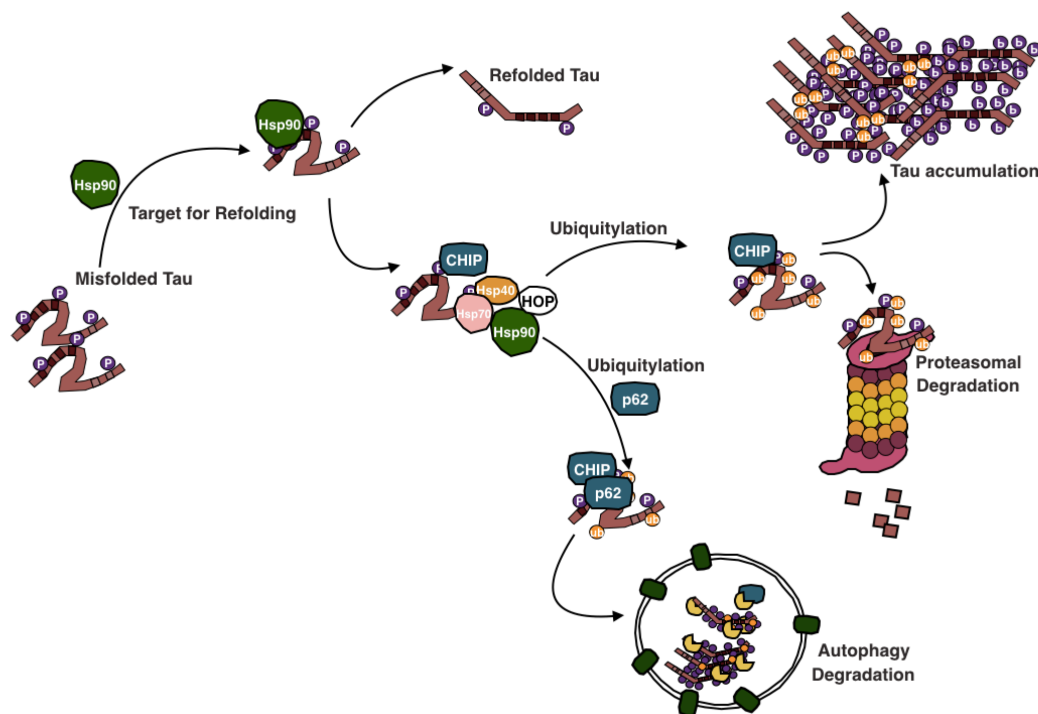

Figure 11. Tau degradation through chaperone-mediated and ubiquitin-proteasome system. Misfolded Tau is described to be targeted by the molecular chaperone, Hsp90, for refolding (Dickey et al. 2007). When Hsp90 is unable to refold abnormal Tau (Tau becomes hyperphosphorylated and changes its conformation, making it difficult for refolding), other chaperones like CHIP, HOP, Hsp70 and Hsp40, ubiquitylates Tau to target the protein for proteasome degradation (Petrucelli et al. 2004; Shimura et al. 2004; Koren et al. 2009). In AD, hyperphosphorylated Tau was described to be resistant to proteasome degradation (drawn by Silva JM).

Molecular chaperones, such as Heat Shock Protein 70 (Hsp70), Hsp90 and co-chaperone CHIP are the main proteins responsible for Tau ubiquitination and degradation by the proteasome (Petrucelli et al. 2004; Shimura et al. 2004; Koren et al. 2009). Misfolded Tau is recognized by Hsp40, Hsp70 and CHIP proteins while 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. 2013) (**Figure 11**). Hsp90 is a molecular chaperone that is involved in the folding and stabilization of many client proteins, and is known to play an important role in Tau degradation

(Dickey et al. 2007). Furthermore, CHIP appears to be a key player in Tau degradation, where overexpression of CHIP in the rat hippocampus has been shown to enhance Tau degradation, while its deletion leads to accumulation. Together, Hsp90 and CHIP, are major factors for Tau refolding or degradation by the proteasome. However, Tau hyperphosphorylation has been shown to make Tau protein resistant to degradation by the ubiquitin-proteasome pathway. After its hyperphosphorylation, Tau becomes polyubiquitinated; apparently, this does not lead to its clearance by the proteasome (**Figure 11**) because, as suggested, the rate of accumulation of polyubiquitinated Tau maybe greater than the ability of the proteasome to digest it. Inhibition of the proteasome with lactacystin or MG132 leads to accumulation of hyperphosphorylated Tau and also its ubiquitylation (Pappas et al. 2007; Demontis and Perrimon 2010). Many studies have reported that the activity of the proteasome and the levels of chaperones and co-chaperones are severally altered in AD brains, which can be related with an age-related decrease in the proteasome activity or chaperone response, leading to an increase in Tau aggregation (Tseng et al. 2008; Jinwal et al. 2011; Salminen et al. 2011).

Autophagy

Larger aggregates of tau are not likely to be accessible by the proteasome but can be degraded by the lysosomal pathway. Autophagy, meaning “self-eating” in Greek language, is an intracellular degradation pathway responsible for digestion and recycling of nutrients via autophagosomes. Autophagosomes are membranous structures that engulf cytoplasmic proteins and organelles and deliver them to the lysosome for degradation exhibiting an essential role in maintaining cellular homeostasis. Much of the studies on autophagy focus on macroautophagy (from now on, referred to as autophagy) which is characterized by three principal steps: initiation, elongation and maturation (**Figure 12**). Autophagy is initiated through changes in the phosphorylation state of individual components of a stable complex termed ULK (ULK1, ULK2, Atg13, FIP200 and Atg101) (Chan et al. 2009; Mizushima 2010). ULK1 phosphorylation is mainly regulated by the mTOR complex (TORC1). Various cell stress signals can suppress TORC1, activating ULK1 and ULK2 and turning autophagy on. In neurons, the regulation of mTOR pathway balances protein synthesis vs autophagic protein degradation influencing neuronal function, mainly through alterations in synaptic signaling (Stoica et al. 2011), dendritic arborization (Jossin and Goffinet 2007) and myelination (Narayanan et al. 2009). The ULK1 phosphorylation triggers translocation of Beclin-

1 complex to a pre-autophagosomal structure that initiates autophagosome elongation (Fimia et al. 2007; Di Bartolomeo et al. 2010).

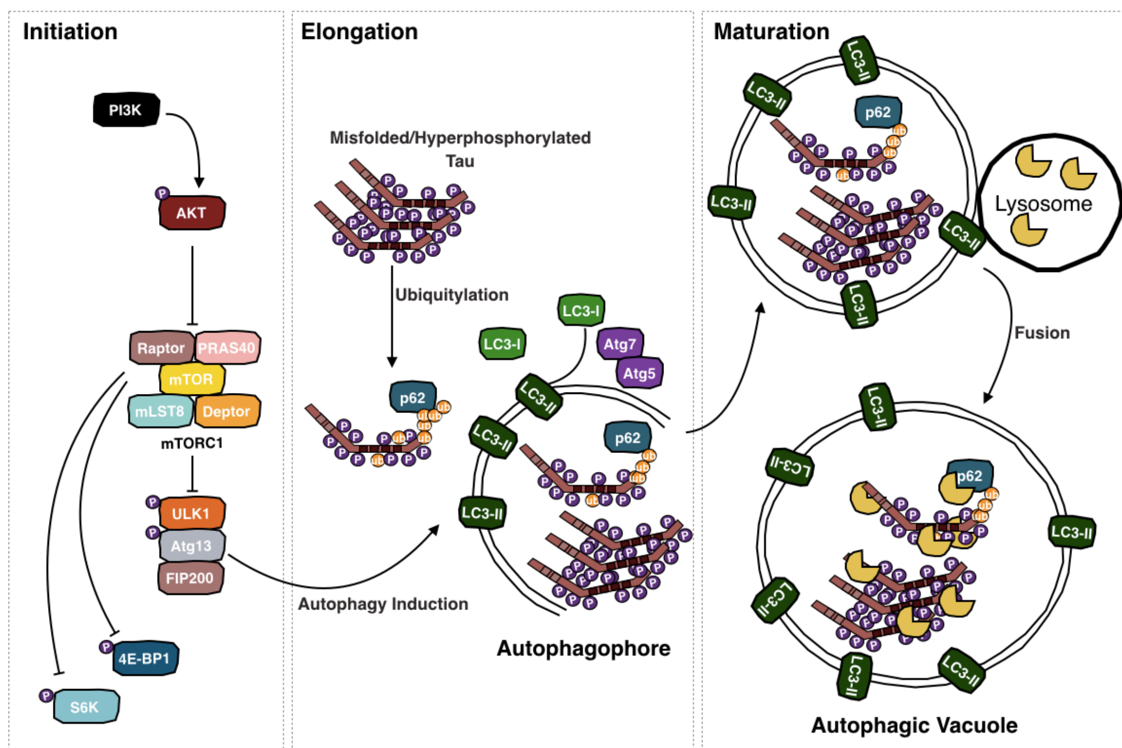

Figure 12. Autophagy-mediated clearance of hyperphosphorylated Tau. The induction of PI3K pathways leads to the inhibition of mTOR signaling, which triggers the initiation of autophagic signaling by ULK (initiation step). Then, hyperphosphorylated Tau is targeted for autophagy degradation through p62-dependent ubiquitylation. After targeting of the substrates, LC3-I is converted to LC3-II which, together with several Atg proteins, it triggers the formation of the autophagophore and consequent sequestration of the Tau aggregates for degradation (elongation step). In the final step of autophagy pathway (maturation step), autophagosomes fuses with lysosomes to initiate the process of degradation (drawn by Silva JM).

The second step in autophagic process is the elongation of the membrane and its closure around the "cargo", which involves several Atg proteins, like Atg7 and Atg5, and a set of proteins that catalyze the conversion of PtdIns to phosphatidylinositol 3-phosphate (PtdIns3P). Moreover, autophagic receptors and adaptors target the substrates to the autophagic core machinery, such as the microtubule-associated light chain 3 (LC3) (Filimonenko et al. 2010). LC3-II is one of the best characterized and widely used autophagosome markers in mammals (Mizushima and Yoshimori 2014) as LC3-II levels correlate with the number of autophagosomes in cells and thus, a reliable index of autophagic flux in cells and tissues. Autophagic receptors, such as p62 and HDAC6, recognize and facilitate the elimination of ubiquitylated proteins (Shaid et al. 2012).

The last step of autophagy process is the maturation, where autophagosomes fuse with lysosomes to start the degradation process. Cells increase the opportunities for lysosomal fusion by directing autophagosomes to move along the MTs towards perinuclear microtubule organizing center (MTOC) of the neuron, where lysosomes are most abundant (Lee et al. 2010; Korolchuk et al. 2011). MTs exhibit an important role in this step as MT disassembly leads to dynein-dynactin complex alterations triggering a massive build-up of autophagosomes in neuronal processes (Boland et al. 2008; Kimura, Noda, and Yoshimori 2008). The completion of the autophagy process requires lysosome digestion of autophagic cargo and the release of metabolites for reuse and signaling function.

Alterations in different step of autophagy process have been linked to Tau pathology. Mice deficient for the autophagy-related genes Atg5 and Atg7 exhibit severe neurodegeneration and ubiquitin-positive inclusions (Hara et al. 2006; Komatsu et al. 2006). AD patients present impaired initiation of autophagy and an excess of autophagic vacuoles is dystrophic neurites, probably due to an impaired targeting of the autophagosomes to the lysosome (Boland et al. 2008; Lee et al. 2010). Furthermore, the implications in axonal transport of hyperphosphorylated Tau could lead to an inhibition of Tau degradation through autophagy, impairing the fusion of autophagosomes and lysosomes. Moreover, several inhibitors of autophagy, that act in the different steps of the process, such as NHCl, chloroquine, 3-methyladenine (3-MA), and cathepsin inhibitors, delayed Tau degradation and enhanced the formation of high molecular weight species of Tau (Hamano et al. 2008; Wang et al. 2010). On the other hand, autophagy inducers, like rapamycin facilitate the degradation of insoluble forms of Tau and protect against its toxicity (Ravikumar et al. 2004; Majumder et al. 2011). Together these findings support the idea that an impairment of autophagic pathway is fundamental to the progression of Tau-driven neurodegeneration and Tau aggregation pathology.

RNA binding proteins and SGs

The classical process of pathological protein aggregation contrasts with the tightly regulated and reversible process of aggregation that occurs as an intrinsic aspect of the biology of RNA binding proteins (RBP). The function of RBPs can be divided into two types of activities: nuclear and cytoplasmic. In the nucleus, RBP regulate mRNA maturation, including splicing, helicase activity, polymerase elongation and nuclear export (Heyd and Lynch 2011). In the cytoplasm, they regulate RNA transport, silencing, translation and degradation (Liu-Yesucevitz et al. 2011). Furthermore, RBPs

regulate transcript activity by forming RNA granules, macromolecular complexes containing RBPs and mRNA transcripts, consolidated to form granules by protein/protein interactions. The granules vary by molecular composition and function, mediating RNA degradation (Krichevsky and Kosik 2001), RNA transport in neurons [for movement transcripts from the soma into dendrites and axons] (Thomas et al. 2011) and activity-dependent local protein synthesis in the synapse [for controlling synaptic plasticity and memory] (Hoeffer and Klann 2010).

Stress granules (SGs) are one type of RNA granules that are generated in response to stressful conditions (e.g. oxidative stress), allowing the fast production of cytoprotective proteins. SG formation is mediated by phosphorylation of eIF2α which induces the translocation of many RBPs from the nucleus to the cytoplasm (Kedersha et al. 1999), such as TIA-1 or GTPase-activating protein SH3-domain binding protein (G3BP) (**Figure 13**). Indeed, overexpression of these SG-related proteins induces the formation of SGs in the absence of stress and their absence impairs SG assembly (Tourrière et al. 2003; Gilkes et al. 2004; Ohn et al. 2008). Mutations in RBPs also increase the propensity of these proteins to aggregate forming SGs. Cytoskeletal machinery facilitates the aggregation of RBPs to form SGs (**Figure 13**). One of these molecules is HDAC6, which is required for this process, through the deacetylation of tubulin, reducing microtubule-dependent motility, and thereby promoting the consolidation of cellular complexes, like SGs, autophagosomes and aggresomes (Lee et al. 2010a; Lee et al. 2010b; Hoover et al. 2010).

While primarily protective, SGs can eventually become pathological and neurotoxic under prolong period of their induction. Indeed, SGs are correlated with several neurodegenerative disorders, although the underlying mechanisms are largely unknown. In AD and FTDP-17 human brain, the load of SGs inclusions is high, exhibiting a density that is equal or even greater that the load of NFTs while SGs proteins (e.g. TIA-1 and TTP) identify most NFTs (Vanderweyde et al. 2012). Similarly, in a mouse model of Tau pathology (e.g. P301L-Tau Tg mice), Tau aggregates are often, if not always, co-localized with SGs. Furthermore, the aggregation of pathological Tau stimulates SGs formation, enhancing their stability and maturation, and, in a vicious cycle, SGs formation accelerates aggregation of pathological, aggregated Tau (Vanderweyde et al. 2012; Wolozin 2012). Importantly, recent studies point towards the direct involvement of TIA-1 and Tau protein, and that Tau is indeed important for TAI-1 normal interactions with other proteins, and Tau regulates TIA-1 distribution and accelerates SG formation (Vanderweyde et al. 2016). However, the mechanisms behind this interaction are still unclear.

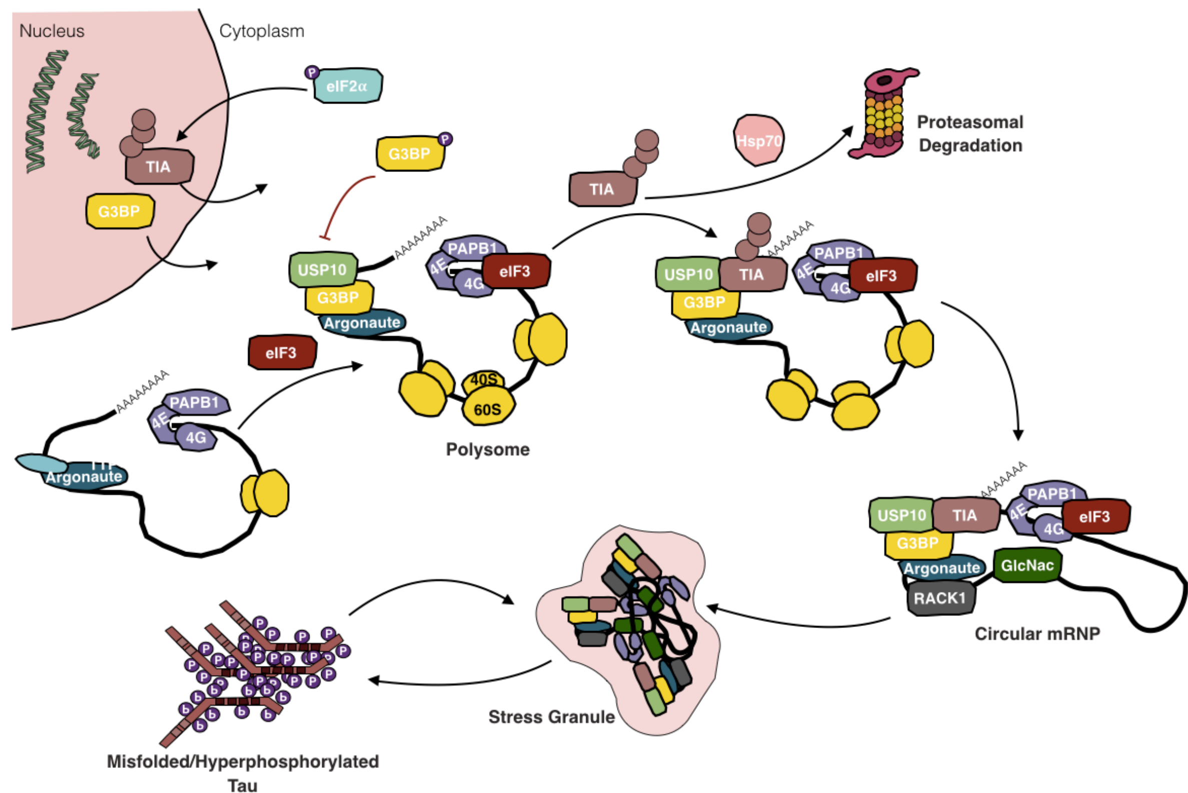

Figure 13. Stress Granules (SGs) assembly and Tau aggregation. The phosphorylation of eIF2α transiently stalled initiation complexes recruit TIA-1 and TIA-R (represented together as TIA) and elongation ribosomes run off the transcript, converting the polysome into a circular mRNP. The aggregation of TIA and G3BP-USP10 complex and the further modification of ribosomes by N-acetylglucosamine (GlcNAc), promote the assembly of these circular mRNPs into SGs. Inhibition of G3BP interaction with USP10 by phosphorylation inhibits SGs formation; other partners of G3BP, such as HDAC6 and caprin1, are also involved in SG formation, however the nature of the important of this association for SG formation is still unclear. Furthermore, Hsp70 interaction with TIA-1 decides whether TIA is targeted for proteasome degradation or initiates SGs assembly. Moreover, the aggregation of pathological Tau stimulates SG formation, and SG formation accelerates Tau aggregation (drawn by Silva JM).

1.5 ENVIORNMENTAL STRESS AS A RISK FACTOR FOR TAU-DRIVEN NEURODEGENERATION

AD is a multifactorial disorder with a highly complex pathophysiology and unknown causes. Several risk factors have been associated to the development of the disorder with aging being the most predominant. In addition, genetic mutations in specific genes (e.g. ApoE, APP, PS1, PS2), gender and cardiovascular problems have also been found to increase the risk of AD (Fratiglioni et al. 1997; Stein 2001; Turner et al. 2003; Reitz and Mayeux 2014). Recently, strong evidence supports a detrimental role of chronic environmental stress and stress hormones, glucocorticoids

(GC), in the onset and progression of the disease, with several AD patients exhibiting high GC (cortisol) levels (Swaab et al. 1994; Hartmann et al. 1997). But how stress and stressful stimuli affect the brain and its function?

1.5.1 Stress and HPA axis

Stress may be broadly defined as a disruption of homeostasis following the application of internal or external challenges ('stressors'). Stressors are recognized and perceived by the brain through the release of different molecules (e.g. adrenaline, corticosteroids) as an adaptive mechanism to the adverse challenges (McEwen 2007; de Kloet, Karst, and Joëls 2008; Joëls and Baram 2009). Furthermore, stress generates a cascade of events that coordinate a complex and integrated response that lead the organism to adapt to the changes in the environment, restoring homeostasis. This response implies that the organism is able to reply effectively when needed, and then terminates it afterwards.

The first and fast response involves a rapid activation of the autonomic nervous system, leading to the release of epinephrine and norepinephrine from the adrenal gland; these molecules act very quickly through an elevation of basal metabolic rate, blood pressure and respiration, and by increasing the blood flow in the vital organs (**Figure 14**). In the second part of the stress response, the hypothalamus–pituitary–adrenal (HPA) axis is activated, resulting in the production of corticosteroids (cortisol in humans and corticosterone in rodents) which serve as an inhibitory feedback signal back to hypothalamus and pituitary (**Figure 14**). HPA axis is the major neuroendocrine circuit in the brain.

Corticosteroids action on brain is mediated by glucocorticoids and mineralocorticoids receptors. In the mammalian brain, mineralocorticoid receptors (MRs) have higher affinity for corticosteroids and they are the first to be occupied (even at low corticosteroid levels). On the other hand, glucocorticoid receptors (GRs) have ~10 fold lower affinity. Accordingly, on basal conditions or non-stressful conditions, GRs are partially occupied and become increasingly occupied when corticosteroid levels are elevated (as it occurs in stressful conditions (de Kloet, Karst, and Joëls 2008). GRs have a ubiquitous distribution in the brain, where their density is higher in the PVN, in neurons that arise from aminergic pathways and limbic neurons that modulate PVN function (Bali and Kovács 2003; Figueiredo et al. 2003; Herman, Mueller, and Figueiredo 2004). MR and GR co-expression is found in hippocampal pyramidal cell in CA1 and DG, in the amygdaloid and lateral septal nuclei, and in some cortical regions. Interestingly, GRs and MRs function as transcriptional

regulators through the alteration of genes expression (Trapp et al. 1994; Nishi et al. 2001; Gesing et al. 2001; Chen et al. 2010). Using large scale gene expression profile methods (e.g. SAGE and microarrays), it was observed that MRs and GRs differentially regulate gene expression exhibiting broad effects on brain. Specifically, they play a role in cell metabolism, structure and synaptic transmission (Datson et al. 2001), altering the expression of enzymes and receptors of amines and neuropeptides, growth factors and cell adhesion molecules (Schaaf et al. 1998; Hansson et al. 2000; Sandi 2004; Sabban et al. 2006;). Indeed, stress and GC lead to alterations in the dendritic structure of neurons (Wossink et al. 2001) and impact on adult neurogenesis, having a role in neuronal plasticity (Cameron and Gould 1994; Hu et al. 1997; Richetin et al. 2015). MRs maintain the excitability and stability of networks, while GR activation leads to a delayed suppression or normalization of network activity, facilitating the retention of information, allowing an increase in calcium influx into the cell (Oitzl and de Kloet 1992; Sandi et al. 2005; Cacucci et al. 2007). However, this increase and sustained calcium influx can prime neurons for the detrimental stress effects which are observed when exposure to stressful conditions is extended for longer periods. Indeed, chronic stress can become maladaptive resulting in damages in brain structure and function (Joëls and Baram 2009; Sousa and Almeida 2012).

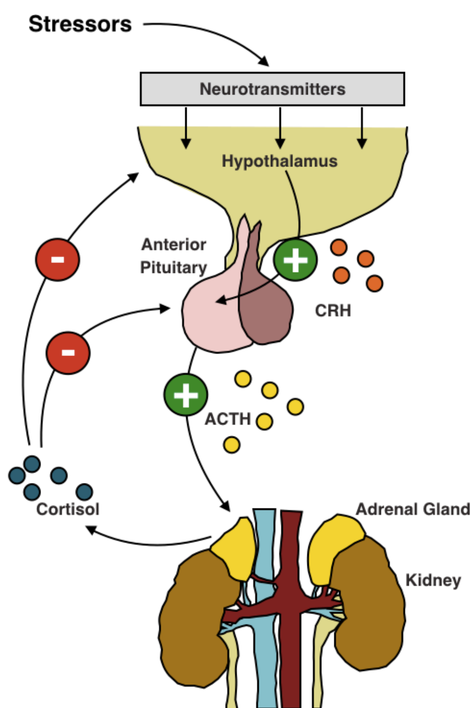

Figure 14. Stress and hypothalamus-pituitary-adrenal (HPA) axis. Upon a stressful stimulus, activation of the HPA axis where hypothalamus releases corticotropin-releasing hormone (CRH) to the anterior pituitary. Then, the pituitary secretes the adrenocorticotropin hormone (ACTH) which, in its turn stimulates

the production of glucocorticoids (GC) [cortisol in humans and corticosterone in rodents] and their release from the adrenal glands to the circulating blood. Feedback inhibition of HPA axis is achieved by the GC on the hypothalamus and anterior pituitary. Stress response coordinates emotional, cognitive and autonomic inputs. This coordination determines the magnitude and specificity of an individual to respond to the stressor. The receptor system that mediates the slow and genomic actions of corticosteroids has several features (drawn by Silva JM).

1.5.2 Stress, Glucocorticoids and GR signaling

As mentioned above, GRs are ligand-induced transcription factor that, upon hormone (corticosteroid) binding, translocate from the cytoplasm to the nucleus where it regulates the transcription of many genes. GR activation, translocation to the nucleus and gene transcription activity, is mediated and regulated by a large multiprotein complex based on Hsp90/Hsp70 chaperone machinery. This ATP-dependent process includes several co-chaperones and is critical for receptor translocation to the nucleus and DNA binding activity of GRs (Grad and Picard 2007). Within the nucleus, Hsp90 plays a critical role in both the GR movement to transcription targets and the disassembly of regulatory complexes, as the hormone levels decrease. Furthermore, the interaction with the chaperone machinery is also important for GR ubiquitination and further proteasomal degradation, mainly through the interaction of CHIP to Hsp70/Hsp90 complex (Ballinger et al. 1999). The balance between interaction with Hsp70/Hsp90 complex for nuclear translocation and the interaction with CHIP for degradation could be in the basis of an overactivation of GR signaling in the pathophysiology of stress. Differential composition of the complex of GR-related molecular chaperones and co-chaperones can modulate the sensitivity of GRs and thus, in the vulnerability of individuals to stress-related pathologies. For example, FK506 binding protein 51 (FKBP5) and BCL-2 associated athanogene (BAG1) have been implicated in the development of stress-related disorders e.g. depression and anxiety (Song, Takeda, and Morimoto 2001; Lekman et al. 2008) where, several reports have also related the association of FKBP5 polymorphisms with response to antidepressant drugs (Binder et al. 2004; Lekman et al. 2008; Kirchheiner et al. 2008). In addition, GR signaling is also essentially regulated by the molecular chaperone Hsp90 and its acetylation by HDAC6. It was shown that inactivation of HDAC6 leads to an hyperacetylation of Hsp90, compromising GR maturation, affecting ligand binding, nuclear translocation and transcriptional activation (Kovacs et al. 2005). This offers a clear cross-point between HDAC6 function and the stress response, through HDAC6-mediated Hsp90 deacetylation that is crucial for GR activation.

1.5.3 Chronic Stress and Tau-related Neurodegeneration

While stress and stress response are adaptive and important for maintenance of mental and physical health, exposure to prolong stress may become maladaptive resulting in damages in brain and many peripheral tissues (Joëls and Baram 2009). Indeed, elevated GC levels and GR activation are associated with the detrimental effects of chronic stress on neuronal and brain function (Sapolsky et al. 1990; Stein-Behrens et al. 1994). Human and animal studies demonstrate that exposure to chronic stress or high GC levels results in smaller hippocampal volumes and similar volumetric reductions in the prefrontal cortex (PFC) (Cerqueira et al. 2005; Cerqueira et al. 2007; Rothman and Mattson 2010). Furthermore, chronic stress and high GC levels lead to significant decreases in the total length and number of spines in the apical dendrites of neuronal population in both hippocampus and PFC (Cerqueira et al. 2007; Sotiropoulos et al. 2008). Altogether, the above structural and synaptic deficits triggered by stress and GC are thought to underlie the stress/GC-evoked deficits in hippocampal-dependent reference memory and PFC-dependent behavioral flexibility (Sotiropoulos et al. 2011); however, the underlying mechanisms of these stress/GC neuroplastic effect remain poorly understood.

Similarly, to chronic stress, AD brain also exhibits neuronal atrophy and synaptic loss followed by cognitive deficits, suggesting a link between the two pathologies (Sotiropoulos and Sousa 2016). Indeed, clinical studies report high GC levels in AD patients, which were positively correlated with their memory deficits (Elgh et al. 2006). Furthermore, chronic stress is also suggested to lower the age of onset of the familial form of AD (Simard, Hudon, and van Reekum 2009), highlighting the potential implication of chronic stress and GC in the pathogenesis and/or progression of AD (Hartmann et al. 1997; Sotiropoulos et al. 2008). In line with the above clinical evidence, previous studies from our team and others have shown that chronic stress and/or GC treatment trigger Tau hyperphosphorylation in different AD models, leading to Tau accumulation in neuronal soma and affecting different Tau epitopes (Green et al. 2006; Sotiropoulos et al. 2011) implicated in cytoskeletal pathology and synaptic loss in AD patients (Lauckner, Frey, and Geula 2003). Indeed, clinical studies report a strong correlation between the extent of Tau hyperphosphorylation and severity of impairments in memory, speed of mental processing, and executive functions (Augustinack et al. 2002; van der Vlies et al. 2009). Furthermore, stress is shown to trigger Tau accumulation and aggregation (Sotiropoulos et al 2014), probably by affecting turnover of the protein

(Sotiropoulos et al. 2008). However, our understanding of the underlying cellular signaling and molecular pathways through which chronic stress and GC trigger Tau accumulation and neurotoxic aggregation remains extremely poor.

AIMS

Tau protein is now suggested to be an essential regulator of brain plasticity as well as pathology in and beyond Alzheimer's disease (AD). Accordingly, previous studies suggested that Tau may lie at the core of chronic stress-induced pathological aging of the brain raising Tau malfunction as a critical mechanism through which stress and GC exert their neuro-remodeling and neurodegenerative effects. However, the precise mechanisms by which Tau contributes to brain malfunction and pathology are poorly clarified. Moreover, despite that Tau reduction seems to be a promising therapeutic strategy against AD neurodegeneration, it is still debated whether loss of Tau and its normal function impact of neuronal and brain function. Thus, this PhD thesis aimed to address the following three objectives:

i) Clarify the potential role of Tau malfunction in hippocampal neuroplasticity and damage occurring under prolong exposure to the main stress hormones, GC, known to increase brain vulnerability to disease (**Chapter 2**);

ii) Identify the cellular mechanisms of Tau aggregation and pathology triggered by lifetime, chronic stress (**Chapter 3**).

iii) Obtain clear evidence about the normal Tau function in adult brain as well as the consequences of its loss on neuronal structure and function by creating conditional deletion of *MAPT* in adult animals (**Chapter 4 & 5**);

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CHAPTER 2

TAU MISLOCATION IN GLUCOCORTICOID-TRIGGERED HIPPOCAMPAL PATHOLOGY.

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Tau Mislocation in Glucocorticoid-Triggered Hippocampal Pathology

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Abstract The exposure to high glucocorticoids (GC) triggers neuronal atrophy and cognitive deficits, but the exact cellular mechanisms underlying the GC-associated dendritic remodeling and spine loss are still poorly understood. Previous studies have implicated sustained GC elevations in neurodegenerative mechanisms through GC-evoked hyperphosphorylation of the cytoskeletal protein Tau while Tau mislocation has recently been proposed as relevant in Alzheimer's disease (AD) pathology. In light of the dual cytoplasmic and synaptic role of Tau, this study monitored the impact of prolonged GC treatment on Tau intracellular localization and its phosphorylation status in different cellular compartments. We demonstrate, both by biochemical and ultrastructural analysis, that GC administration led to cytosolic and dendritic Tau accumulation in rat hippocampus, and triggered Tau hyperphosphorylation in epitopes related to its malfunction (Ser396/404) and cytoskeletal pathology (e.g., Thr231 and Ser262). In addition, we show, for the first time, that chronic GC administration also increased Tau levels in synaptic compartment; however, at the synapse, there was an increase in phosphorylation of Ser396/404, but a decrease of Thr231. These GC-triggered Tau changes were paralleled by reduced levels of synaptic scaffolding proteins such as PSD-95 and Shank proteins as well as reduced dendritic branching and spine loss. These in vivo findings add to our limited knowledge about the underlying mechanisms of GC-evoked synaptic atrophy and neuronal disconnection implicating Tau missorting in mechanism(s) of synaptic damage, beyond AD pathology.

Keywords Tau · Glucocorticoids · Synaptic atrophy · Neurodegeneration · Hippocampus

Abbreviations

GC	Glucocorticoids
MT	Microtubules
AD	Alzheimer's disease
MWM	Morris water maze
PSD-95	Postsynaptic density protein 95
DEX	Dexamethasone
GluN2B	NMDA receptor 2B
GluA2	AMPA receptor 2
ANOVA	Analysis of variance
TEM	Transmission electron microscopy

Introduction

Stress, largely through the elevation of circulating glucocorticoids (GCs), impacts on brain structure and function [1–4]. One of the most vulnerable brain areas is the hippocampus, which exhibits remarkable dendritic atrophy and spine loss after GC administration as well as in stress-related pathologies characterized by high GC levels [1, 4]. However, the mechanisms underlying these GC-induced deleterious effects that damage hippocampus structural and functional integrity are still poorly understood.

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Microtubule-associated protein Tau is implicated in cytoskeletal dynamics, as it stabilizes the microtubule (MT) network [5]. While being mainly an axonal protein, Tau was recently found in dendrites and synapses where it is suggested to have novel signaling and scaffolding role(s), regulating synaptic structure and function [5–8]. Recent human and animals studies suggest that Tau hyperphosphorylation and its mislocation in synapses may be related to synaptic pathology in Alzheimer's disease (AD) [9–12]. Our previous work showed that stress and GC trigger Tau hyperphosphorylation in neuronal somata [13, 14], but to date, there is no evidence about the potential impact of dendritic/synaptic Tau on GC-induced dendritic remodeling and spine loss. Thus, in the present study, we used subcellular fractionation-based biochemical analysis and electron microscopy to monitor Tau dynamics and localization in hippocampal neurons of GC-treated rats adding to our understanding of cellular phenomena of GC-driven hippocampal malfunction and dendritic remodeling.

Methods

Animals and Treatment

Three- to four-month-old male Wistar rats (Charles River Laboratories, Spain) were paired housed under standard laboratory conditions (8:00 A.M. to 8:00 P.M.; 22 °C; ad libitum access to food and drink). Half of the animals were receiving daily subcutaneous injections of the synthetic glucocorticoid, dexamethasone (DEX) (300 μg/kg; Sigma D1756; dissolved in sesame oil containing 0.01 % ethanol; sesame oil Sigma S3547) for 14 sequential days, while control animals received daily subcutaneous injections of sesame oil [1]. All experimental procedures were approved by the local ethical committee of University of Minho and national authority for animal experimentation; all experiments were in accordance with the guidelines for the care and handling of laboratory animals, as described in the Directive 2010/63/EU.

Behavioral Test

Spatial reference memory was assessed using the Morris water maze (MWM) test at the end of DEX treatment period. As previously described [1, 13], testing was conducted in a circular black tank (170-cm diameter) filled with opaque water (22 °C) and placed in a dimly lit room with extrinsic clues. The tank was divided into virtual quadrants and had a black, escaping platform (12-cm diameter) placed in one of them. Animals (N=7–8 per group) were asked to find the escaping platform over four consecutive days (four trials/day; 120-s trial period). Note that there are no differences of swimming speed between control and DEX-treated animals (data not shown). On the 5 day (probe test), the animal had to search (120 sec) for the escaping platform that was absent. Swim paths of each animal were monitored and recorded by a CCD camera, using a video tracking system (Viewpoint).

Neurostructural Analysis

As previously described [15], after animal perfusion with saline, half of each brain (N=7–8 per group) were immersed in Golgi-Cox solution for 14 days. After transfer to 30 % sucrose solution, vibratome-cut coronal brain sections (200 μm thick) were used. After development, fixation and dehydration, slides were used to perform three-dimensional morphometric analysis. Dendritic arborization and spines were analyzed in dorsal hippocampus (CA1 area). All branches of neuronal dendritic tree (6–8 neurons/animal) were reconstructed at ×600 (oil) magnification using a motorized microscope (Axioplan2, Zeiss) and Neurolucida software (MBF Bioscience). For spine analysis, proximal and distal apical dendritic segments (30 μm) were randomly selected and spines were counted and further classified in immature (thin) and complex/mature (mushroom, wide/thick, and ramified) categories as previously described [15].

For electron microscope analysis, isolated hippocampi were fixed (4 % PFA; 3 days; 4 °C), transferred to 4 %PFA/0.8 % gluteraldehyde in 0.1 M of phosphate buffer (PB; pH 7.4) for 1 h and then, to 0.1 M PB (4 °C). Vibratome-cut axial sections of the dorsal hippocampus (300 μm thick) were collected, and the entire CA1 area was surgically removed. Tissue was then carefully oriented and embedded in Epon resin and ultrathin sections (500 Å), encompassing the superficial-to-deep axis, were cut onto nickel grids. For Tau-immunogold staining, sections were treated with heated citrate buffer (1×; Thermo Scientific, USA) for 30 min followed by 5 % BSA (Sigma, USA). Grids were incubated overnight with the following primary antibodies diluted in 1 % BSA (in PB): Tau-5 (1:30, Abcam), pSer199/202-Tau (1:50 Abcam), pSer262-Tau (1:20, Santa Cruz Biotechnology), pSer396 (1:50; Abcam), followed by appropriate secondary gold antibody (1:15; Abcam). For manual calculation of density of the above phospho-Tau, more than 50 nonoverlapping TEM (30,000×) of counterstained ultrathin sections (60 nm) were used. Grids were observed on a JEOL JEM-1400 transmission electron microscope equipped with a Orious Sc1000 digital camera. Analysis was performed by an experimenter blind to the samples provenience.

Subcellular Fractionation and Western Blot Analysis

For fractionation, we have used a well-established fractionation protocol [8, 16]. As shown in Fig. 1g, dorsal hippocampi (N=5 per group) were homogenized with 10 volumes of homogenization buffer [sucrose 9 %; 5 mM DTT; 2 mM EDTA; 25 mM Tris pH 7.4; complete protease inhibitor (Roche), and

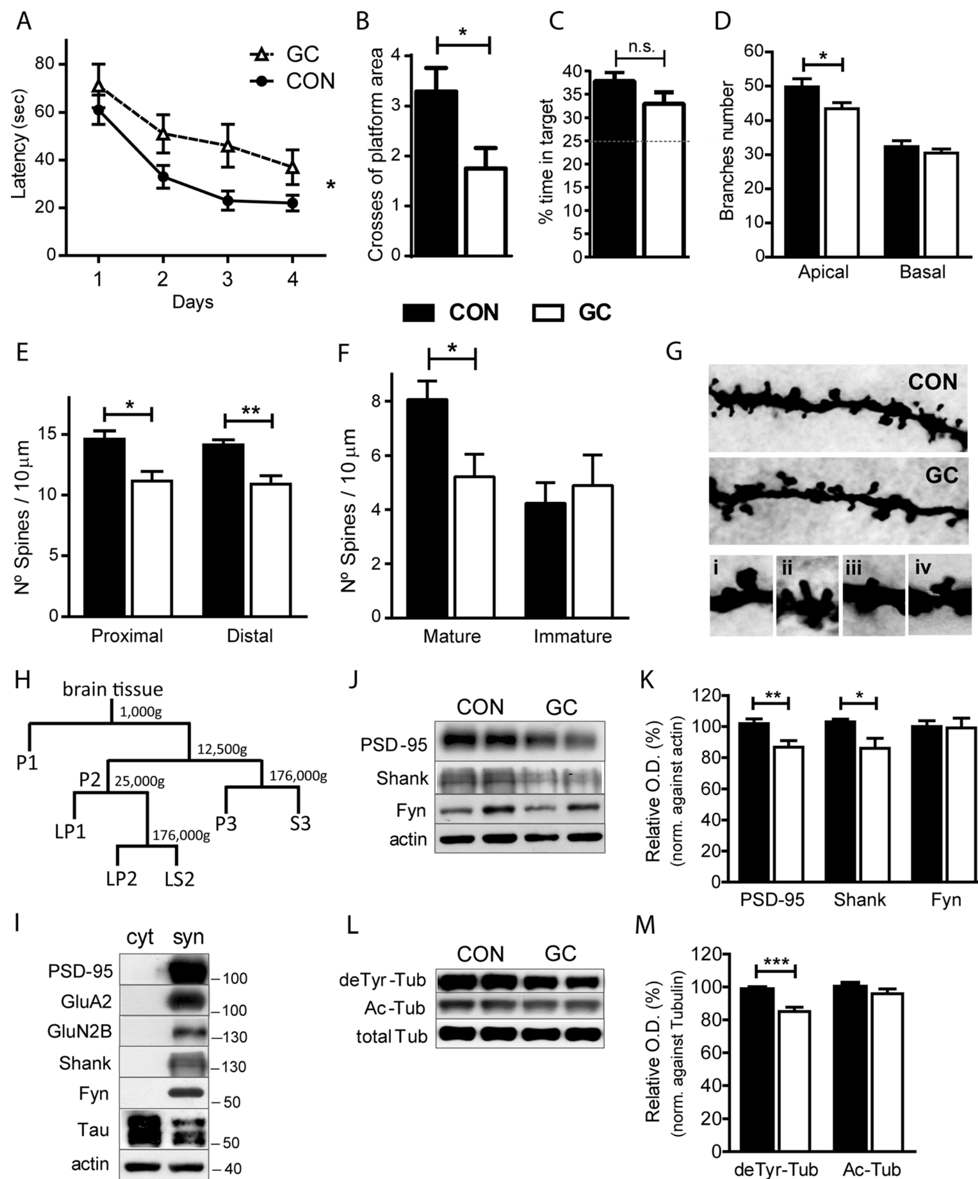

Fig. 1 Glucocorticoid-driven biochemical and structural correlates of neuronal atrophy and memory deficits: **a**–**c** GC-treated animals exhibited increased latency to escaping platform during the four learning days of Morris water maze test compared to control (saline) animals (**a**) as well as reduced numbers crossing from the platform area (**b**) while no difference of percentage of time swam in target quadrant between the two groups was found in probe test (**c**). **d**–**g** Morphometric analysis of Golgi-impregnated hippocampal neurons showed that GC treatment reduced the number of branches in apical but not basal dendrites (**d**) followed by reduced spine density in both proximal and distal parts of apical dendrites (**e**, **g**). Specifically, GC evoked a clear reduction of density of mature/complex spines (**f**, **g** (i, iii, iv)) but not immature/thin ones (**e**, **f** (ii)). **h** Schematic representation of the subcellular fractionation protocol followed in this study for separating P1 (nuclear pellet and debri), P2 (crude synaptosomal fraction), P3 (light membranes), S3 (cytosolic fractions), LP1 (synaptosomal membrane fractions), LP2 (synaptic vesicle-enriched fraction), LS2 (soluble synaptosomal fraction)—for details, see Methods. **i** Western blot analysis of cytosolic and synaptosomal fraction where PSD-related scaffold proteins and receptors are found at synaptosomal, but not at cytosolic, fraction. **j**, **k** Representative blots and quantitative WB analysis of scaffold proteins showing that GC significantly decreased PSD-95 and Shank levels in synaptosomal fraction compared to control animals. **l**, **m** Levels of stable detyrosinated, but not acetylated, tubulin were reduced in dorsal hippocampus of GC-treated animals. All graphical data is shown as group mean±SEM; *$p<0.05$; **$p<0.01$; ***$p<0.001$

phosphatase inhibitor cocktails II and III (Sigma)] using a Dounce glass homogenizer. Post-nuclear supernatant was subjected to centrifugation (12,500*g*) and divided into the crude synaptosomal fraction and synaptosome-depleted fraction. The latest fraction was further subjected to ultracentrifugation (176,000*g*), and separated into light membrane and Golgi fraction (P3) and cytoplasmic fraction (S3) while crude synaptosomal fraction was further lysed hypoosmotically and centrifuged (25,000*g*) for 20 min in order to obtain the synaptosomal membrane fraction (LP1). S3 (1x) and LP1 (2x) fractions were used for Western blot analysis. Samples were electrophoresed on 10 % acrylamide gels and transferred onto

nitrocellulose membranes (Trans-Blot® Turbo™ Blotting System, BioRad). Membranes were blocked in 5 % nonfat milk in TBS-T buffer before incubation with the following antibodies: PSD-95 (1:20000, NeuroMab), GluA2 (1:1000, Abcam), GluN2B (1:1000, Abcam), pTyr1472-GluN2B (1:1000, Cell Signaling) pan-Shank (1:20, NeuroMab), Fyn (1:200, Santa Cruz), detyrosinated-Tubulin (1:1000, Abcam), acetylated-Tubulin (1:500, Abcam), α-Tubulin (1:2000, DSHB), Tau-5 (1:2000, Abcam), p199/202-Tau (1:1000, Abcam), pThr231-Tau (1:1000 Abcam), pSer262-Tau (1:250, Santa Cruz), p396-Tau (1:5000, Abcam), PHF1 (1:1000; kindly provided by Dr. P. Davies, Albert Einstein College, USA) cdk5 (1:2000, Millipore), total GSK3 α/β (1:2000, Invitrogen), phospho-Tyr279/216-GSK3α/β (1:2000, Invitrogen), and actin (1:2000, DSHB). After incubation with the appropriate secondary antibody, antigens were revealed by ECL (Clarity, Bio-Rad). ECL films (GE Healthcare) were used for detection of antigen signal and films were scanned and quantified using TINA 3.0 bioimaging software (Raytest). All values were normalized and expressed as percentages of controls. As previously shown [16], the protocol fractionation efficiency of the hippocampal tissue was confirmed using different synaptic proteins (see above) that were exclusively found in synaptosomal but not in cytosolic fraction (see Fig. 1g).

Statistical Analysis

All data were evaluated by GraphPad software (version 6, La Jolla, CA, USA) using Student *t* test while Morris water maze learning curve data were analyzed by repeated-measurements ANOVA. Differences were considered to be significant if $p<0.05$. Results are expressed as group means±SEM.

Results

Behavioral and Neurostructural Correlates of Prolong Exposure to Glucocorticoids

For evaluating the impact of prolong GC treatment on hippocampus-dependent spatial memory, we used the MWM test and we found a significant increase in the time that GC-treated (GC) animals needed to find the escaping platform, confirming a deficit in spatial reference memory ($p<0.05$) (Fig. 1a). In addition, we found that animals exposed to GC exhibited lower number of crosses at the platform area supporting further the memory-impairing role of GC ($p=0.028$) (Fig. 1b). However, there is no difference between the two groups in the percentage of time that animals swam into target quadrant during probe test (Fig. 1c; $p=0.14$), indicating that at the end of the experiment all animals were able to learn the task.

As neuroplastic changes and synaptic loss are robust correlates of impaired cognitive behavior, we next analyzed the entire dendritic tree of hippocampal CA1 pyramidal neurons using unbiased 3D morphometric analysis of Golgi-impregnated pyramidal neurons. We found that GC treatment altered the dendritic arborization in pyramidal neurons by diminishing the number of branches in apical, but not basal, dendrites ($p=0.03$ and $p=0.37$, respectively; Fig. 1d). Furthermore, spine density was reduced in both proximal and distal segments of apical dendrites ($p=0.017$ and $p=0.007$, respectively; Fig. 1e, g). Moreover, when we clustered the spine analysis in immature (thin) and mature (complex) categories based on morphological characteristics [15] (see Fig. 1g (i–iv)), we found that the density of mature/complex was reduced by GC treatment ($p=0.038$) but no differences were found in immature ones (Fig. 1f). Note that immature (thin) spines are thought to be particularly plastic and linked to learning process while mature spines (e.g., mushroom) are more stable type of spines and believed to be involved in memory formation.

Glucocorticoids Triggers Tau Hyperphosphorylation and Missorting in Hippocampal Synapses

For monitoring the molecular underpinnings of the GC-evoked dendritic remodeling and synaptic loss, we then performed a detailed subcellular fractionation and organelle enrichment protocol which allows us to distinguish between cytosolic (excluding light membrane and Golgi fraction; S3) and synaptosomal fractions (LP1; see Fig. 1h; for more details, see "Methods" sections); as a confirmation of the efficacy of the separation protocol, postsynaptic density proteins (e.g., postsynaptic density protein 95; PSD-95) and synaptic receptors (e.g., GluN2B) were not found in cytoplasmic fraction (Fig. 1i). Next, we compared the synaptic and cytoskeletal proteins whose alterations reflect plastic changes and found that GC treatment reduced total levels of two proteins that play a central role in the assembling of the synaptic molecular components and transmission, PSD-95 and Shank scaffold proteins in synaptosomal fraction ($p=0.008$ and $p=0.015$, respectively; Fig. 1j, k); of notice, Fyn levels were not altered. In addition, we found a significant reduction of stable, detyrosinated tubulin levels ($p<0.0001$), while acetylated tubulin was not different between GC-treated and controls (Fig. 1l, m).

We next monitored the influence of GC on intracellular distribution of Tau proteins and their phosphorylated isoforms. As shown at Fig. 2a, b, our subcellular fractionation-based WB analysis revealed that chronic GC exposure induced a significant increase in total cytosolic Tau levels ($p=0.003$), as detected by the pan-Tau antibody Tau5 (that recognizes both phosphorylated and nonphosphorylated protein isoforms), which indicates a cytoplasmic accumulation of Tau. In addition, we observed a significant increase in cytosolic fractions of normalized levels of phosphorylated

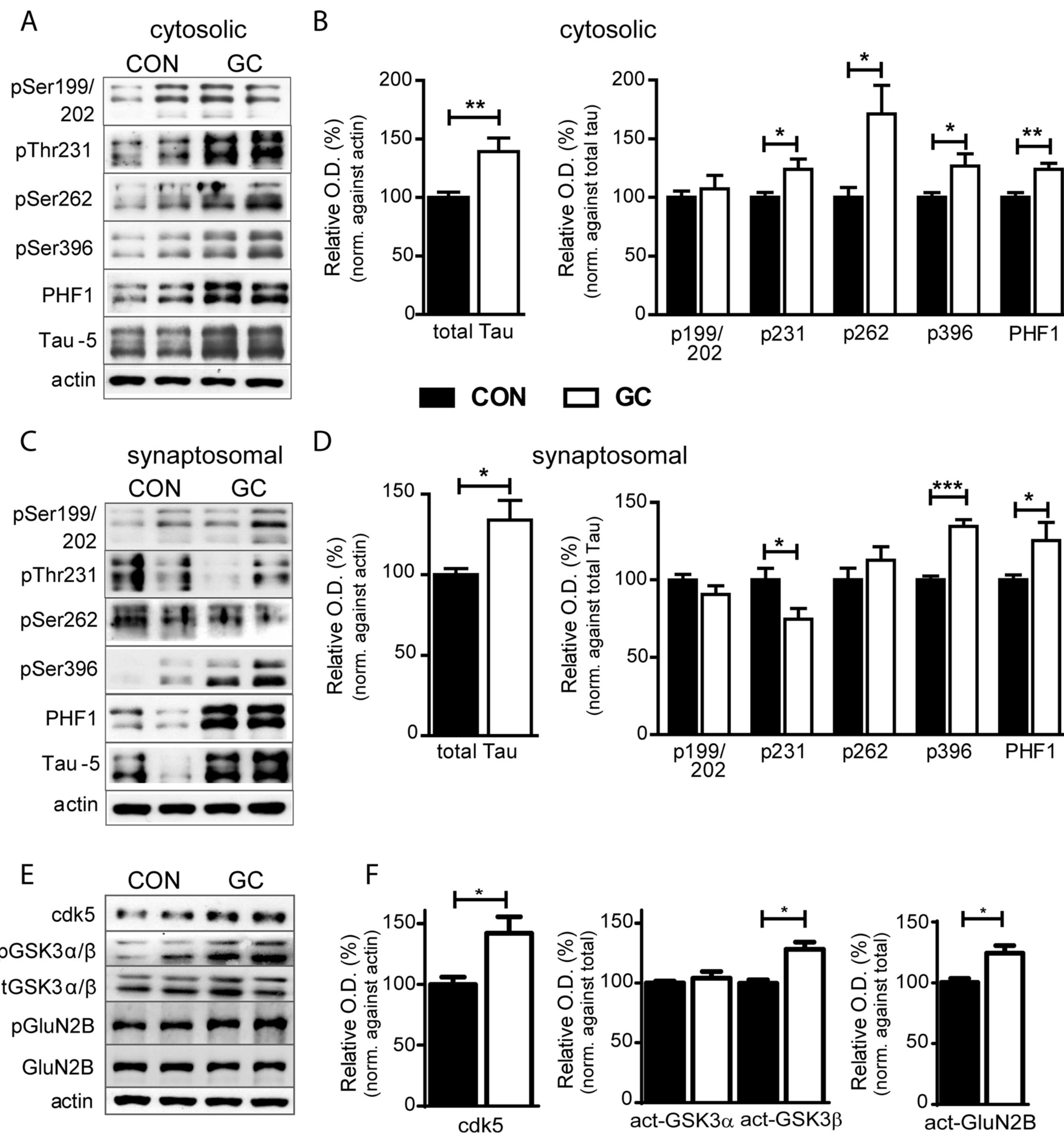

Fig. 2 GC-triggered Tau missorting at hippocampal synapses. **a**–**d** Subcellular distribution of Tau and assessment of its phosphorylation state at different biochemically separated fractions in control and GC-treated hippocampi. GC evoked an accumulation of total Tau protein as detected by increased Tau-5 levels in both cytosolic (**a**, **b**) and synaptosomal fractions (**c**, **d**). In addition, normalized levels of pThr231-Tau, pSer262-Tau, pSer396-Tau, and p396/404-Tau (PHF-1) were increased in cytosolic fraction of GC-treated hippocampi but no GC effect was detected on pSer199/202-Tau. In contrast, synaptosomal levels of pThr231-Tau were decreased by GC while pSer262-Tau remained unaltered. Similarly to cytosol, p-Ser396-Tau and PHF-1 Tau levels were increased after GC treatment while levels of pSer199/202 were not altered. **e**–**f** GC increased protein levels of two key kinases of Tau hyperphosphorylation, cdk5 and active-GSK3β (pTyr216-GSK3β) accompanied by elevated levels of p-Tyr1472-Glu2B receptors. Numeric data shown represent±SEM values as percentage of controls; *$p<0.05$; **$p<0.01$; ***$p<0.001$

isoforms, pThr231-Tau (p=0.023), pSer262-Tau (p=0.015), and pSer396 (p=0.03) in the hippocampus of GC-treated rats, but no effect of GC on pSer199/202 (Fig. 2a, b). Next, our analysis focused on the synaptosomal fraction where we found that overall Tau protein levels were also elevated by GC treatment (p=0.01; Fig. 2c, d) pointing to synaptic accumulation of Tau. Similar to the cytosolic fraction, levels of normalized pSer199/202-Tau were not altered by GC treatment, whereas pSer396-Tau was increased in the synaptosomal fraction ($p<0.001$). In contrast to the cytosolic compartment, pThr231-Tau synaptosomal levels were reduced after GC treatment ($p<0.02$), while no changes were detected on pSer262-Tau levels. Next, we monitored the protein levels of cyclin-dependent kinase 5 (cdk5) and glycogen synthase kinase 3 (GSK-3), two key kinases that essentially contribute to Tau hyperphosphorylation and malfunction and known to be

involved in regulation of synaptic function. We found that GC trigger increased protein levels of cdk5 (p=0.01) but did not change the total levels of the two isoforms of GSK3 (α and β). However, levels of active-GSK3β (phospho-Tyr216-GSK3β) were elevated by GC (p=0.001) while this GC effect was not true for active-GSK3α (phospho-Tyr279-GSK3α) (Fig. 2e). In the light of the recently suggested interaction of Tau with synaptic proteins and receptors [7], we also monitored the impact of GC on these proteins finding an clear increase in the levels of phospho-Tyr1472 GluN2R, a phosphorylation type suggestive to reflect activated GluN2R.

Ultrastructural Evidence of Tau Accumulation and Missorting in Dendrites and Spines of GC-Treated Hippocampus

As Tau intracellular localization and association to MTs and membranes seems to be regulated by its phosphorylation state [9, 11, 17, 18], we next monitor the localization and distribution of Tau, as well as its different phosphorylated forms, using transmission electron microscopy (TEM). As it was recently shown by us and others [8, 19], we found that Tau protein was detected at the rat hippocampal dendrites (Fig. 3 (I, IV)), as well as in synapses (Fig. 3 (II, III)). Moreover, as summarized in Table 1, we found that the density of total Tau was increased in both dendrites and synapses of hippocampal neurons of GC-treated animals (p<0.0001 for both); these findings confirm our molecular data obtained by WB analysis. In addition, chronic GC treatment resulted in increased density of pSer262-Tau at dendrites (p<0.001), but no significant alterations in synapses (p=0.10). Similarly, pSer396-Tau density in dendrites of hippocampus neurons was also significantly increased by GC treatment (p=0.044), but there was only a trend for an increase at the synaptic level. Note that p199/202-Tau levels were not affected by GC in both dendrites and synapses in line with our WB analysis findings. In addition, we found a characteristic PHF1 immunogold staining in hippocampal neurons of GC-treated animals (Fig. 2g (iv)), indicating the presence of Tau aggregates [20]. In summary, electron microscope analysis of different Tau isoforms (both phosphorylation-dependent and independent ones) suggest that GC trigger Tau accumulation and missorting in dendrites and spines of hippocampal neurons providing further support to the above described biochemical findings.

Discussion

Through its interaction with several cellular partners such as tubulin, F-actin, and Src family kinases, Tau seems to play an important role in mediating alterations in the cytoskeletal structure [21]. Besides its predominant localization at axons, several observations suggest the presence and accumulation of Tau at somatodendritic compartment (e.g., dendritic spines) using cellular or animals models of Tau overexpression [6, 7, 9]. The present study confirms the presence of endogenous Tau in dendrites and synapses of the hippocampus of wild-type, nontransgenic, rats, in line with previous cell culture and in vivo studies [8, 19]. In an extension of our previous findings [13, 14], we now show that GC trigger the accumulation of different isoforms of Tau in both dendritic and synaptic compartments, which is an indicator of neuronal malfunction and pathology. Indeed, accumulation of hyperphosphorylated Tau has been shown to result in destabilization of the dendritic cytoskeleton and compromised intracellular trafficking [22–24]. As we found no GC-evoked changes in mRNA levels of Tau (data not shown), which is in line with previous cellular and animals studies [14, 25], the source of this Tau accumulation could be attributed to GC-induced reduction of Tau turnover [14] probably involving diminished Tau degradation [26]; alternatively, changes in Tau phosphorylation state might disrupt MT-Tau interactions liberating Tau from MTs into the dendritic shafts from where it may diffuse to spines and even to the synaptic compartment [6].

The intracellular distribution of Tau is largely regulated by its phosphorylation status [9, 11, 17, 18]. In agreement with previous studies [13, 14], we found that GC triggers Tau hyperphosphorylation at specific sites (Thr231, Ser262, Ser396, and/or Ser396/404); for example, Tau phosphorylation at the pSer199/202 epitope is not affected by GC suggesting that GC impact of Tau phosphorylation is not global. Moreover, in contrast to pThr231-Tau which plays an important role in early AD pathology, hyperphosphorylation of the 199/202 and 212/214 epitopes of Tau appear at later stages of the disease [27]. Thus, our GC-driven findings may be relevant to the earliest stages of cytoskeletal disturbances where Tau is believed to contribute to synaptic dysfunction and atrophy. This notion is further supported by the fact that GC-triggered dendritic remodeling was limited to reduced dendritic branching and spine loss. It is also pertinent to mention that Tau hyperphosphorylation at epitopes Thr231 and Ser262, both altered by GC, correlates strongly with reduced microtubule binding capacity of Tau and therefore, cytoskeletal disturbances [28, 29]. Consistently, we observed that GC-evoked Tau hyperphosphorylation occurs in tandem with decreased levels of the stable detyrosinated microtubules, suggestive of reduced microtubule stability [30, 31] that may be causally related to the dendritic remodeling that occurs after stress [3, 32].

Interestingly, GC impact on Tau phosphorylation state exhibited a subcellular and distinct pattern. Normalized pThr231-Tau levels were increased in cytosol, but decreased in synaptosomal fraction while p262-Tau levels were increased only in cytosol (not in synaptic fraction detected by WB or at spines as monitored by TEM), suggesting a preferential subcellular localization and/or accumulation of different

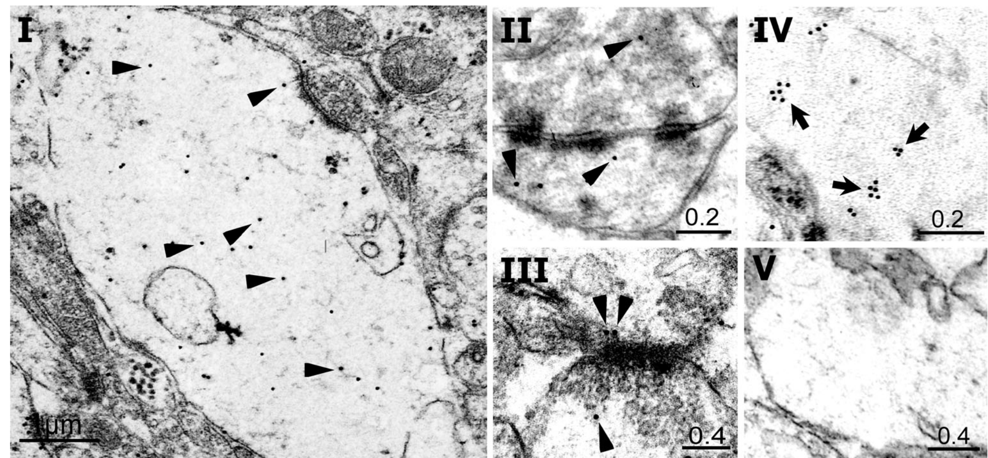

Fig. 3 Ultrastructural detection of different Tau isoforms in hippocampal dendrites and spines. Electron microscope image of Tau-immunogold in dorsal hippocampus showing that Tau protein is detected in dendrites (*I*) and synapses (*II*, *III*) followed by negative controls (*V*). In addition, note a characteristic form of PHF1-detected Tau immunostaining resembling to aggregates in GC-treated neurons

Tau isoforms in both intracellular compartments after GC treatment. It is suggested that different pools of Tau with different phosphorylation state exist within the neuron exhibiting potentially distinct roles. For example, blockage of Tau phosphorylation at Ser396/404 is necessary for Aβ-driven mislocation of Tau at synapses [6], while Tau phosphorylation at pThr231, but not at other epitopes, regulates its synaptic binding to PSD-95 [19]. Interestingly, we found that the levels of PSD95, a crucial protein for synaptic structure and function, were reduced by the exposure to GCs, consistent with our finding of synaptic loss using Golgi-based neuronal structural analysis. Moreover, the reduction of PSD95 levels was accompanied by GC-driven decrease in levels of pThr231-Tau within synaptosomal fraction, further supporting the previously suggested interrelationship between this phospho-Tau isoform and PSD95 [6]. Note that, with one minor exception (p396-Tau), similar results regarding the differential distribution of phospho-Tau isoforms in different parts of the cell were obtained using two different technical approaches (electron microscopy and WB analysis of different subcellular fractions) (see Fig. 2d and Table 1). Nevertheless, the complementary approaches (subcellular fractionation-based biochemical analysis and in-situ ultrastructural detection) allowed a detailed "mapping" of the presence of Tau in different intracellular compartments. Our future efforts will aim to clarify the role of GC on the intraneuronal dynamics of Tau, with particular focus on individual phosphorylation epitopes as well as on the mechanisms through which GC and/or stress interfere with the Tau sorting and clearance machinery in cytosolic and synaptic compartments.

Recent studies show that Tau mislocation at dendritic spine is a common feature observed in AD [11] as well as other Tauopathies (FTP, FTDP-17) [9]; in parallel, increasing attention and support has been given to the essential involvement of Tau missorting in spine toxicity and synaptic pathology [19, 33]. A previous study demonstrated that local Tau missorting induced by Aβ or glutamate was followed by local spine loss and cytoskeletal disruption [24]; importantly, this Tau mislocation at synapses are shown to depend on Tau hyperphosphorylation [19, 34]. Besides Tau hyperphosphorylation, GCs are known to trigger APP misprocessing and Aβ generation [14, 25, 35] while Aβ is shown to trigger Tau hyperphosphorylation by inducing different kinases such as GSK3β and cdk5 [36]. While further studies are needed to clarify the interplay between Aβ, glutamate, and Tau in stress/GC-triggered neuronal remodeling and spine the notion atrophy, the present study provides evidence that supports that GC can also influence the intracellular trafficking of specific phospho-Tau isoforms and their missorting in synapses. Indeed, as previously suggested by studies in animal models of AD, abnormal Tau hyperphosphorylation and synaptic

Table 1 Quantification of immunogold analysis of different phospho-Tau isoforms and total Tau

	Dendritic density		Synaptic density	
	CON	GC	CON	GC
Total Tau (#/μm^2)	10.36±0.52	14.20±0.50***	5.33±0.68	10.48±0.73**
p199/202-Tau (#/μm^2)	11.41±0.69	12,78±1.32	11.74±2.15	9.46±1.46
p262-Tau (#/μm^2)	12.23±0.48	24.19±1.75***	8.82±1.98	6.87±1.82
p396-Tau (#/μm^2)	7.15±0.49	9.42±0.71*	4.46±0.69	5.99±1.20

Electron microscope-based density (per μm^2) of total Tau and different phosphorylated forms of Tau in both dendrites and synapses of hippocampal neurons. Numerical data represent mean±SEM; *p<0.05; **p<0.01; ***p<0.001

missorting results in damage to synaptic structure and function, including postsynaptic receptor targeting and function of excitatory glutamate receptor in dendritic spines and leading to dendritic spine loss.[7, 9, 37]. In line with this, we here showed that prolonged exposure to elevated GC levels upregulate active (phosphoTyr1472) GluN2B receptor units and subsequently, reduced levels of synaptic scaffold/anchor proteins, such as PSD-95 and Shank, and loss of spines. These findings provide new insights into the cellular cascades triggered by GC and, in particular, highlight the potentially important role of Tau hyperphosphorylation in neuronal and synaptic malfunction and atrophy underlying prolong exposure to elevated GC levels and related hippocampal pathology.

In summary, the results reported here represent the first description of GC-induced Tau missorting as a mechanism(s) underlying synaptic atrophy and damage, beyond Alzheimer's disease pathology adding to our knowledge about the poorly understood cellular cascades responsible for stress-related brain pathology.

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Conflict of Interest None of the authors report competing interests.

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CHAPTER 3

CHRONIC STRESS TRIGGERS TAU AGREGGATION THROUGH AUTOPHAGY BLOCKAGE AND STRESS GRANULES INDUCTION.

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Chronic stress triggers Tau pathology through autophagy inhibition and induction of stress granules

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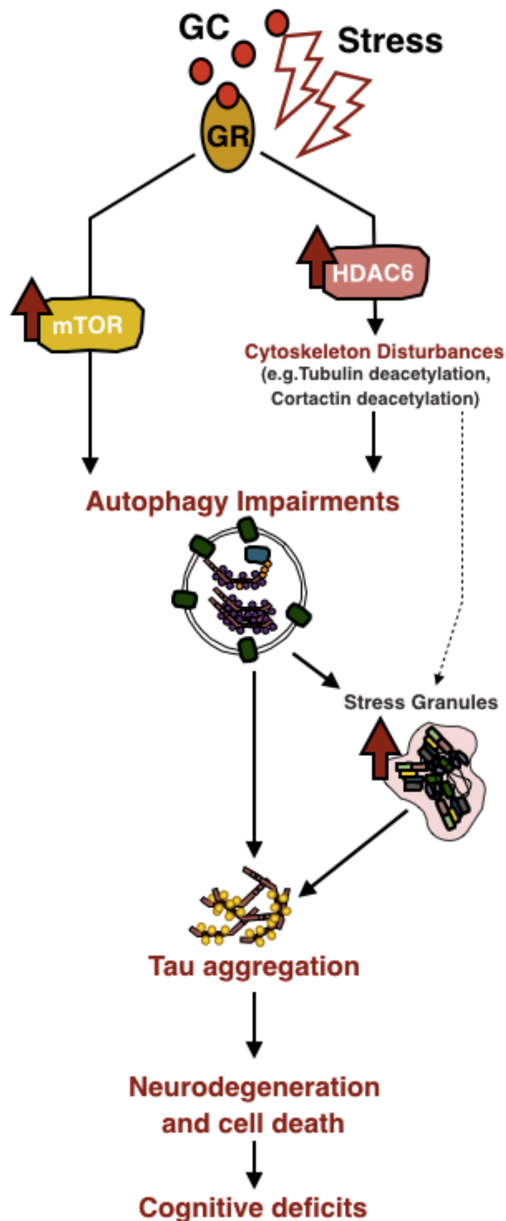
Abstract

Imbalance of neuronal proteostasis associated with misfolding and aggregation of Tau protein is a common neurodegenerative feature in Alzheimer's disease (AD) and other Tauopathies. Consistent with suggestions that lifetime stress maybe an important precipitating factor of AD, we previously reported that environmental stress and high glucocorticoid (GC) levels evoke accumulation of aggregated Tau; however, the underlying molecular mechanisms remain unclear. We now demonstrate that chronic stress and GC trigger an mTOR-dependent inhibition of autophagic process, the cardinal clearance pathway for aggregated proteins, leading to accumulation of Tau aggregates and cell death in mice and cells stably expressing P301L-Tau. Considering the interplay of autophagy with Stress granules (SGs) dynamics, we also show that environmental stress/GC stimulate the induction of SGs, recently shown to promote Tau misfolding, aggregation and neurotoxicity. Notably, pharmacological intervention that stimulates autophagic process (Temsirolimus) attenuates the GC-driven elevation of Tau, SGs and cell death. This work provides novel insights into the mechanisms through which neuronal cells convey the detrimental impact of prolong environmental (HPA-related) stress to intracellular "stress" signaling, causing Tau-driven brain pathology.

Synopsis



Silva et al., demonstrates that prolong exposure to environmental stress and high levels of stress hormones, glucocorticoids (GC), trigger Tau aggregation pathology by the distinct, but inter-relative, cellular cascades of stress granule (SG) formation and mTOR-related autophagy inhibition. Pharmacological stimulation of autophagy attenuated the GC-driven Tau and SG pathology and neurotoxicity pointing to autophagy as an important target.

- Chronic stress and/or prolong GR signaling evokes induction of Histone deacetylase 6 (HADC6) and subsequently reduced acetylation of proteins (e.g. tubulin and cortactin) related to cytoskeletal instability.
- Chronic stress/GC blocked autophagic process in an mTOR-dependent manner in parallel to stress granule formation favoring Tau aggregation and neurotoxicity.
- mTOR-targeted induction of autophagy blocked GC-driven Tau and SG pathology offering neuroprotection.

Introduction

Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder with a complex physiopathology and still undefined triggers. Several risk factors have been associated with the pathology, with recent evidence supporting the role of lifetime stress and main stress hormones, glucocorticoids (GCs) (Launer et al. 1999; Sotiropoulos, Cerqueira et al. 2008). Clinical studies relate distress, high cortisol levels and dysfunction of hypothalamus-pituitary-adrenal (HPA) axis with poor memory scores and earlier disease onset in AD patients (Hatzinger et al. 1995; Rasmuson et al. 2001; J G Csernansky et al. 2006; Simard, Hudon, and van Reekum 2009), highlighting the potential implication of chronic stress and GC in the pathogenesis and/or progression of the disorder. In line with the above clinical evidence, experimental studies from our and other teams have shown that chronic stress and/or exposure to high GC levels, trigger Tau hyperphosphorylation and malfunction, leading to its accumulation and formation of neurotoxic aggregates precipitating AD brain pathology (Green et al. 2006; Sotiropoulos et al. 2011; Sotiropoulos et al. 2014). Despite the exact molecular mechanism that underpin stress-driven pathology remain unclear, experimental evidence suggests that stress/GC reduce Tau turnover (Sotiropoulos et al. 2008), indicating that reduced degradation of Tau may be involved, through dysregulation of molecular chaperones responsible for cellular proteostasis (Sotiropoulos et al. 2014).

Impaired proteostasis associated with misfolded and aggregated Tau has been implicated in increased neuronal vulnerability and neurodegeneration in AD. Furthermore, AD brain is characterized by a massive accumulation of autophagic vacuoles and defects at different steps of the autophagic-lysosomal pathway (Boland et al. 2008; Lee et al. 2010; Armstrong et al. 2014), such as initiation, elongation and maturation. The (macro)autophagy process is critically controlled by several evolutionarily-conserved molecules e.g. the microtubule-associated protein light chain 3 (LC3), autophagy receptor p62 as well as (mammalian target of rapamycin) mTOR (Mizushima 2010; Di Bartolomeo et al. 2010); the latter has been suggested as therapeutic target against AD pathological aggregation and related neurotoxicity (Caccamo et al. 2013; Caccamo, Medina, and Oddo 2013; Vidal et al. 2014). Moreover, inhibition of autophagy-lysosome pathway is also shown to impair the degradation and dynamics of Stress granules (SGs) (Seguin et al. 2014). SGs are dense cytoplasmic aggregates of mRNAs and RNA-binding proteins (RBPs) formed under cellular stress (e.g. heat shock stress) with various proteins being identified as SG-related proteins e.g. T cell intracellular antigen 1 (TIA1), Fused in Sarcoma protein (FUS), GTPase-activating protein-binding

protein 1 (G3BP) (Wolozin 2012). Despite its cytoprotective function, SG formation can become neurotoxic under prolong induction and, therefore, SGs were recently suggested to accelerate Tau aggregation in AD and other Tauopathies contributing to Tau-related neurodegeneration and toxicity (Vanderweyde et al. 2012; Vanderweyde et al. 2016). As mounting evidence demonstrates the selectivity of autophagy in the degradation of Tau aggregates, we hereby monitored the impact of chronic stress and GC on autophagic process and its interplay with SGs mechanisms towards precipitation of Tau pathology.

Material and Methods

Animals and stress protocol

P301L-Tau transgenic female mice, 7-9month old expressing mutated (P301L) human Tau under the CAMKII promoter were used in this study. All experiments were conducted in accordance with the Portuguese national authority for animal experimentation, Direcção Geral de Veterinária (ID: DGV9457). Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and Council. Mice were housed in groups of 4-5 per cage under standard environmental conditions (lights on from 8 a.m. to 8 p.m.; room temperature 22°C; relative humidity of 55%, *ad libitum* access to food and water).

Animals were subjected to chronic unpredictable stress protocol over a period of four weeks before the behavioral testing. The protocol consists of different stressors such as overcrowding, rocking platform, restrain, hair dryer (one stressor per day) that were chosen in a random order to prevent habituation. Stressors were also applied during the behavioral testing period (two hours after animals complete the behavioral task of the day). Biometric evidence of efficacy of the stress protocol were obtained based on measurements of daytime serum corticosterone levels (monitored by a radioimmunoassay kit from ICN, Costa Mesa, CA) and body weight changes. All stressed animals showed significant elevations in daytime serum corticosterone levels ($p<0.05$) and net loss of body weight ($p<0.05$) reflecting the stress efficacy (see **Figure 1**).

Behavior Tests

Open Field (OF) test was conducted in an arena (43.2 cm43.2 cm) with transparent acrylic walls and white floor (Med Associates Inc., St. Albans, VT, USA). Mice were placed in the center of the arena and movement was monitored over a period of 5 min with the aid of two 16-beam infrared arrays. Time spent in the center of the arena was used as an index of anxious behavior. Total distance traveled was used as an indicator of locomotor activity

Elevated-Plus Maze (EPM) was used to access anxious behavior. 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 for 7 min as previously described (Tanemura et al. 2002). 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).

Y-Maze test was used to assess PFC-dependent working memory based on spontaneous alternation task, using the Y-maze apparatus (33cm x 7cm x 15cm). Briefly, animals were placed in the center of the maze and allowed to move freely through the maze during an 7 min session. The number and order of arm entries was recorded. Spontaneous alternations were calculated as the ratio of number of triads (sequence of three consecutive arm entries) and total arm entries.

ReversL Learning in Morris Water Maze (MWM). The test apparatus consists of a swimming circular pool (1m diameter) filled with water (24°C) made opaque with a white bio-safe dye. The cylinder contained a slightly submerged transparent escape platform and placed in a room with landmark (reference) objects. Learning trials (9 days; 3 trials/day; 60-s trial period) start by gently placing mice on the water surface close to the cylinder wall. After subjected to Probe test, animals were tested for reversal learning task, where the platform is moved to the opposite quadrant of swimming pool. Animals performed four trials. Swim paths during these tests were monitored and recorded by a CCDcamera, while data were analyzed using customized software based on Matlab (version 7.2, Mathworks Co Ltd, CA), with an image analysis tool box (Mathworks). Learning was assessed by measuring the distance the animal took to reach the platform (Kimura et al. 2007; Kimura et al. 2010).

Contextual Fear Conditioning was conducted in chambers with dimensions of 20 cm wide, 16 cm deep and 20.5 cm high (Med Associates, St. Albans, VT). A light (CM1820 bulb) mounted directly above the chamber provided illumination. Each chamber was located inside a larger, insulated white plastic cabinet that provided protection from outside light and noise. The behavior of mice was recorded by a video camera and freezing behavior was manually scored using Kinoscope software. Freezing was defined as the complete absence of motion. The fear conditioning procedure was conducted over two sequential days. On day 1, mice were placed in the conditioning white chamber (Context A) and received 3 pairings of light (20 sec) and a co-terminating electrical shock (2 sec, 0.5 mA). The chambers were cleaned with 10% ethanol between animal trials. On day 2, animals were placed in the familiar chamber, context A, in the absence of the light-shock pairings; freezing behavior was measured during 3 min. After this, the animals returned to their home cage. Two hours later, the animals were placed in a new context (context B) and freezing behavior was measured for 3 min, after which the animals returned to their home cages. The context B trial was different from context A in several ways: i) the floor and walls of the chamber were covered by black

plastic inserts; ii) the chamber was scented with vanilla; iii) the chamber ventilation fan was turned on; iv) the experimenter wore a different style and color of gloves, mask and lab coat; v) the chambers were cleaned with H2O between trials; vi) mice were kept in a different holding room before testing and transported in a different cage; vii) the lights of the experimental room were turned on.

Biochemical fractionation and immunoblotting

Hippocampus and medial prefrontal cortex (PFC) were dissected (on ice) and immediately stored at –80°C. For detecting insoluble Tau, we followed a sarkosyl-based fractionation protocol as previously described (Sahara et al. 2002; Kimura et al. 2010). After homogenization in Tris-buffered saline (10mM Tris, 150mM NaCl) with protease and phosphatase inhibitors, lysates were centrifuged at 100.000g. Sarkosyl-insoluble, paired helical filament-enriched fractions were prepared from this pellet 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. 10% Sarkosyl (Sigma, #L-5125) solution was added to the supernatant, and after incubation at 37°C (1h), and centrifugation at 150.000g, the resulting pellet was analyzed the Sarkosyl-insoluble fraction. As insoluble SGs cannot be detected in sarkosyl-insoluble fractions (Vanderweyde et al. 2012), we separate soluble and insoluble SGs homogenizing samples in RIPA buffer (50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM EGTA, protease and phosphatase inhibitors) as previously described (Vanderweyde et al. 2016). Then, homogenates were layered onto 0.32 M sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl, 0.8 M NaCl, 1 mM EGTA) and were centrifuged (20400g, 10 min, 4°C). The supernatants were ultracentrifuge at 112.000g (60 min; 4°C). The supernatant (soluble fraction) was collected and stored at -20°C. The pellet was dissolved in 1% SDS/RIPA buffer and ultracentrifuge at 112000g (60 min; 4°C; 2 times) followed by 1% SDS/TBS buffer dissolution and ultracentrifugation (60 min; 20°C). The final pellet was dissolved in 70% formic acid and centrifuged at 20400g (10 min; 20°C). Then, the supernatant was concentrated in speed vacuum and the resulting pellet dissolved in sample buffer and neutralized with 1.5M Tris-HCl providing the insoluble SG fraction.

The various fractionated samples were electrophoresed using SDS-PAGE gels and semi-dry transferred onto nitrocellulose membranes (Trans-Blot Turbo blotting system, BIORAD). Membranes were blocked with 5% nonfat dry milk in TBST-T buffer and then incubated with the following antibodies: actin (1:2500; ABCAM, #ab8224), LC3 (1:1000; Novus Biologicals, #100-233), SQMTS1/p62 (1:1000;Novus Biologicals, #H00008878-M01), S6K total (1:750; Cell Signaling,

#2708), p-S6K (1:750; Cell Signaling, #9205), p38 total (1:750; Cell Signaling, #9212) and p-p38 (1:750; Cell Signaling,#4511); Tau5 (1:2000; ABCAM, #ab80579), JM (1:1000; kindly gift by Dr A. Takashima, Japan), TLS/FUS (1:500; ABCAM, #84078), EWRS1 (1:1000; ABCAM, #133288), DDX5 (1:1000; ABCAM, #21696), TIA-1 (1:500; ABCAM, #40693), PABP (1:500, ABCAM #21060), tubulin (1:5000, SIGMA #9026), Ac-tubulin (1:1000, ABCAM #24610), Cortactin (1:250, ABCAM #81208) and Ac-cortactin (1:10000, Millipore #09881). After incubation with appropriate secondary antibody, antigens were revealed by ECL (Clarity, Bio-Rad), and signal quantification was achieved using a ChemiDoc instrument and and ImageLab software (Bio-Rad). All values were normalized and expressed as a percentage of control values.

Immunohistological and stereological analysis

As previously described (Kimura et al. 2007), deeply anesthetized animals were transcardially perfused with saline and PFA (4%). After post-fixation, brains were embedded in paraffin and sectioned (4 mm) in coronal plane. Deparaffinised sections were exposed to antigen retrieval by citrate buffer, followed by 0.3% triton X-100 before incubation with antisera against LC3 (1:500; Novus Biologicals) and SQMTS1(p62) (1:250; Novus Biologicals) and the appropriate secondary antibodies, followed by regular DAB protocol. Stained cells were counted and densities were quantified and normalized for total area using the Olympus BX 51 stereological microscope and the Visiopharma integrator system software. For immunofluorescence, tissue sections were deparaffinized, rehydrated and antigen retrieval was performed using citrate buffer (Thermo Scientific). Furthermore, slices were blocked with blocking Solution (5% BSA in TBS-Triton X-100 (0.25%) + 5% normal donkey serum) for 1.5h (RT). Then, incubation with primary antibody in blocking solution was performed overnight at 4ºC, and the appropriate secondaries were used. DAPI staining (1:1000; 10 min) was performed for nuclear staining and then mounted with Immu-Mount (Thermo Scientific, Waltham, MA, USA). The following primary antibodies were used: TIA-1 (1:500; Abcam, #40693), TLS/FUS (1:200, Abcam, #84078) and Tau5 (1:500; ABCAM, #ab80579). Images were collected and analysed by confocal microscopy (Olympus FluoViewTMFV1000). Neuronal densities of hippocampal and PFC areas (DG, CA1 and PrL) were stereologically estimated by counting neurons in cresyl-violet stained serial coronal brain sections, using Neurolucida software (MBF Bioscience, Williston, VT) as previously described (Bessa et al. 2009; Pinheiro, Silva et al. 2015).

P301L-Tau-SHSY5Y cells, treatments and molecular analysis

These studies used human neuroblastoma cell line (SH-SY5Y) stably transfected with P301LhTau-EGFP (Tau40, 2N4R) (kind gift from Professor Juergen Gotz, University of Queensland, Australia). Cells were maintained in DMEM supplemented with 10% FBS 1% G-max and 1% antibiotic (all reagents obtained by Invitrogen); for selection purposes, 3ug/mL Blasticidin S hydrochloride (Sigma, #15205) was added in the medium. Cells were maintained at 37°C and 5%CO_2 while for all experiments, cells were placed on gelatin-coated plates and differentiated for 6-7 days with all-trans retinoic acid (10^{-5}M; Sigma) in differentiation medium (DMEM supplemented with 1% FBS, 1% antibiotics and 1% glutamax). Dexamethasone (DEX; Fortecortin®, Merck, Darmstadt, Germany) was used at a final concentration of 10^{-6}M for 48h, as previously described(I Sotiropoulos, Catania, et al. 2008). Temsirolimus (CCI-779, LC Laboratories; 100uM) was added to the medium 4 hours before the GC addition and kept in the medium during the entire GC treatment; as previously shown (Menzies et al. 2010; Jiang et al. 2014), CCI-779 is not toxic at this concentration. At the end of treatment, Cell viability was assessed MTS assay using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) based on manufacturer instructions. Triplicates of each condition were used and experiment were repeteaed three times. Absorbance was measured at 490nm in an ELISA reader using Microplate Manager 6 software (BioRad). For WB analysis, cell homogenates were prepared in RIPA buffer (50mM Tris HCl, 2mM EDTA, 250mM NaCl, 10% glycerol, proteinase and phosphatase), Aftre sonication and centrifugation (15min; 14.000rpm; 4°C), supernatant samples were analyzed by WB as described above. For IF analsis, cells were cultured in gelatin-coated glass coverslips and fixed in 4%PFA. After permeabilization with 0.1% TritonX-100/PBS, cells were incubated overnight with primary antibodies: LC3(1:200; Novus Biologicals, #100-233) and SQMTS1(p62) (1:200; Novus Biologicals, #H00008878-M01), LAMP2a (1:250; ABCAM, # ab25631), Tau-5 (1:1000; ABCAM, #ab80579), TLS/FUS (1:300; ABCAM, #84078), G3BP (1:500; ProteinTech, # 13057-2-AP), TIA-1 (1:300; ABCAM, #40693), HDAC6 (1:250; ABCAM, #ab1440). After appropriate fluorescence-conjugated secondary antibodies (RT, 30 min) and DAPI staining, cells were analyzed by laser confocal microscopy (Zeiss LSM 510, Carl Zeiss Microimaging, Goettingen, Germany).

Statistical Analysis

Numerical data is expressed as group mean±SEM. All data were evaluated by one-way ANOVA or Student's *t*-test using GraphPad 6.0; differences were considered significant if $p < .05$.

Results

Chronic stress triggers accumulation of neurotoxic Tau aggregates in hippocampus and prefrontal cortex of P301L-Tau Tg mice.

Hippocampus and prefrontal cortex (PFC) are among the first brain áreas, in patients suffering from Alzheimer's disease or other Tauopathies, that exhibit the characteristic accumulation of different abnormal Tau forms (e.g. hyperphosphorylated, truncated and misfolded Tau), which cumulatively precipitate Tau aggregation into neurotoxic insoluble deposits; the later closely correlate with cognitive impairment (Poorkaj et al. 1998; Spillantini TD; Ghetti B 1998; Giannakopoulos et al. 2003). Previous studies have shown that stress and GC triggers aberrant hyperphosphorylation, missorting and misfolding of Tau providing some mechanistic elucidations (Green et al. 2006; Sotiropoulos, Catania, et al. 2008; Lopes et al. 2016). For clarifying the impact of chronic stress on Tau aggregation, the underlying cellular mechanism(s) and its significance on behavioral deficits, the current study used transgenic mice expressing human Tau carrying the aggregation-prone P301L-Tau mutation and subjected them to chronic unpredictable stress.

Stressed P301L-Tau animals exhibited a clear decrease in body-weight in comparison to control (non-stressed) animals (**Figure 1A**) while they also show elevated levels of the stress hormone, corticosterone (**Figure 1A**), providing a clear confirmation of stress protocol efficacy. To access hippocampus- and PFC-dependent cognitive performance, Contextual Fear Conditioning test (CFC), reversal Learning test and Y-maze (YM) were used. While both animal groups showed similar freezing levels in pre-training session of context A, stressed animals exhibited lower levels of freezing the test day (Context A) compared to control animals indicating deficits of associated memory (**Figure 1B**). Note that the stress-driven difference disappears when both groups were tested in another context, non-associated with adverse stimulus (context B) (**Figure 1B**). Furthermore, animals' behavioral flexibility and working memory function were tested using reversal learning and Y-maze test, respectively. During reversal learning test, stressed animals exhibit an increase in the time to reach the platform than control animals, suggesting cognitive deficits (**Figure 1C**). In the Y-maze, stressed mice presented a decrease in the percentage of spontaneous alternations among different arms of the Y-maze apparatus (**Figure 1D**), pointing towards a stress-driven impairment in working memory.

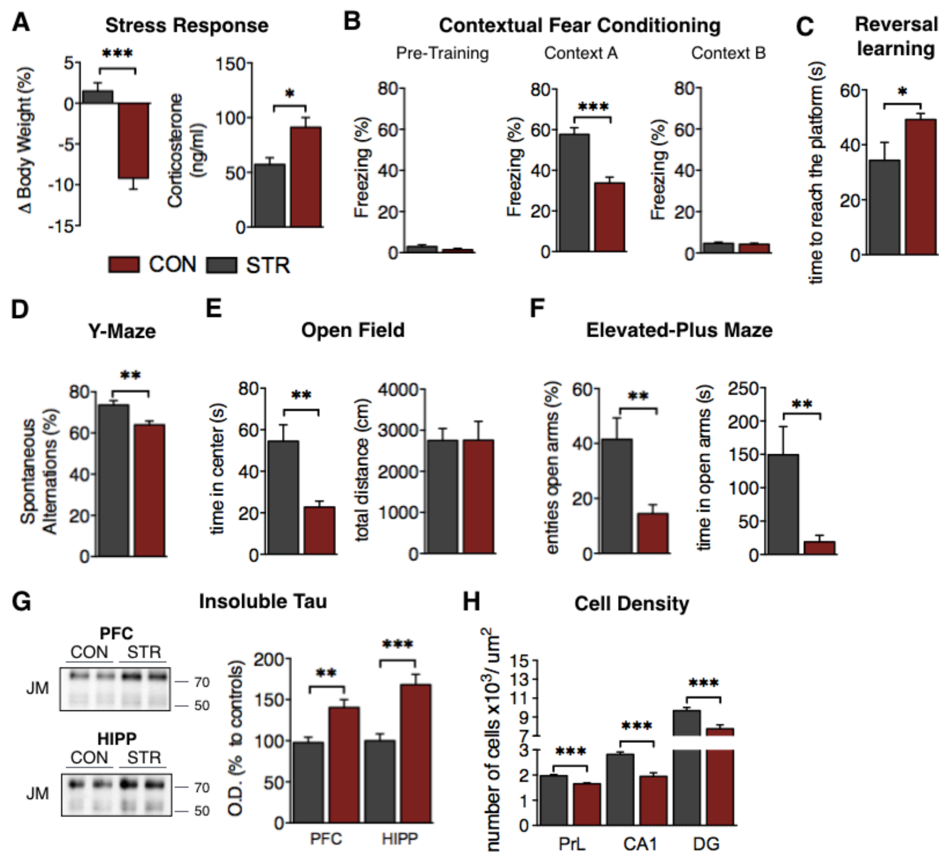


Figure 1. Chronic stress evokes accumulation of neurotoxic Tau aggregates causing cognitive and emotional deficits in P301L-Tau Tg mice. A. P301L-Tg mice exhibited reduced body weight (*p*=0.005) and increased corticosterone (*p*<0.0001) levels after chronic environmental stress. **B.** In contrast to pre-training session (*p*=0.1718), stressed animals exhibited a significant decrease in percentage of freezing time in the test section (context A) of CFC in comparison to control animals indicating associative memory impairment (*p*=0.0002); note that both animal groups exhibit similar freezing levels in context B which is not associated with an adverse stimulus (*p*=0.6406*)*. **C.** Chronic stress increased the time that animals swum to reach the new (opposite) place of the escaping platform indicating PFC-dependent deficits of behavioral flexibility (*p*=0.046). **D.** Stress also reduced percentage of spontaneous alternations in the arms of a Y-maze as compared with control animals pointing to deficits of working memory (*p*=0.0075). **E-F.** Whereas no different in total distance travelled by animals in OF apparatus (*p*=0.9882), stressed animals exhibited a decrease in time spent in the center of the OF arena (*p*=0.0036) (**E**) followed by reduced time (*p*=0.0041) and entries (*p*=0.0019) that animals spend in the open arms of EPM apparatus (**F**); these behavioral parameters suggest increased anxious levels in stressed animals compared with controls. **G-H.** Chronic Stress elevated the levels of insoluble Tau in both PFC and Hippocampus of P301L-Tau mice (Hipp: *p*=00.28; PFC: *p*=0.0005) (**G**); an effect that was accompanied by decreased cell density in PFC (prelimbic cortex; PrL: *p*<0.0001) and hippocampus (DG: *p*=0.0007; CA1: *p*<0.0001) (**H**). All numeric data are represented as mean ± SEM, **p*<0.05; ***p*<0.01; ****p*<0.001.

As neuropsychiatric symptoms, such as anxiety, are frequently observed in AD (Jost and Grossberg 1996), we also evaluate anxious behavior using open field (OF) and elevated-plus maze (EPM). We found that chronic stress decreased the time that animals spent in the center of the OF arena (**Figure 1E**) as well as reduced both animal time and entries in the open arms of EPM apparatus (**Figure 1F**). Note that stress didn't cause any change in locomotion as assessed by total distance travelled in the OF apparatus (**Figure 1E**). The above stress-driven behavioral deficits in P301L-Tau Tg animals were accompanied by increased levels of Sarkosyl-insoluble Tau, in both hippocampus and PFC, of stressed animals as measured by Western Blot analysis (**Figure 1G**). These aggregates are biochemically like those found in the neurofibrillary tangles that characterize AD and other Tauopathies (Wang and Mandelkow 2015). Furthermore, P301L-Tau aggregates are shown to exhibit a major part of neurotoxicity of Tau pathology (Kimura et al. 2010), which is in line with the reduction of cell density that stressed P301L-Tau animals exhibited in hippocampus (CA1 and DG) and PFC (prelimbic cortex) when compared to control P301L-Tau animals (**Figure 1H**).

Stress-driven inhibition of autophagic process through mTOR activation.

A growing body of research has connected autophagy to neurodegenerative pathology, while autophagic clearance has been suggested to exhibit selectivity for the degradation of Tau aggregates (Hamano et al. 2008; Y. Wang et al. 2010; Nassif and Hetz 2012). Accordingly, we have monitored essential molecules involved in the autophagic process (**Figure 2A**). First we measured the levels of LC3 and p62, two sensitive indicators of autophagic activity. Molecular analysis of hippocampus and PFC of P301L-Tau animals showed that chronic stress reduced the levels of LC3 in parallel to increased levels of p62 (**Figure 2B**), suggesting that autophagic activity was significantly reduced. Furthermore, LC3 and p62 staining confirmed the above western blot findings as assessed by reduced density of LC3+ cells and increased p62+ (**Figure 2C**). As many studies describe an essential role of mTOR in protein homeostasis (Wullschleger, Loewith, and Hall 2006) through its involvement in the initiation of autophagic process (**Figure 2A**), we assessed mTOR activity by analyzing direct targets of mTOR, such as p70(S6K) and p38, which are phosphorylated when mTOR is active (**Figure 2A**). We found that chronic stress increased the levels phosphorylated S6K and p38 (**Figure 2D**) with no alterations in the total levels of these proteins. Altogether, these findings suggest that exposure to chronic stress inhibited autophagic process probably at the level of autophagy induction (mTOR-related), that consequently may lead to a potential blockage in the vesicle assembly and sequestration of substrates to the autophagic process (**Figure 2A**).

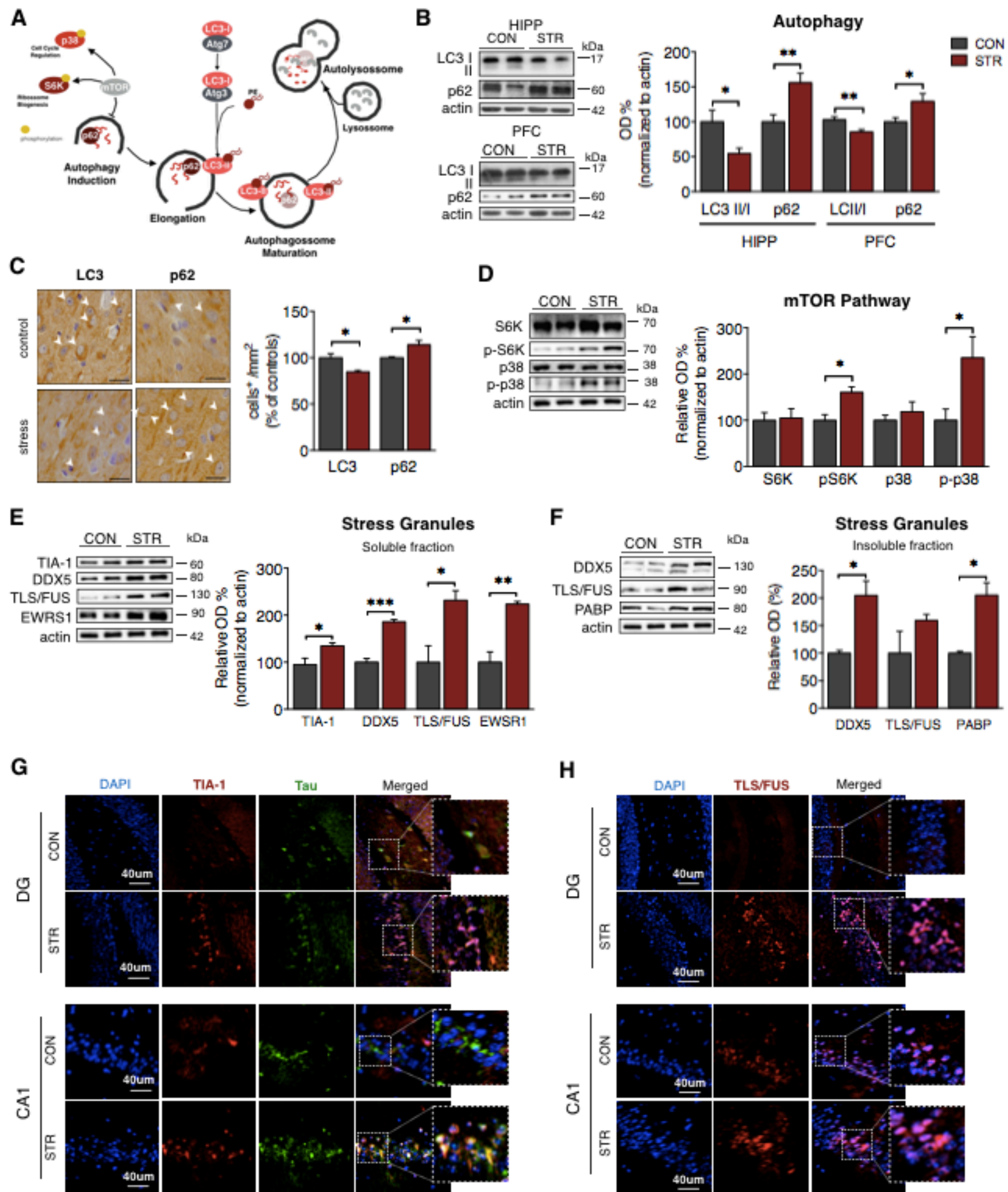


Figure 2. Prolong exposure to enviromental stress inhibits autophagic process and induces Stress granules. A. Schematic representation of autophagy highlighting the role of mTOR, LC3 and p62 in this celullar process. **B-C.** Stressed animals exhibit reduced LC3 (*PFC:* p=0.0036; *HIPP:* p=0.0269)and increased p62 (PFC: p=0.0291; HIPP: p=0.0032)) protein levels as assessed by WB analysis (**B**); these findings were confirmed by corresponding changes in LC3+ (p=0.0276) and p62+ (p=0.0213) cell densities, (**C**) indicating a stress-driven inhibition of autophagic process. **D.** In line with the above findings, the levels of phospho S6K (p=0.0146) and p38 (p=0.0305) proteins were increased by stress, which are indicative of an active mTOR. **E-F.** Chronic stress triggered an increase in the protein levels of several SG markers in both soluble (**E**), TIA-1 (p=0.0133), TLS/FUS (p=0.0173), DDX5 (p=0.0004) and EWRS1 (p=0.0055), as well in insoluble fraction of P301L-Tau mice (**F**), DDX5 (p=0.0201) and PABP (p=0.0110), with no differences observed in TLS/FUS (p=0.0896). **G-H.** Stress causes the appearance and accumulation in cytoplasm of the

SG marker TIA-1 (**G**) and increase in TLS/FUS staining (**H**). All numeric data are represented as mean ± SEM, *p<0.05; **p<0.01; ***p<0.001.

Induction of Stress granule formation by chronic stress in P301L-Tg mice

Besides autophagy, the cellular stress response to the neurodegenerative burden caused by the accumulation of Tau also integrates other mechanisms, with SG formation being among the ones promoting Tau assembly and aggregation (Wolozin 2012; Vanderweyde et al. 2012). As inhibition of autophagy-lysosome pathway is recently shown to impair the degradation and dynamics of Stress Granules (SGs) (Seguin et al. 2014), we next monitored different SG proteins as well as the formation of SGs in P301L-Tau animals under control and stressful conditions. Western blot analysis evaluated cytoplasmic levels of different SGs markers in both soluble and insoluble fractions of P301L-Tau mice homogenates. In line with previous studies (Vanderweyde et al. 2012), we found that control P301L-Tau animals also exhibit SG proteins such as TIA-1, TLS/FUS, EWRS1, DDX5, PABP (**Figure 2E-H**), which indicates the presence of cellular stress conditions probably due to the accumulation of pathological Tau. In addition, chronic stress significantly elevated the levels of TIA-1, DDX5, EWRS1 and TLS/FUS in the soluble fraction (**Figure 2E**), while a similar stress-driven increase was found for TLS/FUS, DDX5 and PABP in the insoluble fraction (**Figure 2F**). Note that TIA-1 was not detected in the insoluble fraction in agreement with previous work (Liu-Yesucevitz et al. 2010; Vanderweyde et al. 2012). Confirming the above results, immunofluorescence staining demonstrated that chronic stress increased TIA-1 (**Figure 2G**) in both DG and CA1 region of the hippocampus, and lead to an increase in the co-localization between TIA-1 and PHF-1 Tau, and we can also observe an increase in the extranuclear staining of TIA after stress, as previously described. We also observe an increase in TLS/FUS staining in both hippocampal regions after stress (**Figure 2G**). Thus, these findings suggest that chronic stress triggers the induction of aggregation-related SG pathology in P301L-Tau mice and accumulation of different SG proteins and their insoluble inclusions.

Glucocorticoids mimic the stress-driven effect on aggregation-enhancing neurodegenerative cascades *in vitro.*

Even though the detrimental effects of chronic stress on neuronal and brain structure and function are largely attributed to glucocorticoids (GC) (de Kloet, Joëls, and Holsboer 2005), and previous work point toward the role of glucocorticoid receptor (GR) in Tau malfunction and pathology (Green et al. 2006; Sotiropoulos, Catania, et al. 2008) other studies exclude GC and GR signaling

from the effect of stress on Tau (Rissman et al. 2012). Thus, we next tested the impact of prolong treatment with high GC levels using the synthetic glucocorticoid Dexamethasone on a neuronal cell line expressing P301L-Tau tagged with GFP. We found that GC treatment (10^{-6}M, 48hr) caused an increase of GFP-labelled-P301L-Tau protein, as well as total Tau levels (**Figure 3A**). In addition, WB analysis revealed that GC effect on Tau occurred in both endogenous and exogenously-expressed human Tau protein (**Figure 3C**). Using both IF and WB analysis we found that autophagic markers such as LC3, p62 as well as the lysosomal marker, LAMP2a are also affected by GC treatment. In line with our *in vivo* findings in stressed P301L-Tau mice, GC treatment in P301L-Tau cells lead to an increase of p62 levels accompanied by a reduction in LC3II and LAMP2a measured by WB analysis (**Figure 3C**). Accordingly, we also observed a decrease in $LC3^{+}$ puncta staining after GC exposure (**Figure 3D**) followed by reduced LAMP2a staining (**Figure 3E**). The above results indicate that GC induced a blockage of autophagic clearance and accumulation of P301L-Tau and wildtype human Tau, which was followed by a reduction in cell viability as accessed by MTS (**Figure 3F**). Furthermore, exposure to GC also resulted in increased cytoplasmic levels of different SG markers such as TIA-1, TLS/FUS, DDX5 and G3BP (**Figure 3G**). IF staining also confirmed the GC-induced increased staining of SGs. Moreover, we could observe an increase in TIA-1 staining and the movement of TIA-1 from the nucleus to the cytoplasm after glucocorticoid exposure (**Figure 3H**); and an increase in G3BP staining in accordance with the WB data (**Figure 3I**).

Importantly, SG formation as well as different parts of autophagic process (e.g. autophagosome maturation) rely on microtubule-based networks and cytoskeletal machinery. One of the molecules involved in these processes is histone deacetylase 6 (HDAC6), which, through its influence on different cytoskeletal molecules (e.g. tubulin, cortactin), may regulate microtubule-dependent motility and, thus, the consolidation of cellular complexes such as SGs and autophagosomes (Lee et al. 2010; Hoover et al. 2010). Thus, we next analyzed the impact of GC on HDAC6 and its cytoskeletal targets. IF staining of HDAC6 showed that GC triggered a great increase of HDAC6 in P301L-Tau cells (**Figure 3J**) which was accompanied by a decrease in acetylation levels of both cytoskeletal targets of HDAC6, tubulin and cortactin (**Figure 3K**); note that similar deacetylation effects was found in P301L-Tg mice after stress exposure (**Supp Figure 1**). Overall, these *in vitro* data highlight the importance of GC in the induction of cellular cascades related to imbalanced Tau proteostasis and subsequent neurotoxicity.

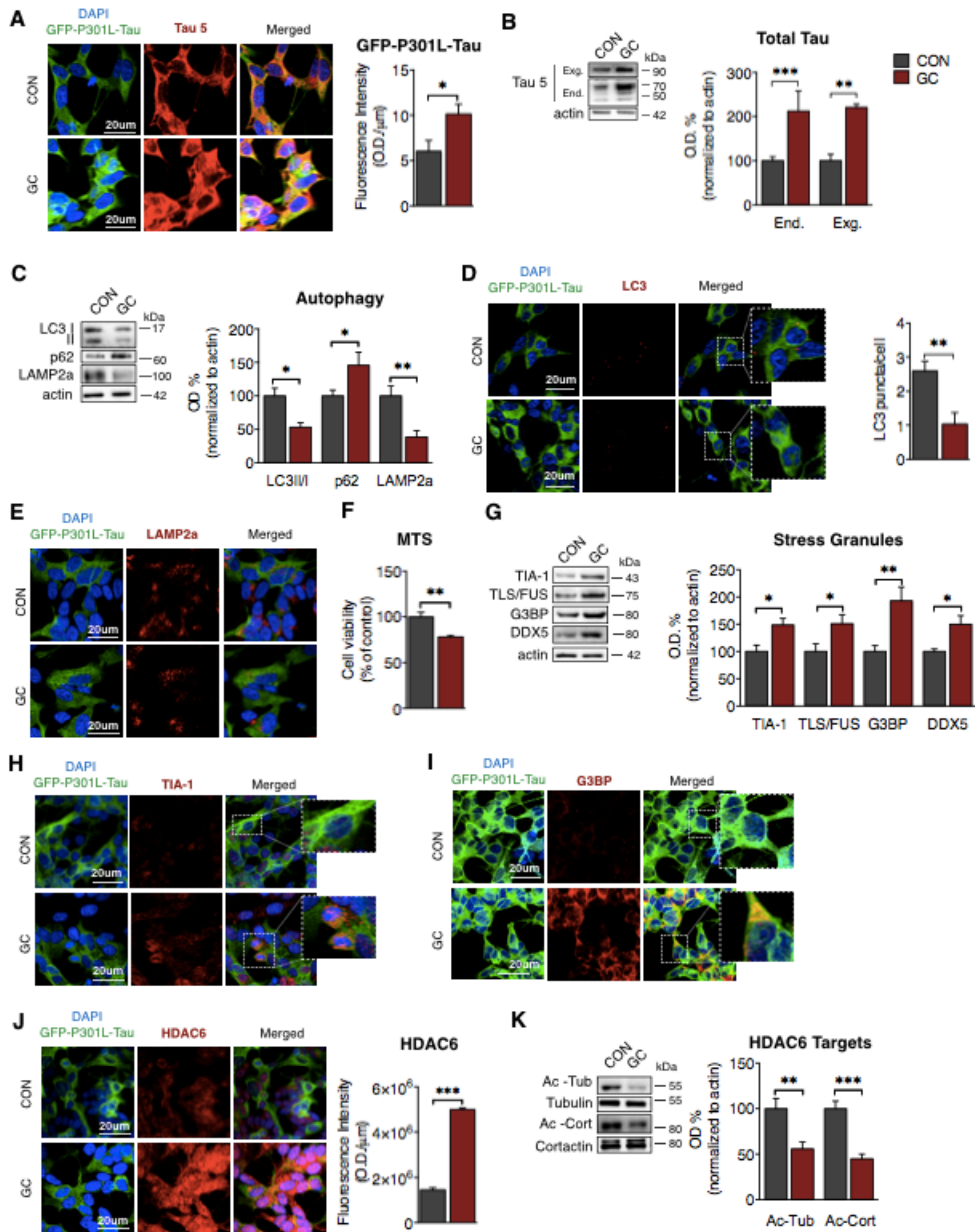


Figure 3. Glucocorticoids mimic the stress-driven effect on aggregation-enhancing neurodegenerative cascades in P301L-Tau-SHSY65Y cells*. A-B.* Similar to stress effect on P301L-Tau animals, GC treatment (10-6M; 48hr) trigger cytoplasmic accumulation of exogenously expressed mutated human Tau (P301L-Tau) and endogenous human Tau (wild-type) as assessed by IF (**A**) (*p*=0.044) and WB (**B**) analysis (End Tau: *p*=0.0007; Exog Tau: *p*=0.0014). **C-F.** GC decreased LC3II (*p*=0.0134) and LAMP2a (*p*=0.0028) levels with parallel increase of p62 levels (*p*=0.0397) (**C**); immunofluorescence analysis confirmed the GC-induced reduction in LC3 (*p*=0.0041) puncta(**D**), and LAMP2a staining (**F**) that was accompanied by reduced cell viability (*p*=0.0041)(**E**) in P301L-Tau cells after GC treatment. **G.** In addition, GC also elevated the cytoplasmic levels of the SG markers, TIA-1 (*p*=0.0438), TLS/FUS (*p*=0.0340), G3BP (*p*=0.0043) and DDX5 (*p*=0.0382) as assessed by WB analysis. **H-I.** IF staining of TIA-1 (**H**) and G3BP (**I**)

showed that GC cause their accumulation and movement to the cytoplasm. **J-K.** IF analysis showed that GC treatment elevated HDAC6 staining in P301L-Tau cells (p<0.0001) (**J**) in parallel with decreased levels of acetylated forms of tubulin (p=0.0043) and cortactin (p<0.0001), two cytoskeletal targets of HDAC6 (**K**). All numeric data are represented as mean ± SEM, *p<0.05; **p<0.01; ***p<0.001.

Pharmacological intervention of autophagy stimulation attenuates GC-driven neurotoxic cascades.

Mounting evidence supports mTOR as an important regulator of protein homeostasis (Di Domenico et al. 2016) while the above *in-vivo* data suggest that the inhibitory effect of stress on autophagic process includes mTOR involvement. Thus, we next clarified whether pharmacological inhibition of mTOR could protect against GC-driven Tau-related neurotoxicity ameliorating the autophagic blockage and induction of SGs. For that purpose, we used a rapamycin analog, Temsirolimus (CCI-779) shown to be safe and recently approved by USA and European Drug authorities (Malizzia and Hsu 2008; Hudes et al. 2008). While treatment of CCI-779 alone had no effect (data not shown), co-treatment of CCI-779 with GC blocked the reduced cell viability caused by GC (**Figure 4A**) providing neuroprotection against GC toxicity. Furthermore, we monitored the levels of autophagic process observing that GC+CCI co-treatment reverted the changes that GC evoked on different autophagy-related molecules. Specifically, co-treatment increased LC3II levels up to control ones, with similar reverse changes in p62 and LAMP2a protein levels (**Figure 4B**). Additional confirmation was also obtained by LC3 staining, where LC3$^+$puncta in GC+CCI treated cells were increased up to control levels (**Figure 4C**), suggesting that CCI blocked the GC-evoked inhibition of autophagic process. Moreover, CCI treatment attenuated the GC-driven elevation of exogenously (P301L-human Tau) and endogenous (wildtype human Tau) Tau, as shown by both WB (**Figure 4D**) and IF approaches (**Figure 4E**). Interestingly, CCI-779 also blocked the impact of GC on elevation of different SG markers such as TLS/FUS, DDX5 and G3BP (**Figure 4F**). Further confirmed by immunofluorescence, where we observe a decrease in the staining of TIA-1 and G3BP (**Figure 5G-H**). Conclusively, the above findings suggest that mTOR inhibition could attenuate the GC-evoked neurodegenerative cascades underlying Tau neurotoxicity.

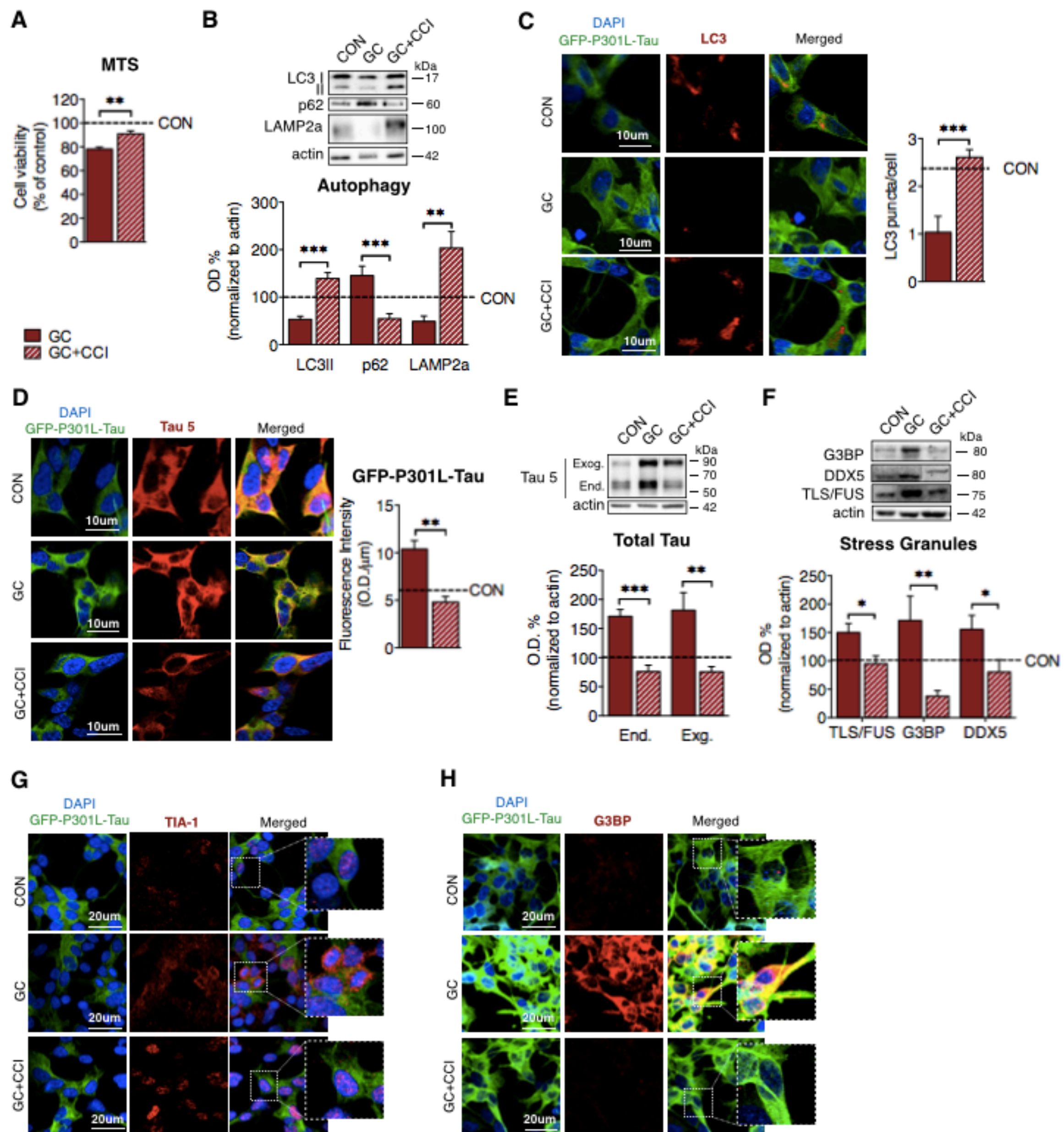


Figure 4. mTOR-driven pharmacological stimulation of autophagy blocked GC-triggered Tau and SGs induction. A-C. CCI-779 treatment blocked GC-driven decreased cell viability (p=0.0027) (**A**) in P301L-Tau cells and increased LC3II (p=0.0009) and LAMP2a (p=0.0021) proteins levels, while decrease p62 ones (p=0.001)(**B**); similarly, cells treated with CCI-779 and GC exhibited elevated LC3II puncta comparable to control cells (p=0.0012)(**C**). **D-E.** CCI-779 co-treatment also attenuated the accumulation of exogenous (p<0.0057) and endogenous (p<0.001) human Tau protein levels (**D**), and decreased fluorescence intensity (p=0.0021) (**E**). **F-H.** Interestingly, the GC-driven increase of SG markers, TLS/FUS (p=0.0227), G3BP (p=0.0075) and DDX5 (p=0.0458), were attenuated by co-treatment with CCI-779 (**F**); IF staining of TIA-1 (**G**) and G3BP (**H**) confirmed the blockage of GC-driven induction of SG by CCI-779. All numeric data are represented as mean ± SEM, *p<0.05; **p<0.01; ***p<0.0001.

Discussion

The molecular mechanisms and cellular events that facilitate the transformation of the highly soluble and monomeric Tau protein to its misfolded, aggregated and insoluble form has become the center of interest for Alzheimer's Disease (AD) pathology, as Tau malfunction was recently suggested to mediated, at least partly, the Aβ toxicity in AD brain (Roberson et al. 2007; Ittner and Gotz 2011). Many preclinical and clinical trials focus on Tau anti-aggregation strategies while different compounds are being tested against accumulation of aggregated Tau during the last years (Jiang et al. 2014; Soeda et al. 2015; Novak et al. 2016). Despite the significant advances in our understanding of Tau-mediated neurodegeneration, the exact mechanisms that facilitate Tau pathology as well as their interaction with different risk factors that may precipitate the disease remain poorly understood and unclear.

Prolong stressful life experiences and excessive glucocorticoid (GC) exposure are suggested to increase susceptibility to brain pathology with increasing attention to its implication in AD. Clinical studies report high cortisol levels in AD patients, indicative of altered hypothalamus-pituitary-adrenal (HPA) axis (Hatzinger et al. 1995; Rasmuson et al. 2001), while the increase of cortisol levels is negatively associated with memory scores in AD patients (Csernansky et al. 2006). Furthermore, chronic stress is also suggested to lower the age of onset of the familial form of AD (Simard, Hudon, and van Reekum 2009), highlighting the potential implication of chronic stress and GC in the pathogenesis and/or progression of AD (Yang et al. 2014). Direct support of the neurodegenerative potential of chronic stress is provided by different experimental studies, including some of ours, showing that stress and GC trigger different parameters of Tau pathology such as aberrant hyperphosphorylation, somatodendritic accumulation (Green et al. 2006; Sotiropoulos et al. 2011) and synaptic missorting (Lopes et al. 2016; Pinheiro, Silva et al. 2015). In addition, we have recently demonstrated that chronic stress also induces truncation and misfolding of Tau leading to the formation of neurotoxic Tau aggregates (Sotiropoulos et al. 2014), but the underlying mechanisms are unknown. We hereby demonstrate for the first time that chronic stress as well as GC inhibit autophagic process, providing an explanation for the accumulation and aggregation of Tau under stressful conditions. Indeed, this notion is in line with previous *in vitro* and *in vivo* work showing that stress/GC reduced Tau turnover (Sotiropoulos, Catania, et al. 2008) and deregulates molecular chaperones responsible for Tau degradation (e.g. Hsp90 and Hsp70) (Sotiropoulos et al. 2014). Moreover, activity of autophagy is also affected in brains of patients and animals of Tauopathies

highlighting a specific relationship between autophagy deficits and Tau pathology (Ambegaokar and Jackson 2012). Autophagy has been indicated as the main degradation pathway in AD brain (Hamano et al. 2008; Wang et al. 2010; Inoue et al. 2012;) as numerous reports have suggested that, although proteasomal substrates (Brown et al. 2005; Feuillette et al. 2005), Tau inclusions and aggregates may not be accessible to the ubiquitin-proteasome system (Hara et al. 2006; Boland et al. 2008).

Autophagy is a highly regulated process which is initiated by changes in phosphorylation states of individual components such as the ULK (Unc51-like-kinase) complex, mainly regulated by mTOR. Interestingly, mTOR signaling is altered in AD (Lafay-Chebassier et al. 2005; Li et al. 2005; Caccamo et al. 2010), with the levels of mTOR and its downstream targets, including p70(S6K), being increase in human AD brains (Caccamo et al. 2013; Jiang et al. 2014). Accordingly, our current findings demonstrate that chronic stress and GC increased the levels of phosphorylated S6K and p38, indicators of active mTOR signaling. Together with the reduced LC3II/LC3I ratio and accumulation of p62, the above findings suggest that chronic stress inhibits autophagic process by activating mTOR pathway; note that chronic stress is shown to trigger mTOR in hippocampus (Polman et al. 2012). As mTOR activation is associated with increased total Tau levels in AD brains (An et al. 2003; Pei and Hugon 2008), it is highly plausible that part of the stress/GC-driven Tau accumulation maybe attributed to mTOR. In line with previous studies showing that decrease in mTOR signaling can revert Tau pathology (Menzies et al. 2010; Jiang et al. 2014), we also demonstrate that the use of the mTOR inhibitor, CCI-779, blocks the GC-driven Tau-related neurotoxicity and induction of aggregation-related cascades (see below). Altogether, the above findings point to mTOR and autophagy involvement in the cellular mechanisms through which GC may trigger accumulation of Tau and its aggregates.

Recent work from AD and FTDP human brains and Tau Tg mice has causally implicated the formation of SGs in the development and progression of Tau pathology (Wolozin 2012). SGs are dense cytoplasmic aggregations of mRNAs and RNA-binding proteins (RBPs) formed under cellular stressors. While our understanding about SG formation is limited, SGs constitute a protective mechanism against cellular stress allowing the protection of mRNA and the fast production of cytoprotective proteins (Wolozin 2012). However, prolonged SG induction can become pathological and neurotoxic, and indeed, is related with several neurodegenerative diseases (Vanderweyde et al. 2012), as SGs are suggested to accelerate Tau aggregation while, in a vicious cycle, Tau stimulates SG formation with TIA1 exhibiting a leading role in Tau misfolding and aggregation (Vanderweyde et

al. 2016). Note that both hyperphosphorylation and aggregation-prone mutation of Tau can enhance, but are not required for, SG formation (Vanderweyde et al. 2016). The current study reveals for the first time that chronic stress and GC increased the levels of different SG markers (e.g. TIA-1, FUS, DDX5, G3BP) in soluble and insoluble fractions of P301L-Tau animals or cells. Note that TIA-1 is shown to directly interact with Tau and stimulates Tau inclusion (Vanderweyde et al. 2016). Moreover, the aggregation of TIA-1 is regulated by molecular chaperones and it is blocked by Hsp70 overexpression resulting to inhibition of SG formation (Mazroui et al. 2007). Note that chronic stress reduces Hsp70 levels providing a potential cascade through which TIA-1 is increased under stressful conditions (Sotiropoulos et al. 2014). As SG formation is induced by the translocation to the cytoplasm (Kedersha et al. 1999) and the increase expression of different SGs proteins (Tourrière et al. 2003; Gilkes et al. 2004; Ohn et al. 2008), our findings suggest a novel role for SG biology in the stress/GC-driven neuronal pathology. Indeed, similarly to AD brain, we have shown that Tau missorting and dendritic accumulation is part of chronic stress/GC hippocampal pathology (Pinheiro, Silva et al. 2015; Lopes et al. 2016;). Dendritic and synaptic missorting of Tau is recently suggested to facilitate formation of SGs as part of the translational stress response (Vanderweyde et al. 2016) opening a wide range of avenues for research and therapeutic exploration focusing on RNA-protein intraneuronal trafficking and function in stress-related pathologies.

Although inhibition of autophagy is shown to impair the degradation and dynamics of SGs (Buchan et al. 2013; Seguin et al. 2014), the existence of a direct interaction between autophagy and SGs is still debatable. However, cytoskeletal machinery facilitates the aggregation of RBPs to form SGs and have an important role in autophagy maturation and autophagosome/lysosome fusion. One of the molecules involved in these processes is histone deacetylase 6 (HDAC6), which, through the deacetylation of tubulin, reduces microtubule-dependent motility and thereby promotes the consolidation of cellular complexes such as SGs and autophagosomes (Lee et al. 2010; Hoover et al. 2010). Recent work implicates HDAC6 in the formation of SG in AD brain as HDAC6 seems to localize and interact with SG proteins under cellular stress; interestingly, HDAC6 is a SG component interacting with G3BP (Seigneurin-Berny et al. 2001; Kwon 2007). Additionally, pharmacological inhibition or genetic ablation of HDAC6 abolished SG formation (d'Ydewalle, Bogaert, and Van Den Bosch 2012) while the expression of HDAC6 significantly increases in the hippocampus and other brain regions of AD patients and animal models of the disease (Ding, Dolan, and Johnson 2008; Perez et al. 2009; Zhang, Sheng, and Qin 2013). Our findings show that GC increased HDAC6 levels, resulting in reduced acetylated levels of HDAC6 cytoskeletal targets e.g. tubulin. Reduced acetylation

of tubulin is associated with microtubule instability, which is also suggested to cause enlargement of SGs (Chernov et al. 2009). Interestingly, recent reports in mice show that HDAC6 inhibition increases resilience to stress through Hsp90 hyperacetylation, disabling GR translocation from the cytoplasm into to the nucleus (Espallergues et al. 2012; Jochems et al. 2014).

Conclusively, the current studies shed more light onto the underlying molecular mechanisms through which chronic stress and GC may damage protein and neuronal homeostasis precipitating Tau pathology and neurodegeneration.

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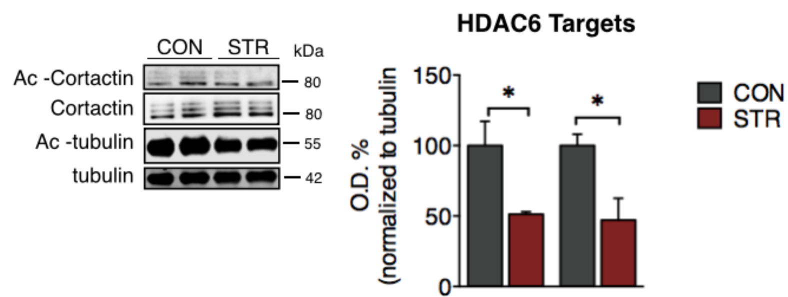
Supplementary Information

Supplementary Figure 1



Supplementary Figure 1. Stress leads to deacetylation of HDAC6 cytoplasmic targets in P301L-Tg mice. Chronic stress reduced acetylation of tubulin (p=0.0160) and cortactin (p=0.0482), two cytoskeletal targets of HDAC6, in brain of P301L-Tau Tg mice.

CHAPTER 4

UNMASKING THE ROLE OF TAU IN THE ADULT BRAIN: THE GENERATION OF A CONDITIONAL KNOCK-OUT MODEL.

Unmasking the role of Tau in adult brain: the generation of a conditional knock-out model

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Abstract

Tau is mainly a neuronal protein, widely expressed in nervous system. While originally described to be associated with and regulate microtubule assembly, Tau is now known to interact with various cytoskeletal and other proteins (e.g. actin, Fyn) and thus, be involved in many cellular processes such as axonal growth, cargo trafficking and more recently synaptic plasticity. Furthermore, under different pathological conditions, e.g. Alzheimer's disease, Tau is causally associated to cytoskeletal deficits, dendritic atrophy as well as neuronal and synaptic malfunction. All these findings highlight Tau as an essential protein in both neuronal function as well as pathology. Surprisingly, the constitutive Tau knock-out (KO) mouse models available exhibit no obvious behavioral, neurostructural or functional alterations suggesting a gap of knowledge on the real Tau function. Avoiding the suggested developmental compensation mechanisms found in constitutive Tau-KO models, we aimed to generate an inducible conditional Tau-KO mouse line. We hereby report the creation of a novel transgenic mouse, named Tau-lox which expresses the endogenous (mouse) *mapt* gene flanked by two LoxP sequences; this mouse line was further crossed with tamoxifen-inducible CaMKIIa-driven $CreER_{T2}$ mouse line. Both Tau-lox as well as Tau-lox/CaMK mouse exhibit no developmental, neurological or behavioral alterations, as assessed by Milestone, SHIRPA and other behavioral tests. In addition, Cre induction by tamoxifen administration lead to a severe reduction of Tau protein levels in different forebrain regions of Tau-lox/CaMK, 4 weeks after drug injection. Conclusively, this novel Tau-lox mouse line will provide an excellent *in vivo* model for dissecting the function of Tau as well as the consequences of its loss in adult or aged brain providing temporal as well as brain area- or cell-specific flexibility in Tau reduction/deletion.

Introduction

Tau was discovered in 1975 as a protein that induces microtubule formation (Weingarten et al. 1975; Goedert et al. 1989). It is predominantly expressed in neurons of the central and peripheral nervous system. The microtubule-associated protein Tau gene (MAPT) is located on chromosome 17q21 and consists of 16 exons spanning 135 kb (Neve et al. 1986). Exons 2, 3 and 10 of MAPT pre-mRNA are alternatively spliced to produce six isoforms in the adult human brain which differ by the presence of a 29-amino acid repeat in the amino-terminal half of the protein (0N, 1N or 2N), and of either three (3R isoform) or four microtubule-binding repeats (4R isoform) in the carboxyl- terminal half. Through its phosphorylation, which is a physiological and dynamic process known to regulate its function, Tau is thought to have a key cytoskeletal role based on its ability to bind to microtubules (MTs), promoting their polymerization and thus, regulate their network (G. Lee, Cowan, and Kirschner 1988; Goedert et al. 1989). It is also reported that Tau is involved in axonal growth and development as well as axonal and dendritic cargo transport, a critical event for neuronal and synaptic function (Oddo et al. 2003; Polydoro et al. 2009; Mondragón-Rodríguez et al. 2012). Recently, Tau was shown to be implicated in signaling pathways related to synaptic plasticity and structure, interacting with Fyn and PSD-95 (Ittner et al. 2010 ; Frandemiche et al. 2014).

Under different pathological conditions, e.g. Alzheimer's disease (AD) and frontotemporal dementia (FTD), Tau is abnormally hyperphosphorylated in many sites, leading to impaired MT-binding capacity and potentially reduction or loss of Tau normal function, through its detachment from MTs consequently causing cytoskeletal deficits (Cuchillo-Ibanez et al. 2008; Vossel et al. 2010; Rodríguez-Martín et al. 2013). In addition, hyperphosphorylated Tau is also missorted to dendrites and spines triggering synaptic malfunction and excitotoxicity (Ittner et al. 2010; Hoover et al. 2010; Lopes et al. 2016). Furthermore, the (hyperphosphorylated) unbound Tau generates both soluble and insoluble oligomers that form big inclusions and pathological aggregates, known to have deleterious effect on different aspects of neuronal function, causing cell death; this Tau-related neurotoxicity is believed to be issued by the gain of toxic tau function (Morris et al. 2011; Wang and Mandelkow 2015). Importantly, Tau is also shown to mediate, at least partly, the detrimental effects of amyloid beta (Aβ) on neuronal and brain function, related to AD pathology (Rapoport et al. 2002; Roberson et al. 2007), glutamate-driven excitotoxicity (Roberson et al. 2007; Zempel et al. 2010), and stress and glucocorticoids (GC) (Pinheiro, Silva et al. 2015; Lopes et al. 2016). Conclusively, the above findings suggest that Tau protein has an essential role in different aspects of neuronal function and pathology.

Despite the above evidence supporting the importance of Tau protein in neuronal and brain function, Tau knock-out (KO) mouse lines available nowadays don't present major alterations in behavior, neuronal structure or survival. Three out of four created constitutive Tau-KO models don´t exhibit MT alterations (reviewed in (Morris et al. 2011)). In addition, Tau binding to microtubules regulates axonal transport and cargo transport, but neurons or animals lacking Tau don't exhibit any axonal abnormalities, highlighting a significance gap of knowledge about the real role of Tau in neuronal function. This paradoxical in vivo phenotype of Tau-KO animals is suggested to be partly attributed to developmental compensatory mechanisms associated with other microtubule-associated proteins (MAPs). This notion is supported by studies showing that MAP1a protein expression is increased in the first months of life of Tau-KO animals (Harada et al. 1994; Dawson et al. 2001; Tucker, Meyer, and Barde 2001), whereas simultaneous KO of *mapt* and *map1b* display severe phenotype and lethality within 4 weeks (Takei et al. 2000).

Based on this background, we created a conditional Tau-KO mouse line avoiding the developmental compensatory mechanisms suggested to occur in previously generated (constitutive) Tau-KO mouse lines, using the Cre/LoxP site-specific recombinase system that has been widely used to carry out conditional gene deletion in mice.

Material and Methods

Generation of MAP-LoxP-containing constructs

The Mapt targeting vector was prepared by recombination as previously described by Lee and colleagues (Lee et al. 2001). Briefly, 9 kb of Ant1 genomic sequence containing exon 4 including approximately 4.8 Kb and 4.15 kb of intron 3 and 4 sequence was retrieved from the RP23-344E9 BAC (obtained from the BACPAC Resources Center, Children's Hospital Oakland Research Institute, Oakland, CA) by gap repair. The first loxP site was inserted into intron 3 approximately 0.5 kb upstream of exon 4 and the second loxP site together with the Frt-PGKneo-Frt cassette was inserted approximately 0.35 kb 3' of exon 4. The targeting vector was then linearized by NotI digestion, phenol/chloroform purified, precipitated and then resuspended in PBS (**Supplementary Fig. 1**). The linearized targeting vector was then electroporated into ES cells derived from F1(129Sv/C57BL6j) blastocyst. ES cell culture and electroporation were performed as described by Wurst and Joyner (Wurst 1993). Drug (G418 and Ganciclovir) resistant colonies were picked and grown in 96-well plate. Targeted ES clones were identified by long range nested PCR using Platinum HiFi Taq purchased from Invitrogen according to the manufacturer.

Generation of transgenic animals MAPT^{tm1nsis}

Chimeric animals were generated by aggregation of ES cells with CD1 morula according to Nagy and colleagues (Nagy et al. 1993). Chimeric males were bred with ROSA26-Flpe female (Jax stock no: 009086) to remove the PGKneo cassette and generate F1 pups with Mapt floxed allele. Positive pups were identified by PCR genotyping using two different primer pairs, LoxP gtF/R and Frt gtF/R (**Figure 1**). (**Supplementary Fig. 2**)

Housing conditions

All animals were maintained under standard laboratory conditions: an artificial 12h light/dark cycle (lights on from 8:00 to 20:00 hours), with an ambient temperature of 21±1°C and a relative humidity of 50–60%; control mice, from now on referred as Standard Diet(SD), were given a standard diet (4RF25 during the gestation and postnatal periods, and 4RF21 after weaning, Mucedola SRL, Settimo Milanese, Italy) and water ad libitum. Tamoxifen treated animals received the 4RF25 diet along the weeks of treatment. Health monitoring was performed according to FELASA guidelines, confirming the Specified Pathogen Free health status of sentinel animals maintained in

the same animal room. All procedures were conducted in accordance with European regulations (European Union Directive 86/609/EEC). Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity – Direcção Geral de Veterinária (DGV). The joint Animal Ethics Committee of the Life and Health Sciences Research Institute approved all the protocols performed.

Mouse breeding to obtain conditional KO animals

Mapt transgenic animals were breed to B6;129S6-Tg(Camk2a-cre/ERT2)1Aibs/J (JAX Laboratory, #012362 stock), that express a tamoxifen-inducible Cre-recombinase under the control of the mouse Camk2a (calcium/calmodulin-dependent protein kinase II alpha) promoter region, specific for forebrain regions. When Camk2a-CreER$_{T2}$ transgenic mice are bred with mice containing loxP-flanked sequences, tamoxifen-inducible Cre-mediated recombination will result in deletion of the floxed sequences in the Camk2a-expressing cells of the transgenic mutant offspring (**Figure 5**) (See **Supplementary Figure 3**, for detailed predicted Mapt transcript before and after Cre excision).

Genotyping and Primers

Transgenic MAPT^{tm1nsis} mice were genotyped using two pairs of primers: LoxP gtF (5'-GTCCCAGGTGATTCCTCCAC-3') and LoxP gtR (5'- CCAGCCTAGCTCAGGCTATAGC-3'), which detects a fragment of 348 bp specific to the wildtype and 439 bp specific to the floxed allele, in hemizygous animals, the 2 bands are observed; and Frt gtF (5'-GAGATCTAGGCTCAGTAAACC-3') and Frt gtR (5'-CTCAGCAACCGAGGCCACCTGC-3') detects a fragment of 255 bp specific to the wildtype allele and 352 bp specific to the 3' Frt/LoxP site of the floxed allele.

For Tg(Camk2a-cre/ERT2)1Aibs/J allele, the genotyping was based on 2 pairs of primers: 10447-F (5'-AGCTCGTCAATCAAGCTGGT-3') and 8990-R (5'-CAGGTTCTTGCGAACCTCAT-3'), which gives a band of 184 bp for the transgene; and for an internal control oIMR7338-F (5'-CTAGGCCACAGAATTGAAAGATCT-3') and oIMR7339-R (5'-GTAGGTGGAAATTCTAGCATCATCC-3'), which gives a band of 324 bp.

Developmental characterization – Milestones

Assessment of neurobehavioral neonatal development included the execution of a range of well-described tests used to evaluate neurologic parameters such as motor, reflexes and

strength/coordination development (Hill, Lim, and Stone 2008; Lim et al. 2008). This procedure was designed to allow a fast throughput; so that several litters can be examined daily within a relatively short period of time (Hill, Lim, and Stone 2008; Lim et al. 2008). The day of birth was considered as postnatal day (PND) 0 while from that day on, mice were daily examined for the acquisition of developmental milestones and weight gain until PND 21. For fast identification of each mouse, pups were marked on the first days after birth with a respective color in the dorsal part and on PND 5, toe clipping was done. Every day, the home cage was moved into the testing room and left to habituate for at least 30 min. During the experiment, the pups were left in the same room as the mother and the time of separation was minimized. The execution of each test was random, as well as the animals order. Each test was performed in the same range of time of each day during the 21 days long protocol. Testing read-out focuses on the time to accurately perform, or respond to, a stimulus or posture. The time animal spent to execute the test was registered and later converted to dichotomic scores (**Supplementary Table. 1**). The animal is considered to exhibit a mature response on a specific test when the highest score is observed for two consecutive days (Hill, Lim, and Stone 2008; Lim et al. 2008), in **Supplementary Table 2** are described the range of days where a mature response is expected in normal development.

Morphometric characterization – SHIRPA

SHIRPA is a three-stage protocol, which allows for the comprehensive analysis of the behavioral phenotype of mice. Using SHIRPA, we can examine a wide range of behavioral, neurological and physiological measures in the same cohort of animals. Use of such a comprehensive battery of tests suggest that subtle phenotypic changes will be picked up. The first stage consists of a primary observational screen, where modified Irwin profile is used allowing direct comparison of any phenotypic variations which may be found. The second stage is a comprehensive screening battery that includes open field and assessment of locomotor activity. The third stage is more sophisticated test that it is usually used to access various behavioral dimensions such as anxiety, depression and cognition (see also behavioral testing). In this work, we used the first and second stage of the protocol.

Behavior Testing

Open Field (OF) test was conducted in an arena (43.2 cm43.2 cm) with transparent acrylic walls and white floor (Med Associates Inc., St. Albans, VT, USA). Mice were placed in the center of

the arena and movement was monitored over a period of 5 min with the aid of two 16-beam infrared arrays. Time spent in the center of the arena was used as an index of anxious behavior. Total distance traveled was used as an indicator of locomotor activity.

Elevated-Plus Maze (EPM) was used to access anxious behavior for 5 min as previously described. 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 using 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).

Y-Maze (YM) assesses the number of spontaneous alternation. Each mouse was placed at the end of one arm and allowed to move freely through the maze during a 7min session. The number of alternations were analyzed, defined as consecutive entries into all three arms without repetitions in overlapping triplet sets. The percentage of alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries). In the Y-maze, mice tend to explore the maze by systematically entering each arm. The ability to alternate requires that the mice remember which arms have already been visited. Therefore, alternation behavior is a measure of working memory.

Tail suspension Test (TST) assesses depressive-like behavior. The method is based on the observation that a mouse suspended by the tail shows alternate periods of climbing and immobility. The animal is suspended by the tail for 5min, and the time that the animal stays immobile or climbing is measured.

Forced Swim Test (FST) assesses depressive-like behavior. The method is based on the observation that a mouse exposed to a water container shows alternate periods of swimming and immobility. The animal is placed in a glass cylinder with 30cm in depth of water for 7min. The time that the animal stays immobile in the water during the last 5min of the test is measured as immobility.

Induction with tamoxifen

Tamoxifen (Sigma, #T5648) was dissolved in a corn oil (Sigma, #C8267) with 10% ethanol, at a concentration of 20 mg/ml of tamoxifen. For initial screening of the transgenic lines, 4mg/day of TM was injected intraperitoneally into double transgenic animals at 2 months of age, for 5 consecutive days, during two or three weeks, with 1 week interval between treatment weeks (see

experimental design in **Figure 6**). Animals were sacrificed 2 or 4 weeks after the last tamoxifen injection. PFC, Hippocampus, rest of Cortex and cerebellum were dissected for molecular analysis.

Western Blotting

At the end of experimental design, animals were decapitated and brains were excised immediately. Prefrontal cortex (PFC), Hippocampus, rest of Cortex and Cerebellum were dissected (on ice) and immediately stored at –80°C. After homogenization in RIPA buffer (50mM TrisHCl, 2mM EDTA, 250mM NaCl, 10% glycerol)) with phosphatase inhibitors (Phosphatase inhibitor cocktail 2, Sigma #5726; Phosphatase inhibitor cocktail 3, Sigma #0044) and protease inhibitors (Roche #11697498001), lysates were centrifuged at 13000rpm for 15min and supernatant was collected. Samples were quantified using Bradford Assay method. After SDS–PAGE electrophoresis of 20ug of sample, and semi-dry transfer, all membranes were incubated in different antisera actin (1:2500; ABCAM, #ab8224) and Tau5 (1:2000; ABCAM, #ab80579)) while blots were revealed by enhanced chemiluminescent (ECL, BioRad) using Chemidoc®BioRad detection system.

Statistical Analysis

Normality tests were performed for all data analyzed. Statistical analysis between two groups was made using Student's t-test. One-way or two-way analysis of variance (ANOVA) was used when appropriate. Bonferroni's post hoc multiple comparisons were used for group differences determination. Numerical data is expressed as group mean±SEM and differences were considered to be significant if $p < .05$.

Results

The creation of MAPT-cKO mice based on Cre-LoxP system

To create the conditional Tau-KO mouse line, we used Cre-LoxP system. We created the mouse line with loxP sites flacking MAPT gene, named $Tau^{loxP/loxP}$. $Tau^{loxP/loxP}$ mouse model was created inserting the first loxP site into intron 4, approximately 0.5kb upstream of exon 4 and the second loxP site approximately 0.35kb 3' of exon 4, using Frt-PGKneo cassette (**Supplementary Fig. 1**). The targeting vector was linearized and electroporated into ES cells (**Supplementary Fig. 2**) and the chimeric animals were bred with ROSA-26-Flep female mice to remove the PKG-neo cassette (**Figure 1**). When obtained, $Tau^{loxP/loxP}$ as well as its heterozygous $Tau^{loxP/+}$ and WT littermates ($Tau^{+/+}$) were characterized during development and adult age before further use for crossing with Cre-recombinase mouse line (Cre-CaMKII) mice as described below.

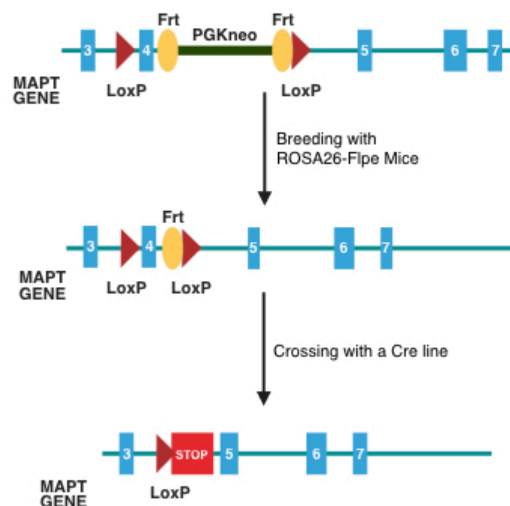

Figure 1. $Tau^{loxP/loxP}$ mice generation using genetic manipulation. Chimeric mice, with Frt-PGKneo-Frt-LoxP inserted previously in a MAPT LoxP in the intron 4 sequence, were crossed with ROSA26-Flpe mice to remove the PGKneo cassette, and further backcrossed to obtain the final $Tau^{loxP/loxP}$ mice. After crossing with a Cre line and further Cre activation, occurs the formation of a STOP codon in the *mapt* gene, which will stop the transcription and protein translation.

MAPT cKO mice present no developmental, phenotypical or behavioral alterations

To access any possible influence of the insertion of loxP sites in the *mapt* gene, we used several protocols during development or adult age of our mouse lines, $Tau^{loxP/loxP}$, $Tau^{loxP/+}$ and their WT littermates $Tau^{+/+}$. In the post-natal days (PND) that precede winning, from PND0 to PND21, animal's

developmental characteristics were analyzed using developmental milestones protocol. Milestones protocol is a widely-used protocol for assessment of neurobehavioral during neonatal development, with the execution of a variety of well-described tests used to access neurologic parameters such as motor, reflexes and strength/coordination development. This protocol allows for a fast screening of all litters to be examined daily within a relatively short period. A high number of litters was used in order to avoid individual cage influencing factors such as differential parental and dominance hierarchy. Animals of both sexes were analyzed together using standard multivariate analysis. No major gross and observable deficits were observed and all litters presented normal development.

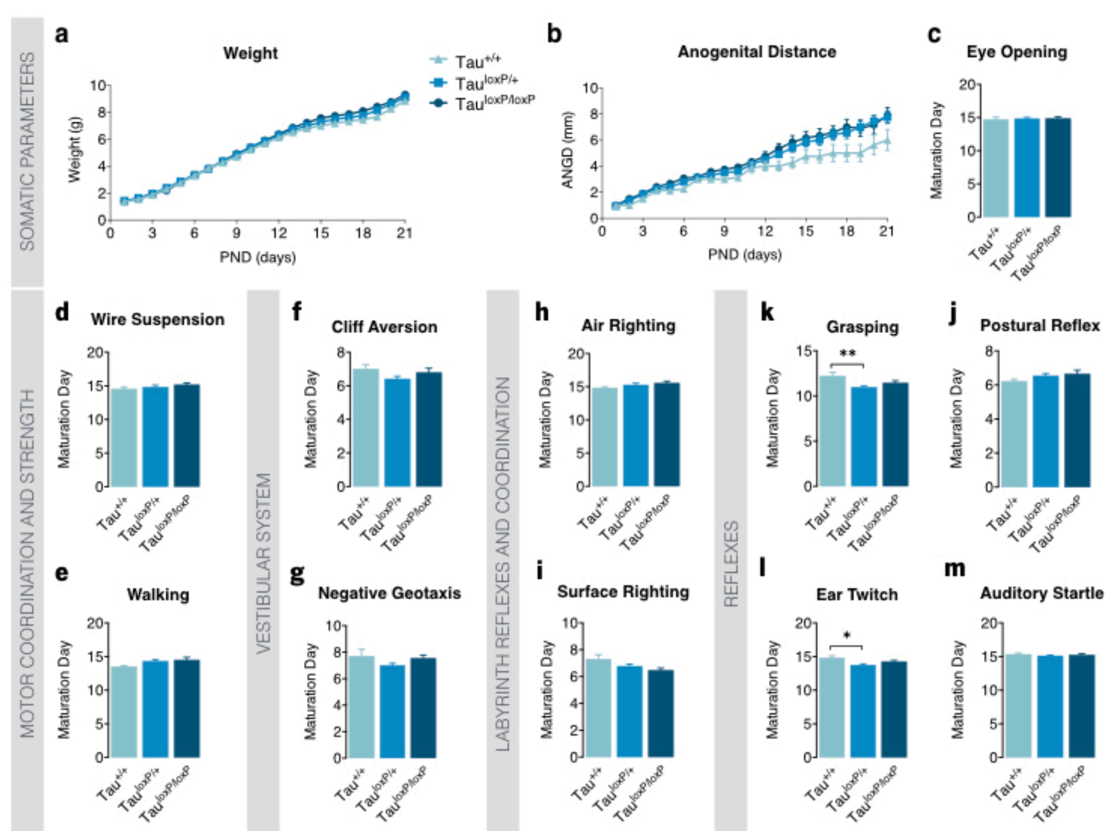

Figure 2. Developmental characterization of Tau$^{loxP/loxP}$ mice. Milestones protocol was used to access developmental and neurological profile of homozygous (Tau$^{loxP/loxP}$), heterozygous (Tau$^{loxP/+}$) and WT littermates (Tau$^{+/+}$), between PND1 to PND21. (**a-c**) Somatic parameters presented no differences between genotypes along the behavior assessment. (**d-e**) Motor coordination and strength was accessed by wire suspension (**d**) and walking (**e**), with no differences observed. (**f-g**) Vestibular system presented no impairments in neither of the genotypes, in both cliff aversion test (**f**) and negative geotaxis (**g**). (**h-i**) Labyrinth reflexes presented no differences in air righting (**h**) or surface righting (**i**). Regarding general reflexes, we observed an early maturation in grasping in heterozygous animals (**k**) and the same in ear twitch (**l**), where in both postural reflex (**j**) or auditory startle (**m**) no differences were observed. All numeric data are represented as mean ± SEM, **p*<0.05; ***p*<0.01; ****p*<0.0001.

Regarding somatic parameters, throughout the 21 days we found no differences in the body weight ($F_{(2,41)}$=0.6575, p=0.5235)(**Figure 1a**) or anogenital distance (AND) ($F_{(2,37)}$=2.788, p=0.0745)(**Figure 1b**) among groups. Another important somatic parameter is eye opening where we observe no differences between genotypes ($F_{(2,41)}$=0.06131, p=0.9406) (**Figure 2c**). In motor parameters, no differences were observed in wire suspension test ($F_{(2,41)}$=0.747, p=0.4675), that measures strength (**Figure 2d**), or walking ($F_{(2,41)}$=1.889, p=0.1641) that accesses motor coordination (**Figure 2e**). Regarding vestibular system, neither cliff aversion test ($F_{(2,41)}$=2.549, p=0.0905) (**Figure 2f**) or negative geotaxis ($F_{(2,41)}$=1.787, p=0.1802) (**Figure 2g**) present any significant differences among the three genotypes, indicating that all the animals acquire spatial perception and coordination around the same PND. On the evaluation of labyrinthic reflexes and coordination, no differences were observed in air righting ($F_{(2,41)}$=1.292, p=0.2870) (**Figure 2h**) or surface righting ($F_{(2,41)}$=1.923, p=0.1591) (**Figure 2i**). In other tests used to access neurological reflexes, we observed that $Tau^{loxP/+}$ mice present and early maturation day in grasping ($F_{(2,41)}$=4.873, p=0.0126; p=0.0064) (**Figure 2k**) and in ear twitch ($F_{(2,41)}$=4.718, p=0.0143) (**Figure 2l**) but this is not true for $Tau^{loxP/loxP}$ mice (p=0.1402, p=0.2208 respectively). In auditory startle ($F_{(2,41)}$=0.3694, p=0.6934) (**Figure 2m**) and postural reflex ($F_{(2,41)}$=1.282, p=0.2884) (**Figure 2j**) no differences were observed. These data indicate that the insertion of loxP in *mapt* in one or two alleles caused no delay in various developmental parameters suggesting that maturation occurs in the normal and expected time window.

We next used SHIRPA protocol as an effective primary screen for identifying subtle neuromuscular alterations and distinguish qualitative differences between genotypes. We observed that $Tau^{loxP/loxP}$ mice present differences in the body weight gain when compared to WT littermates (p=0.0382), contrarily to $Tau^{loxP/+}$ ($F_{(2,42)}$=3.562, p=0.0372) along the 24 weeks of analysis (**Figure 3a**). Regarding the parameters that evaluate the vestibular system, we observe no differences between genotypes in the negative geotaxis ($F_{(2,42)}$=1.720, p=0.1915) (**Figure 3b**) or vertical pole test (VPT) ($F_{(2,42)}$=0.0927, p=0.9117) (**Figure 3c**). In addition, there were no differences in the number of wall leanings in the ($F_{(1,42)}$=2.590, p=0.0869) (**Figure 3d** evaluated in the viewing jar. Furthermore, we also found no major differences in the locomotor activity monitored at the open field ($F_{(2,42)}$=0.9376, p=0.3996) (**Figure 3e**). As shown in **Figure 3f**, no differences were detected between all animal groups in wire maneuver test ($F_{(2.42)}$=0.3961, p=0.6754) which monitors the ability of animals to walk on a suspended wire. Furthermore, the muscular strength was evaluated

by grid test, that allows us to access the capacity of the animals to be hang in a cage grid ($F_{(1,2)}$=1.661, p=0.193) (**Figure 3g**).

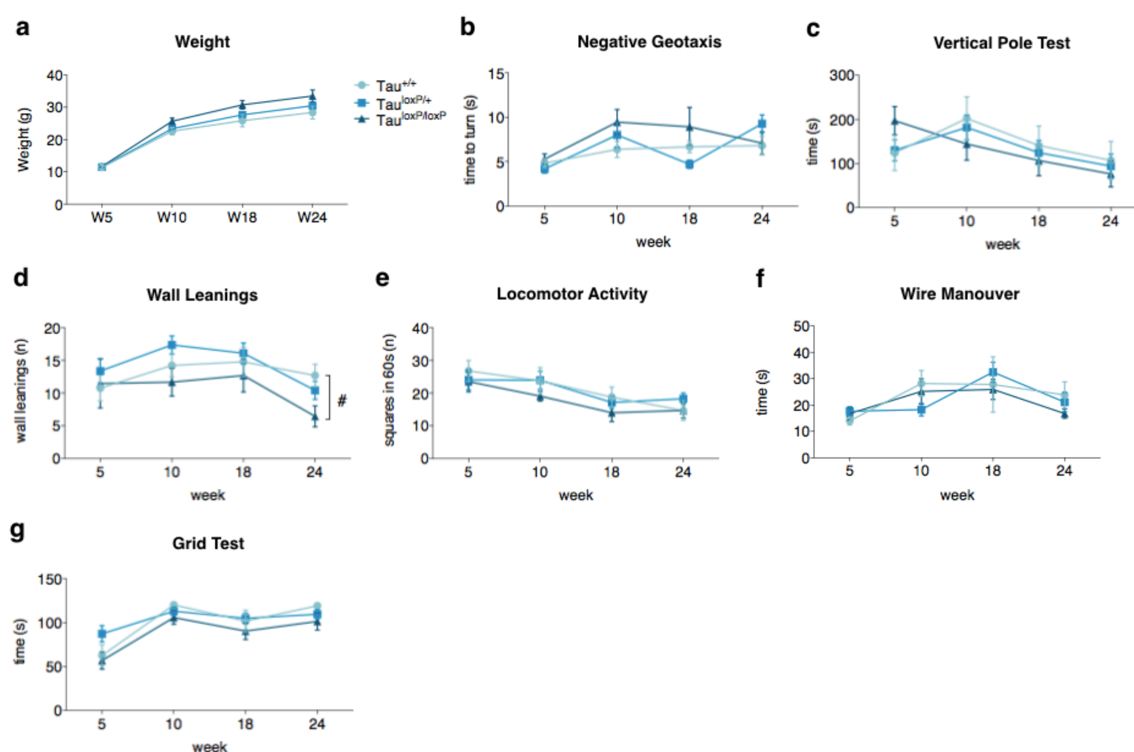

Figure 3. SHIRPA protocol for characterization of Tau$^{loxP/loxP}$ mice until the age of 24 weeks old. SHIRPA protocol was used to screen cerebellar and motor performance as well as overall sensory function. (**a**) Both genotypes present normal weight variation comparing to control. In negative geotaxis (**b**) no differences were observe between groups, as well as in Vertical Pole Test (**c**). Using viewing jar, we could access exploratory behavior where the number of wall leanings (**d**) was not affected by the presence of loxP alleles presence along the 24 weeks. In the arena, we evaluated locomotor activity where we didn't observe any differences between genotypes (**e**). In the wire manoeuver test (**f**) as well as in the grid test (**g**) no differences were observed between groups. All numeric data are represented as mean ± SEM, *p<0.05; **p<0.01; ***p<0.0001.

After performing the developmental and phenotypic characterization of Tau$^{loxP/loxP}$ and Tau$^{loxP/+}$, we evaluated the three dimensions of adult behavior at 26 weeks of age, involving cognition, emotion and anxiety. For assessing anxiety, we used elevated plus maze (EPM) and open field (OF) tests. As shown in **Figure 4**, both transgenic mouse lines and their WT littermates exhibit no significant differences in the time spent ($F_{(2,22)}$=0.9426, p=0.4048) and entries ($F_{(2,22)}$=1.211, p=0.3171)(**Figure 4a**) in the open arms of EPM apparatus while similar results were obtained in OF, where no differences were observed in the time animals spent in the center of the OF arena ($F_{(2,22)}$=0.5784, p=0.5691)(**Figure 4b**). Note that total distance that animals travelled in OF was similar among the

three groups ($F_{(2,22)}$=0.2526, p=0.7790) suggesting no locomotion deffects (**Figure 4b)**. To access learned helplessness and anhedonia, two core parameters of depressive-like behavior, forced swim and tail suspension tests (FST and TST, respectively) as well as sucrose preference test (SPT) were used. Once more, both transgenic lines were similar to their WT littermates in the time spent immobile in FST ($F_{(2,22)}$=0.8017, p=0.4613)(**Figure 4c**) and in TST ($F_{(2,22)}$=0.2526, p=0.9751)(**Figure 4d**), and no differences were observed in the sucrose preference ($F_{(2,22)}$=2.080, p=0.1488) (**Figure 4e**). Furthermore, we also use Y-maze for cognitive monitoring, where all animal groups exhibit similar percentage of spontaneous alternations ($F_{(2,22)}$=0.6585, p=0.5275) (**Figure 4f**). The above sets of data demonstrate that $Tau^{loxP/loxP}$ and $Tau^{loxP/+}$ present no behavioral differences on memory, depression and anxiety that accompanied the aforementioned absence of any development or neurological abnormalities in these animals compared to their WT littermates.

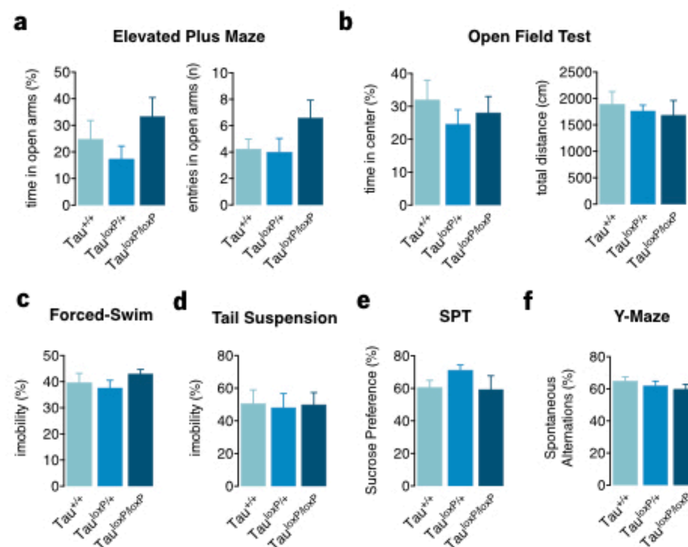

Figure 4. Behavior characterization of $Tau^{loxP/loxP}$, $Tau^{loxP/+}$ and WT littermates. (**a**) No differences among animals of all genotypes were found in time and entries that animals spent in the open arms of the elevated plus maze apparatus, indicating no differences in levels of anxiety among these animal groups. (**b**) Similarly, $Tau^{loxP/loxP}$, $Tau^{loxP/+}$ animals spent the same time as control littermates in the center of the OF arena confirming the EPM-based conclusion of no anxiety differences among all animals, and no differences in total distance travelled in OF among all groups indication no changes in locomotion. (**c-d**) Similar time of immobility in FST and TST between animals of all genotypes indicate absence of differences among groups in learned helplessness.(**e**) Anhedonic behavior was accessed by SPT exhibiting no differences of sucrose preference among animals. (**f**) Cognition was monitored using Y-maze test where, once more, no differences in percentage of spontaneous alterations were observed between genotypes. All numeric data are represented as mean ± SEM, **p*<0.05; ***p*<0.01; ****p*<0.0001.

Tamoxifen activation of Cre leads to Tau protein deletion in forebrain regions

As $Tau^{loxP/loxP}$ animals (from this point, called Tau-lox) present no side-effects of transgenesis, we further crossed them to the widely used CAMK2a-$CreER_{T2}$ mouse line (JAX Laboratory; **Figure 5**) that express the postnatal forebrain neuron-specific Camk2a-Cre promoter, bound to a mutant form of the ligand binding domain of the estrogen receptor (ER_{T2}). This system theoretically will allow us to delete *mapt* gene in the adult brain after tamoxifen administration and induction of Cre-recombinase activity (**Figure 5**). The offspring male animals of Tau-lox x CAMK2a-$CreER_{T2}$ cross were used in the next experiments of this study. Both Tau-lox/CaMK and Tau-lox animals (as control littermates) was treated with tamoxifen at 2months of age. As previously described, different target genes or tissues demand different number of tamoxifen injections. Based on previously published protocols for tamoxifen administration, we used a dose of 4mg per day for 5 consecutive days (Andersson et al. 2010; Whitfield, Littlewood, and Soucek 2015) following two scheme of injections: i) 10 i.p. injections scheme, where tamoxifen was injected for two sets of 5-day injections with one week in-between (injection-free) period, in order to avoid/minimize side-effects of prolong and continuous tamoxifen administration (mainly on digestive system) (Huh et al. 2012; Moon et al. 2014); ii) 15 i.p. injections scheme where tamoxifen injections were divided in three sets of 5-day injections (**Figure 6a**). Since Tau is a long-lived protein with the major part of the protein to be bound and localized on axonal MTs, we monitored Tau levels in two different time points, 2 and 4 weeks after the last tamoxifen injection (**Figure 6a**).

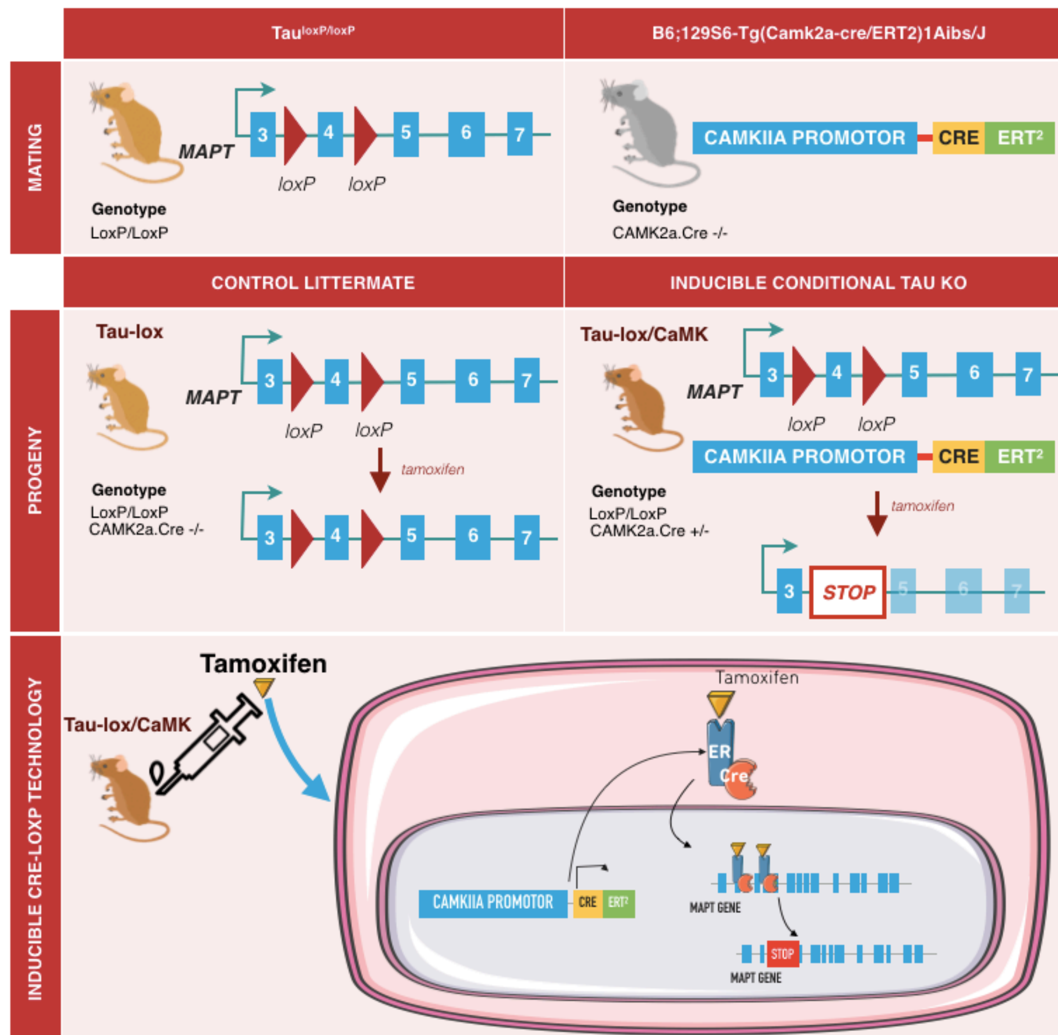

Figure 5. Mating scheme for obtaining Tau-lox/CaMK mice and experimental design. Tau$^{loxP/loxP}$ mice were crossed with B6;129-Tg(Camk2a-cre/ERT2)1Aibs/J, an inducible Cre line originating, Tau$^{loxP/loxP}$ animals that don't express Cre recombinase, here used as control littermates (Tau-lox), and Tau$^{loxP/loxP}$ animals that express inducible Cre recombinase (Tau-lox/CaMK), which are the inducible conditional Tau-KO mice. Tamoxifen administration to Tau-lox/CaMK causes CreER$_{T2}$ activation which triggers the nuclear internalization of Cre, giving rise to the recombination of both loxP sites in mapt gene. This recombination gives rise to a stop codon that blocks the generation of Tau transcription.

During and after the period of tamoxifen administration, we monitored the weight of the animals (**Figure 6b**). Body weight was not different between groups along the weeks of treatment ($F_{(2,42)}$=3.562, p=0.0872). As CAMK2a-Cre expression is mainly observed in the forebrain region, we used cerebellum, a hind limb region, as a control area for our mouse line (**Figure 6c**). As shown in **Figure 6d**, levels of Tau protein in cerebellum are not affected at both 10 and 15 i.p. Tau-lox/CaMK groups ($F_{(2,9)}$=0.3793, p=6948). In contrast, pre-frontal cortex (PFC), hippocampus as well as whole cortex of Tau-lox/CaMK animals exhibit a progressive, time-dependent reduction of Tau levels in

comparison to Tau-lox (control, $Tau^{+/+}$ animals). Specifically, Tau levels in PFC were reduced in both 10 i.p. (p=0.0406) or 15 i.p (p=0.160) Tau-lox/CaMK groups, exhibiting 60-70% reduction ($F_{(2,9)}$=6.322, p=0.0193) at 2 weeks post-injection time point (**Figure 6e**). At 4 weeks post-injection, this reduction of Tau levels was further elevated (80-90% reduction) in both 10 and 15 i.p. Tau-lox/CaMK groups (for both p<0.0001) when compared to Tau-lox group ($F_{(2,9)}$=46.68, p<0.0001) (**Figure 6f**). In the hippocampus, the 10 (p=0.0006) and 15 i.p (p<0.001) Tau-lox/CaMK animals present a 30—45% reduction of Tau levels compared to Tau-lox ($F_{(2,9)}$=36.81, p<0.0001) at 2 weeks post-injection time point (**Figure 6g**), while this reduction was further increased to 60-65% at the later time point of 4 weeks ($F_{(2,9)}$=19.65, p=0.0005) (**Figure 6h**). When analyzing overall cortex, 2 weeks after injection, we found a 30-35% and 40-45% significant decrease of Tau levels in 10 i.p. and 15 i.p. Tau-loxP/CaMK groups, respectively ($F_{(2,9)}$=107.9, p<0.0001) (**Figure 6i**); this tau reduction was enhanced at 4 weeks post-injection time point approaching 65-70% reduction in both Tau-loxP/CaMK 10i.p. (p=0.0042) and Tau-loxP/CaMK 15i.p. (p=0.0021) ($F_{(2,9))}$=13.30, p<0.001) (**Figure 6j**). Based on the above data, we conclude that the scheme of 15 days i.p. injections in Tau-lox/CaMK animals results in severe reduction of Tau levels after 4 weeks of the last tamoxifen injection. Thus, the use of the 15 i.p. injection scheme in Tau-lox/CaMK animals followed by more than 30 days post-injection period seems to be the optimal experimental set-up for conditional deletion of Tau in adult brain.

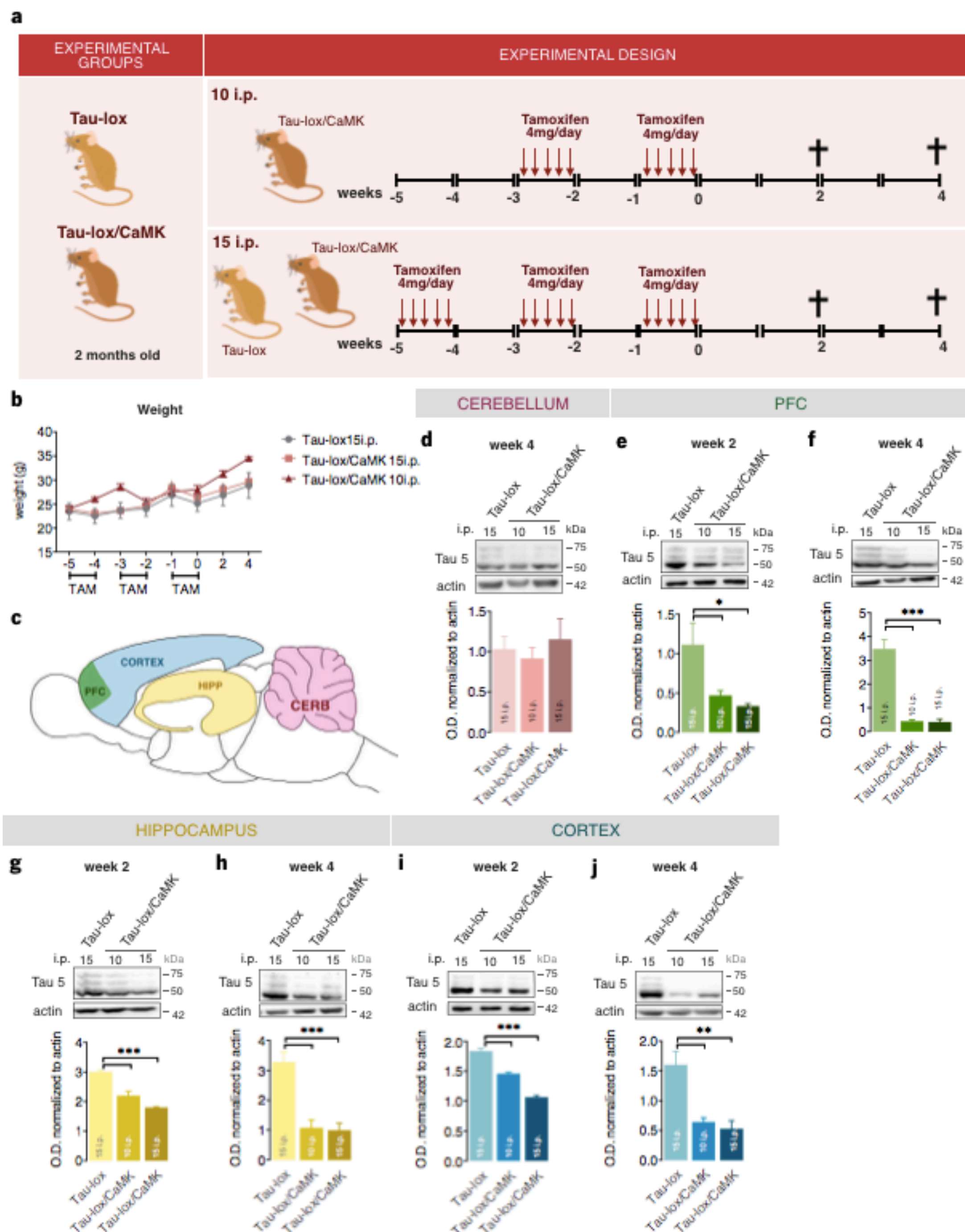

Figure 6. Tamoxifen-induced conditional deletion of Tau in forebrain of Tau-loxP/CaMK mice. (**a**) Tau-lox/CaMK groups received 10 i.p. or 15 i.p. tamoxifen injections in sets of 5 day injections – while Tau-lox (control) mice received 15 i.p. tamoxifen injections. (**b**) Weight variation along tamoxifen administration protocol was monitored, and no differences were observed between groups. (**c**) Schematic representation of the brain areas of interest for molecular analysis of Tau protein levels. (**d**) Cerebellum, as a hindbrain region, was used as a control and no differences were observed. (**e-f**) Tau protein levels were progressively reduced in both 10 and 15 i.p. Tau-lox/CaMK groups at 2 and 4 weeks post-injection time point reaching the level of 70-80% Tau reduction in PFC. (**g-h**) In hippocampus we observe a decrease of 30% after 2 weeks and deletion reached 60-70% 4 weeks after the last injection. (**i-j**) In the cortex we observe at 2 weeks a deletion of 40-50% that increases to 60% after 4 weeks. All numeric data are represented as mean ± SEM, *p<0.05; **p<0.01; ***p<0.0001.

Discussion

Since its discovery in 1975, microtubule-associated protein Tau has been described to have various and important function from neuronal homeostasis related to both cell structure as well as function. The creation of different transgenic animals overexpressing various forms of Tau or fragments of it, have been of clear importance to improve our limited understanding about the involvement of Tau protein in brain pathology, as in AD or other neurodegenerative disorders called Tauopathies. However, except for very few studies reporting some alterations in mechanisms of synaptic plasticity (LTP or LTD) in constitutive Tau-KO animals (Kimura et al. 2014; Ahmed et al. 2014), the creation of several constitutive KO mouse lines didn't offer the expected in vivo evidence about the function of Tau in neuronal and brain function. The four available Tau-KO mouse lines present no behavioral or neurostructural phenotype failing to present MT stabilization problems, deficits in axonal trafficking or any significant neuronal atrophy. Previous studies have provided explanations related to the presence of developmental compensation events by other microtubule-associated proteins. This developmental compensation may mask the consequences of loss of Tau function. This notion is particularly relevant as Tau-related pathology and neurodegeneration in AD brain is suggested to be attributed to both hyperphosphorylation-evoked loss of (normal) Tau function and aggregation-dependent gain of toxic function for Tau. Still, the impact of the loss of normal Tau function in adult or aged brain is not studied, probably due to lack of experimental models that could allow the conditional deletion of *mapt* gene in an age dependent manner, avoiding the loss of Tau during brain development. Thus, this study generated a LoxP/Cre-based mouse model for conditional deletion of *mapt* gene that provides the temporal flexibility in Tau deletion in adult or aged brain overcoming the suggested compensation mechanisms during the critical developmental period.

In recent years, Cre integrase from bacteriophage P1 has become an essential tool for conditional gene activation and inactivation in mouse. Indeed, Cre recombinase efficiently catalyzes recombination between two of its consensus 34 base pair DNA recognition sites, loxP sites, in any kind of DNA or cellular environment (Rossant, Bernelot-Moens, and Nagy 1993; Wood et al. 1993; Nagy et al. 1993). One of the powerful uses of this technology is the conditional removal or activation of gene function, as Cre-mediated recombination leads to the precise excision of an essential region within a gene, affecting protein transcription. Using this system, we created the first Tau loxP flank model, $Tau^{loxP/loxP}$, through the insertion of the loxP recombination sites in the intron 4 of the mouse

mapt gene. One of the great advantages of the Cre/loxP recombination system is that there is no need for additional co-factors or sequence elements for efficient recombination regardless of the cellular environment. In addition, it is highly unlikely that an exact loxP site is represented outside of the phage genome. Consequently, introducing it through transgenesis into the eukaryotic genome restricts the Cre-mediated recombination to the exogenous loxP site, assuring an exact recombination and clear deletion dependent on Cre-recombinase promotor, providing the option of tissue or even cell specificity based on the expression specificity of the promotor that controls Cre. In accordance, our findings suggest that the presence of loxP sites in mapt gene didn't cause any developmental, neurological and any kind of phenotypical alterations that were accompanied by absence of behavioral anomalies in adult animals.

Several groups have described various approaches of controlling the spatial and/or temporal expression of the Cre recombinase. One of the most relevant to the current work is a fusion gene created between Cre and a mutant form of the ligand-binding domain of the estrogen receptor (ER_{TM}). This mutation of ER prevents binding to its natural ligand, estradiol, at normal physiological concentrations, but renders the ER_{TM} domain responsive to tamoxifen (Fawell et al. 1990; Danielian et al. 1993; Littlewood et al. 1995). Fusion leads to ER_{TM}-dependent cytoplasmic sequestration of Cre by Hsp90 (Picard et al. 1990; Indra et al. 1999), thereby preventing nuclear export of Cre and Cre-mediated recombination without ER activation. Thus, we have excluded the use of female animals in the Tamoxifen experiments in order to avoid potential side effects due to female hormones and female sensitivity to estrogen and tamoxifen. We crossed our $Tau^{loxP/loxP}$ mice with a Cre-recombinase mouse line that expresses Cre fused with mutant human estrogen receptor (ER), and most specifically ER_{T2}, which is ~10 fold more sensitive than $CreER_{TM}$, for both nuclear translocation and recombinase activity. This ligand-regulated "switch" for turning on recombinase activity, dependent on tamoxifen administration, can be associated with specific promoters and enhancer elements that allow both temporal and spatial control of recombinase activity. In this case, we used a Cre mouse line that expresses $CreER_{T2}$ under the promoter CAMK2a, a neuronal promoter, specific for forebrain regions. This system allowed us to delete *mapt* gene in region-, cell- and time-specific manner, whereas the only disadvantage of the system is that the excision reaction is effectively irreversible, due to the loss of the circular reaction product. We started tamoxifen administration in 2 months old mice that express Cre-recombinase, Tau-lox/CaMK, and control littermates, Tau-lox. We observed that in the prolonged protocol of tamoxifen administration presented higher decrease in Tau levels that was further enhanced at 4 weeks post-injection time-point. We also observed that Tau deletion

was restricted to forebrain regions, since no deletion was observed in the cerebellum, a hindbrain region. Altogether, this study presents the first tamoxifen-induced conditional Tau-KO model that presents 70-80% reduction of Tau levels in most forebrain areas using the Cre-CamKII-ER_{T2}. Additionally, $Tau^{loxP/loxP}$ mice will provide a novel powerful experimental model to study the real function of Tau in different brain areas and circuits as well as in specific cell types (e.g. neuronal vs. glial) in different time-windows of animal lifespan.

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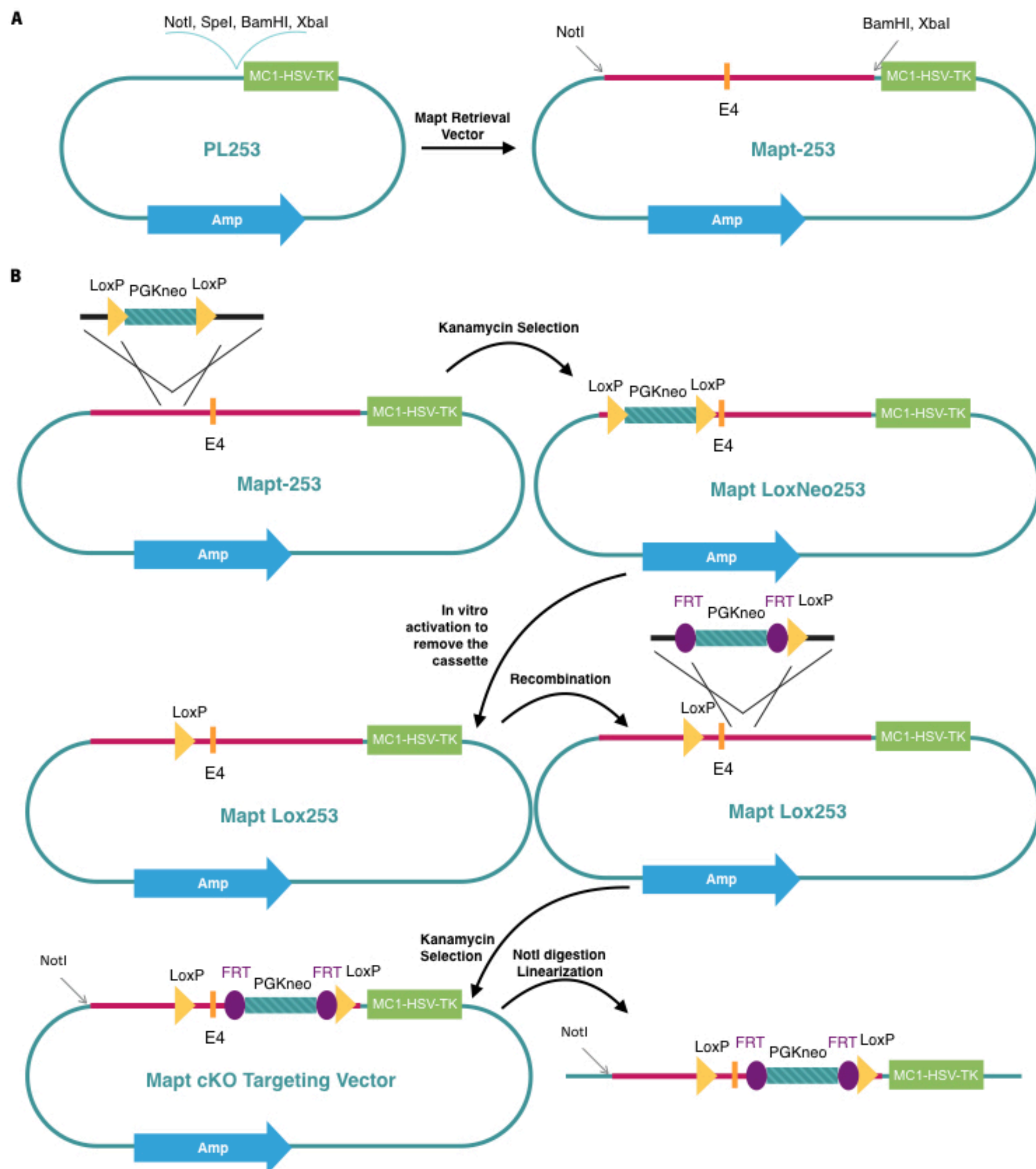
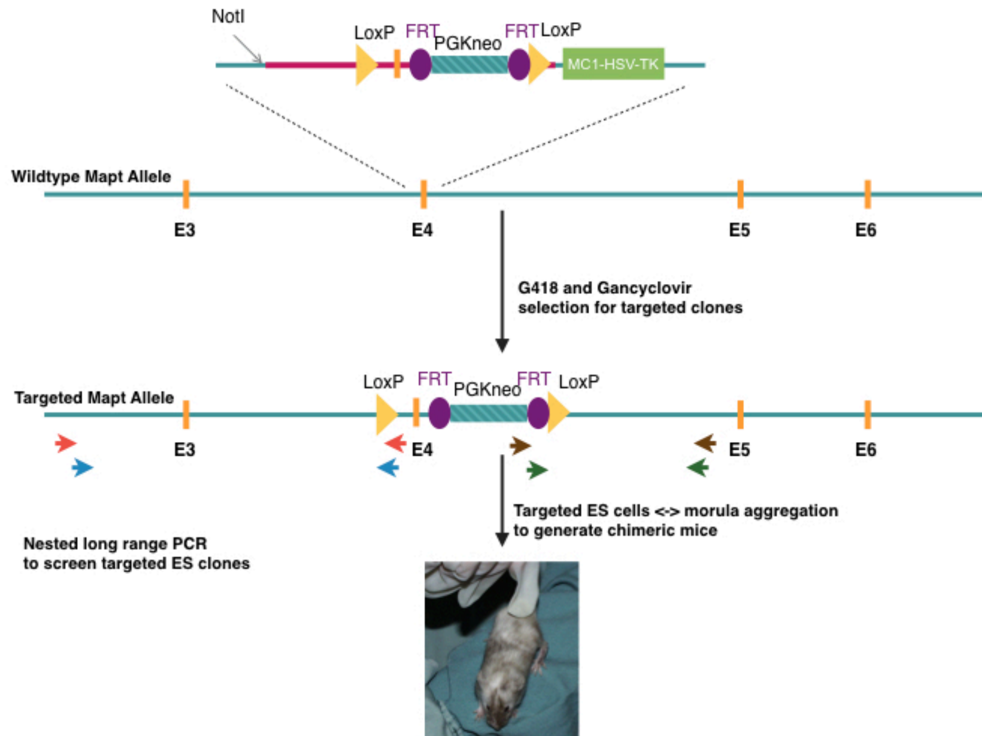
Supplementary Information

Supplementary Figure 1



Supplementary Figure 1. Creation an dlinearization of the target vector with Mapt flox. “FRT-PGK-gb2-neo-FRT-loxPP” cassette was designed to allow kanamycin/neomycin selection in prokaryotic and eukaryotic cells, respectively, combining a prokaryotic promoter (gb2) for expression of kanamycin resistance in *E.coli* with a eukaryotic promoter (PGK) for expression of neomycin resistance in mammalian cells. The prokaryotic promoter gb2 is a slightly modified version of the Em7 promoter; mediating higher transcription efficiency than the generally used Tn5 promoter. The promoter of the mouse Phosphoglucokinase gene (PGK) was used as the eukaryotic promoter. A synthetic polyadenylation signal terminates the kanamycin/neomycin expression. The cassette is flanked by FRT sites for later excision by Flp-recombinase. An additional single loxP site is located at the 3’ end of the cassette. Unique NotI and XhoI sites flank the cassette for convenient cloning with restriction sites, allows for linearization of the vector for further ES cells electroporation.

Supplementary Figure 2



Supplementary Figure 2. Targeting Vector (floxed exon 4) to generate chimeric mice. The linearized targeting vector was electroporated into ES cells derived from F1(129Sv/C57BL6j) blastocyst. Chimeric animals were generated by aggregation of slected ES cells with CD1 morula according to Nagy and Rossant (1993).

Supplementary Figure 3

Supplementary Figure 3. MAPT amino acid sequence alterations after Cre excision. (**a**) MAPT transcript and amino acid sequence. (**b**) Predicted MAPT transcript and amino acid sequence after Cre excision, when crossed with a Cre mouse line.

Supplementary Table 1

Supplementary Table 1. Conversion of time intervals registered for each test into dichotomy scores in milestones protocol. Neonatal mouse pups were daily examined from PND0 to PND21 for each parameter and time to react to each parameter was assessed and then converted to a score. When the animal reached the highest score for 3 consecutive days a mature response was registered. (Hill Lim, M.A., and Stone, M.M. 2008).

		Scores			
		0	1	2	3
Somatic Parameters	Weight	-	-	-	-
	A.N.D	-	-	-	-
	Eye Opening	both eyes closed	one eye open	both eyes open	-
Labyrinthic reflexes and coordination	Surface Righting	no response within 30 s	rights itself but slowly (10-25 s)	rights itself but it takes up to 10 s to do it	rights itself immediately in less than 1 s
	Air righting	lands on its back	lands on the surface with all four paws		
Vestibular system	Negative Geotaxis	does not move at all within the 30 s period	turns its body 180° to the 'head up' position in a period time inferior to 30 s		
	Cliff Aversion	reezes/does not respond within the 30 s test-period	turns very slowly back to the surface (10-25 s)	avoids the cliff, but it still takes some time to turn, up to 10 s	turns back in less than 1 s
Neurological Reflexes	Auditory Startle	does not jump	jump		
	Postural Reflex	not present	present		
	Ear Twitch	not present	present		
	Grasping	no grasping	places its paw on the cotton bud, but it does not hold on firmly	places its paw on the cotton bud with more force, but when the cotton bud is pulled, cannot hold it	grasps the cotton bud very firmly
Motor Coordination and strength	Wire Suspension	falls immediately	grasp the bar with all four limbs (maximum time set of 30 s)		
	Walking	no locomotion	pivoting – moving around with the help of the head and forelimbs, but not using the hind limbs	crawling – moving on all four limbs, dragging the belly over the surface	walking – mature locomotion with the body supported completely by the four limbs

Supplementary Table 2

Supplementary Table 2. Parameters evaluated during developmental assessment in milestones protocol. Neonatal mouse pups were daily examined from PND0 to PND21 on a battery of developmental tests assessing strength, coordination and the appearance of reflexes. This data sheet for developmental milestones, indicates the group of the parameters and the range of days between which the mature response is expected in normal conditions.

Measure		Average age for response (days)	Range (days)
Somatic Parameters	Weight	-	-
	A.N.D	-	-
	Eye Opening	13	7-17
Labyrinthic reflexes and coordination	Surface Righting	5	1-10
	Air righting	18	16-21
Vestibular system	Negative Geotaxis	7	3-15
	Cliff Aversion	8	2-12
Neurological Reflexes	Auditory Startle	15	11-21
	Grasping	7	3-15
	Postural Reflex	13	5-21
	Ear Twitch	10	6-14
Motor Parameters	Wire Suspension	15	9-21
	Walking	15	9-21

CHAPTER 5

CONDITIONAL LOSS OF TAU IN THE ADULT BRAIN CAUSES ANXIOUS AND DEPRESSIVE PATHOLOGY.

Manuscript in preparation

Conditional loss of Tau in the adult brain causes anxious and depressive pathology

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Abstract

Mounting evidence of the last decades supports a fundamental role for Tau protein in neuronal structure and function; however, adult (constitutive) Tau knock-out (KO) animals exhibit no major abnormalities raising doubts about the real role of Tau in the brain. Hereby, we demonstrate that conditional deletion of *mapt* in forebrain of adult mice triggers anxious and depressive-like behavior accompanied by neuroplastic deficits. Moreover, specific Tau deletion in the central amygdala partially mimics the above deficits, suggesting that loss of Tau in adult brain is detrimental.

Main Text

Neuronal pathology in Alzheimer's disease and other neurodegenerative disorders is attributed to deficits in Tau protein and its homeostasis (Wang & Mandelkow, 2015) that are related to gain-of-toxic function (e.g. insoluble neurotoxic aggregates of Tau) as well as loss-of-normal function (e.g diminished microtubule-binding capacity due to Tau hyperphosphorylation) (Trojanowski & Lee, 2005). While many transgenic mouse lines overexpressing wild-type or mutant Tau have provided mechanistic evidence about how Tau accumulation and aggregation cause neuronal damage (Götz, 2001; Morris, Maeda, Vossel, & Mucke, 2011), the impact of loss of (normal) Tau and its function(s) in adult brain remains enigmatic and puzzling. Several studies support a fundamental role for Tau protein in neuronal structure and function as Tau interacts with different cellular proteins and thus, is involved in a variety of cellular processes such as microtubule (MT) stabilization, axonal growth, cargo trafficking and more recently synaptic signaling and plasticity (Ittner et al., 2010; Morris, Maeda, Vossel, & Mucke, 2011; Kimura et al., 2014). However, the *in vivo* significance of these functions is still uncertain, as adult animals of different constitutive Tau-knockout (KO) models don´t exhibit MT alterations, axonal transport abnormalities or neurostructural and behavioral deficits (Ke et al., 2012). The above controversy highlights a significance gap of knowledge about the real role of Tau in adult brain that could be attributed to developmental compensation mechanisms by other microtubule associated proteins (MAPs) (Harada et al., 1994) that may mask the consequences of Tau deletion.

Avoiding any developmental impact of *mapt* gene or Tau protein loss, we, hereby, present a novel conditional Tau-KO model using the Cre-LoxP system which offers us the temporal flexibility of *mapt* gene deletion in adult mouse brain. First, the Tau-lox mouse line created (see materials and methods; **Supplementary Fig. 1**) was crossed with mice expressing tamoxifen-inducible *CaMK2a-driven Cre-recombinase* (CAMK2aER$_{T2}$) (Madisen et al., 2010) (**Supplementary Fig. 2**). To assess whether the presence of the *LoxP* sites in *MAPT* gene or *Camk2a-CreER*$_{T2}$ transgene had any influence on the developmental, neurological and behavioral profile of these animals, Tau-lox (control littermates) and Tau-lox/CaMK animals were subjected to an extensive battery of tests including Milestones (**Supplementary Figure 3**) and SHIRPA protocol (**Supplementary Figure 4**) as well as cognitive and mood tests, presenting no differences among all groups in all parameters monitored (**Supplementary Figure 5**).

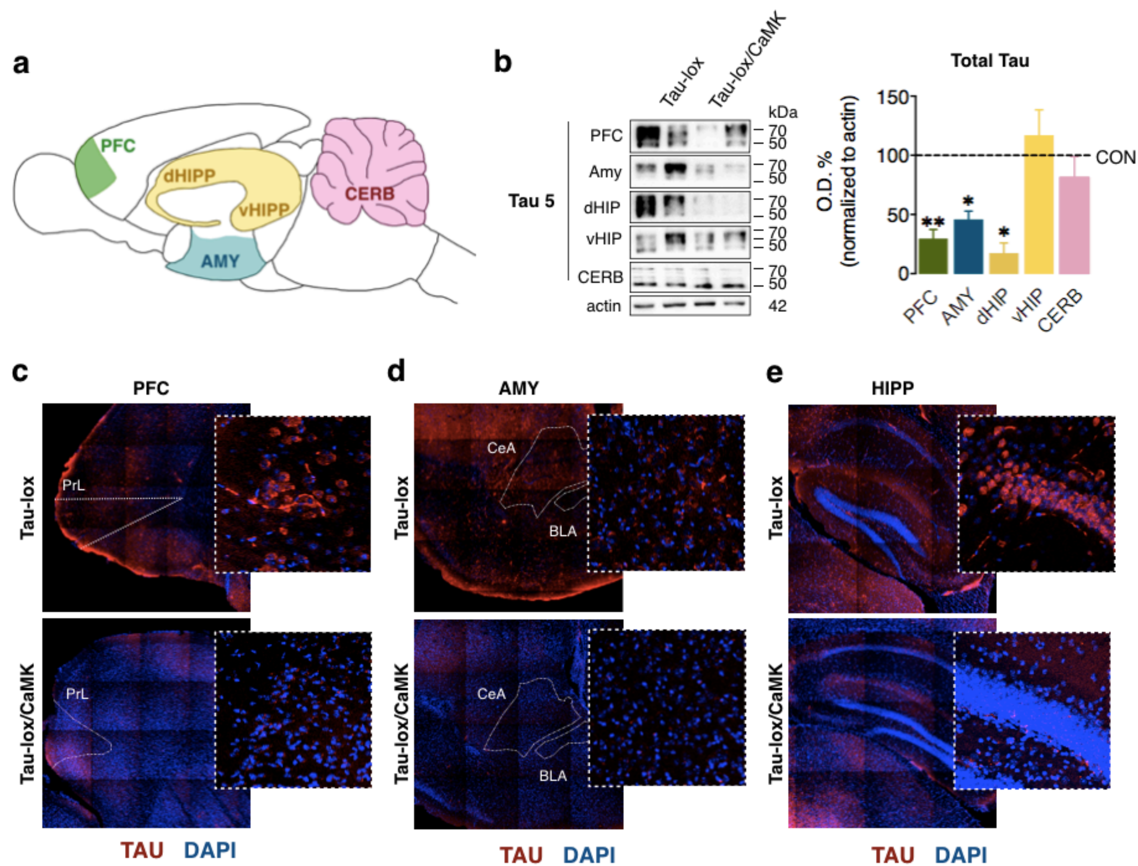

Figure 1. Conditional deletion of Tau in forebrain regions of adult Tau-lox/CaMK mice after tamoxifen injection. (**a**) Schematic representation of mouse brain showing the different brain areas analyzed for Tau protein levels. (**b**) Severe reduction of Tau protein levels is observed in PFC ($t_{(20)}$=2.927, p=0.008), amygdala (AMY) ($t_{(11)}$=2.663, p=0.022) and dorsal hippocampus (dHIP) ($t_{(9)}$=2.417, p=0.038) of Tau-lox/CaMK mice 6 weeks after the last tamoxifen injection in comparison to Tau-lix (control) littermates; no differences are found in cerebellum ($t_{(26)}$=0.819, p=0.42) or ventral hippocampus (vHIP) ($t_{(29)}$=0.628, p=0.534). (**c-f**) Immunofluorescence analysis of Tau protein expression in the brain of Tau-lox and Tau-lox/CaMK mice showing a severe reduction or loss of Tau protein levels in PFC (**d**), Amygdala (**e**) and hippocampus (**f**) of Tau-lox/CaMK mice. All numeric data represent mean±SEM; *p<0.05 and **p<0.01.

Next, Tau-lox and Tau-lox/CaMK animals (2 months old) were intraperitoneally (i.p.) injected with tamoxifen following a 5-weeks long injection protocol (for detailed experimental design see **Supplementary Figure 2**). Consistent with *Camk2a-Cre* expression in forebrain regions of the adult mouse (Madisen et al., 2010), Tau protein levels were not affected in hindbrain (e.g. cerebellum) of Tau-lox/CaMK animals (**Figure 1b**). However, Tau-lox/CaMK animals exhibited severe reduction of protein Tau levels (65-90%) in different forebrain regions such as prefrontal cortex (PFC), amygdala (AMY) and dorsal hippocampus (dHIP) compared to their Tau-lox littermates, 6

weeks after the last tamoxifen injection (**Figure 1b**), in accordance we observed a reduction in mRNA levels (**Supplementary Figure 6**). Severe reduction in Tau levels was confirmed by immunofluorescence staining where the clear axonal and somatic Tau staining of Tau-lox neurons is severely diminished or lost in Tau-lox/CaMK neurons (**Figure 1c-f**). These findings are in accordance with previous studies that described that Cre-LoxP system does not cause homogeneous deletion throughout the cell-targets in the area of interest and even have differentially activation patterns in littermates, causing a partial reduction of protein and mRNA levels of the target gene (Heffner et al., 2012; Turlo, Gallaher, Vora, Laski, & Iruela-Arispe, 2010).

As Tau is suggested to interact with different proteins involved in cytoskeletal integrity (Brandt & G??tz, 2016), we next monitored neuronal morphology performing Golgi-based 3D neuronal reconstruction in different brain areas. We observed a significant decrease in total dendritic length of pyramidal neurons in prelimbic (PrL) and infralimbic (IL), areas of medial PFC, of Tau-lox/CaMK mice when compared with their control littermates (**Figure 2a-b**). Similarly, other areas of Tau-lox/CaMK mice exhibit neurostructural alterations such as central amygdala (CeA) (**Figure 2c**) and DG neurons of the dorsal hippocampus (dDG) (**Figure 2e**), but not basolateral amygdala (BLA) (**Figure 2d**) or DG from ventral hippocampus (vDG) (**Figure 2f**). Interestingly, the neurons of the above brain areas that present dendritic atrophy also exhibited increased spine density (**Figure 2a-f**). Furthermore, the analysis of the neurosynaptosomes revealed that Tau-lox/CaMK mice exhibit increased levels of PSD-95 and HOMER in amydgala and PFC (**Figure 2h-i**), two essential proteins of synaptic structure whose level alterations could reflect the above-mentioned increase of spine density as previously described (Harris & Stevens, 1989; Kimura et al. 2007). However, Shank and cortactin protein levels are decreased in Tau-lox/CaMK mice compared to Tau-lox mice (**Figure 2h-i**). Shank has been identified as having important function at the synapse, including glutamate receptor trafficking, regulating actin cytoskeleton through its interaction with cortactin (Naisbitt et al., 1999; Macgillavry et al. 2016), and synapse formation, transmission and plasticity (Tu et al., 1999; Arons et al., 2012; Grabrucker et al., 2011; Harris, et al. 2016). Furthermore, the direct interaction of both proteins (Wu & Parsons, 1993; Huang et al., 1997), Shank and cortactin, may function as mediators of cytoskeleton remodeling in neurons, linking to NMDA receptor activation in post-synaptic sites with actin network remodeling (Naisbitt et al., 1999).

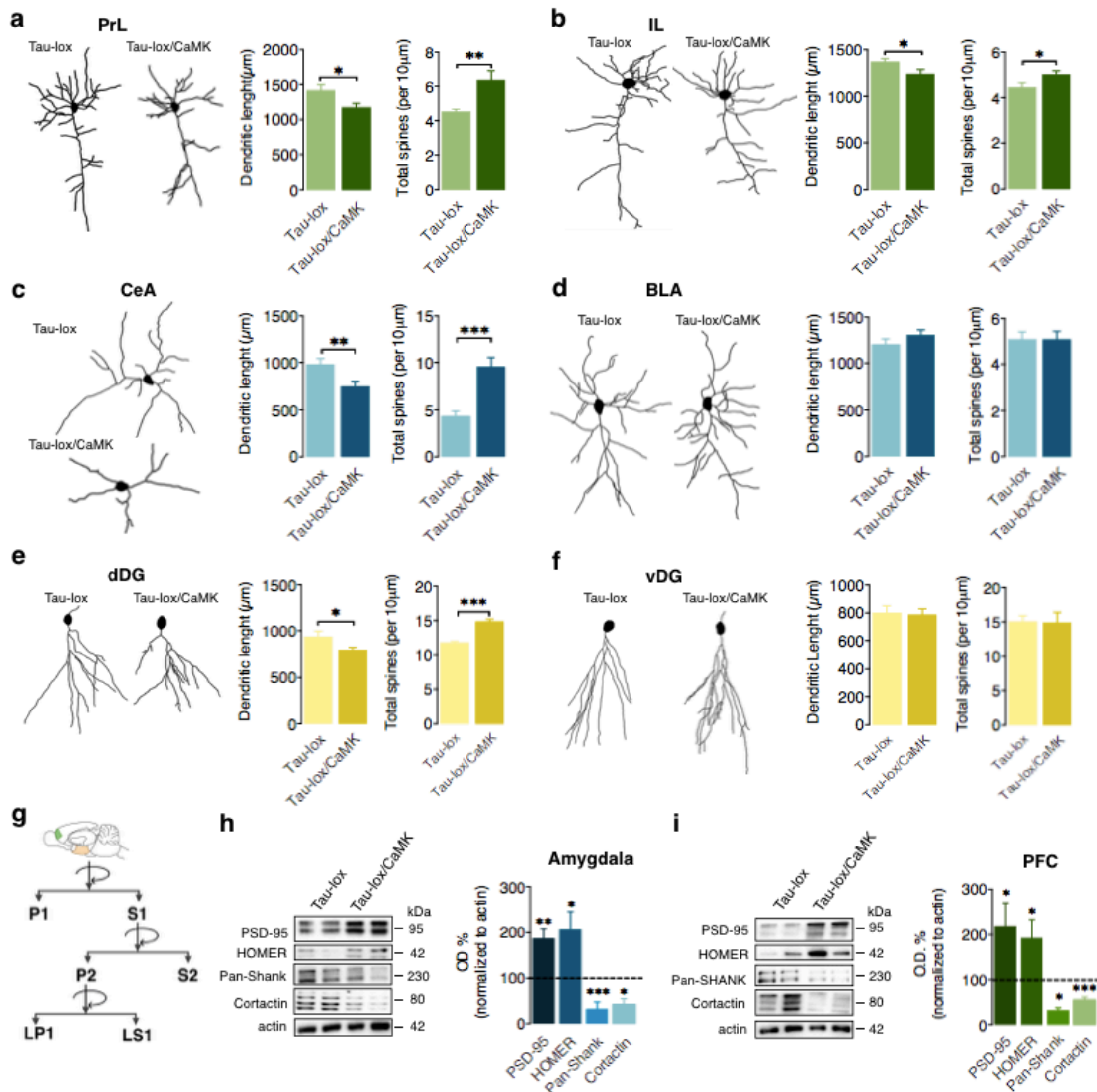

Figure 2. Tau-lox/CaMK mice exhibit neurostructural alterations in adult brain (**a-b**) In comparison to their Tau-lox (control; CON) littermates, Tau-lox/CaMK mice exhibit a decrease in dendritic length and increase in spine number in the prelimbic (PrL; $t_{(41)}$=2.389, p=0.021; $t_{(41)}$=2.195, p=0.033, respectively) and infralimbic (IL) ($t_{(60)}$=2.128, p=0.037; $t_{(47)}$=2.224, p=0.031) region of the mPFC. (**c**) Neurons of central (CeA), but not basolateral (BLA), amygdala present reduced dendritic length and increased spine number in comparison to Tau-lox neurons (CeA $t_{(40)}$=3.079, p=0.0037; $t_{(42)}$=5.962, p<0.0001, respectively; BLA $t_{(44)}$=1.362, p=0.18; $t_{(38)}$=0.164, p=0.987). Dorsal, but not ventral, hippocampal neurons of Tau-lox/CaMK mice present similar neurostructural characteristics (dDG dendr. length $t_{(46)}$=0.216, p=0.035; spine density $t_{(48)}$=7.258, p<0.0001) (vDG $t_{(45)}$=0.226, p=0.822; ($t_{(43)}$=0.0142, p=0.988). (**g**) Schematic representation of the fractionation protocol used to separate neurosynaptossomes (LP1). (**h-i**) Neurosynaptosomal analysis from amygdala and PFC showed a clear increase of PSD-95 (Amy $t_{(18)}$=3.889, p=0.0011; PFC $t_{(15)}$=2.462, p=0.027); and Homer levels (Amy $t_{(29)}$=2.704, p=0.011; PFC $t_{(24)}$=2.123, p=0.044) while, levels of Shank (Amy $t_{(16)}$=4.948, p=0.001; PFC $t_{(10)}$=2.804, p=0.018) and cortactin (Amy $t_{(17)}$=2.775, p=0.013; PFC $t_{(32)}$=4.105, p=0.0003) were severely reduced. All numeric data represent mean±SEM; *p<0.05, **p<0.01 and ***p<0.001.

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As neurostructural alterations in brain areas such as PFC, hippocampus and amygdala usually underlie changes in behavioral performance (Sousa & Almeida, 2012), we next evaluated the three different dimensions of behavior monitoring anxiety, depression and cognition using an extended battery of behavioral tests. Our results show that Tau-lox/CaMK mice present increased levels of anxiety compared to Tau-lox animals as assessed by decreased entries and time that animals spend in the open arms of the elevated plus maze (EPM) test (**Figure 2a**), decrease time and distance travelled in the center of the open field (OF) arena (**Figure 2b**) and reduced time spend in the light chamber of Light/Dark box (LDB) (**Figure 2c**). The Tau-lox/CaMK anxious behavior is also confirmed by the novelty suppressed feeding (NSF) test, where Tau-lox/CaMK mice spent more time to reach the center of the arena (**Figure 2d**). Furthermore, when compared to Tau-lox mice, Tau-lox/CaMK animals present increased immobility time forced-swim (**Figure 2e**) and tail-suspension test (**Figure 2f**) indicating increased levels of learned helplessness, an essential parameter of depressive symptomatology. Moreover, we also monitor anhedonia, a core feature of depressive behavior, showing that Tau-lox/CaMK animals exhibit decreased sucrose preference in sucrose-preference test (**Figure 2g; Supplementary Figure 7a**) as well as preference for sweet pellets in the sweet-drive test (**Figure 2h; Supplementary Figure 7b**) suggesting increased anhedonic behavior. Moreover, we monitor cognitive performance showing that animals present no recognition or spatial memory deficits, as shown by novel place and object recognition tests as well as Morris Water Maze test.

As morphological and functional alterations in amygdala are shown to be essential to regulate limbic neuronal circuits towards the establishment of anxious-like behavior, we next monitor the impact of *mapt* deletion specifically in the CeA, found to be atrophic in Tau-lox/CaMK. For that purpose, we used a viral approach, in which we injected AVV5-CMV-Cre-GFP bilaterally in the CeA of Tau-lox mice (**Figure 3o**). Similarly, Tau-lox/CaMK animals, specific CeA *mapt* deletion evoked anxious behavior as shown by decrease time spent and entries in the open arms of EPM (**Figure 3p**), as well as decrease time in the OF center (**Figure 3q**), with no effects on depressive-like behavior or cognitive performance (**Supplementary Figure 8**).

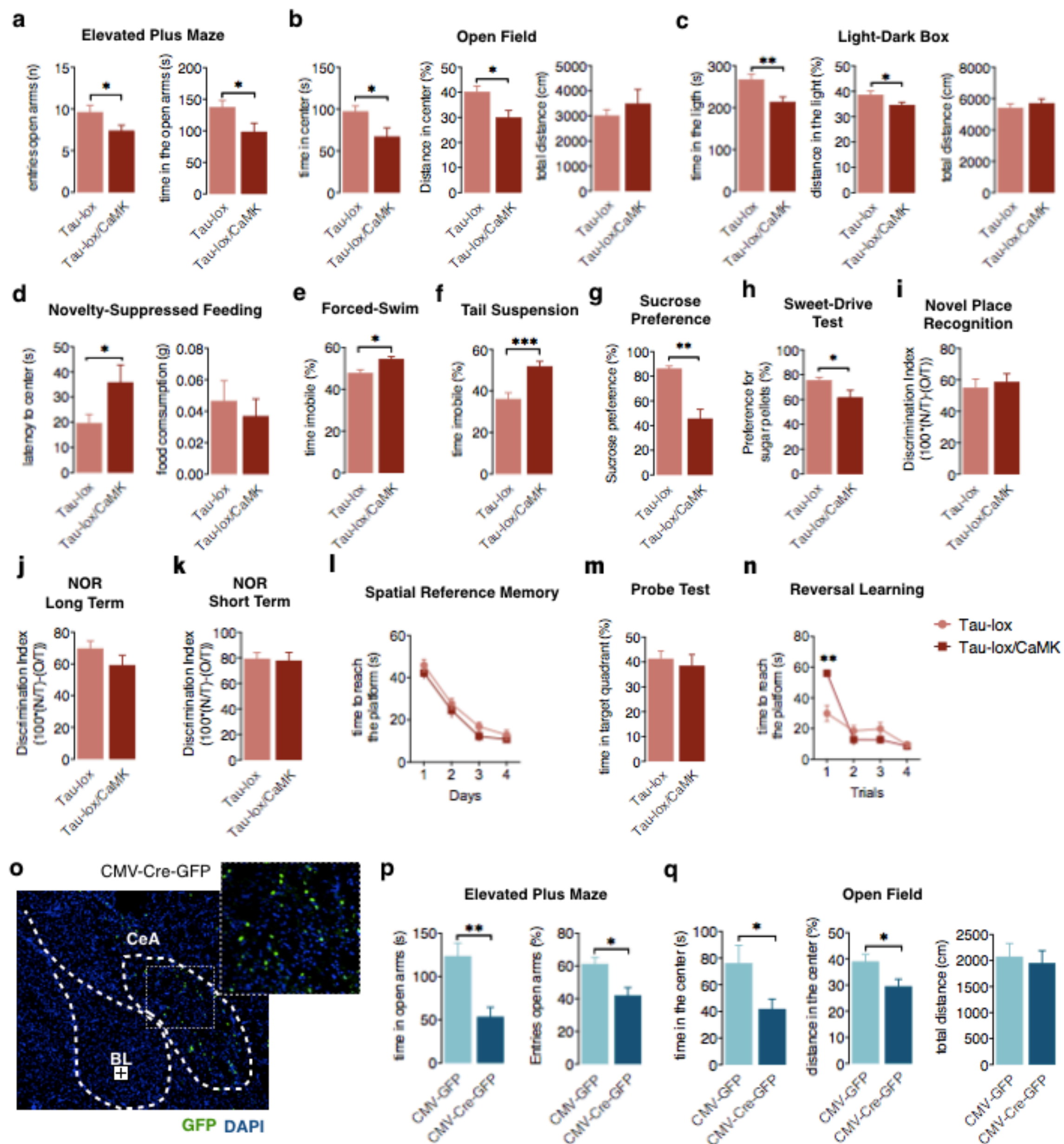

Figure 3. Conditional deletion of Tau in the adult forebrain leads to anxious and depressive-like behavior. (**a-d**) Tau ablation evoked anxious behavior as assessed by reduced entries ($t_{(27)}$=2.082, p=0.046) and time ($t_{(27)}$=2.206, p=0.036) that Tau-lox/CaMK animals spent in the open arms of Elevated plus maze (EPM) apparatus in comparison to tau-lox (control) littermates (**a**). In addition, Tau-lox/CaMK mice present decreased time and distance travelled in the center of the Open field (OF) arena (**b**) (OF time $t_{(16)}$=2.351, p=0.031; distance $t_{(16)}$=2.741, p=0.014) and light part of Light/Dark (L/D) box apparatus (L/D time $t_{(34)}$=2.950, p=0.005; distance $t_{(34)}$=2.167, p=0.373) indicating increase anxiety. Note that both OF/L/DF box tests show no locomotor differences between groups of both genotypes as assessed by total distance (OF $t_{(16)}$=1.585, p=0.132; L/D $t_{(34)}$=0.799, p=0.429). In the novelty suppress feeding test (**d**), Tau-lox/CaMK mice spend more time to reach the center ($t_{(18)}$=2.185, p=0.042) of the arena, but no differences are observed in food consumption ($t_{(18)}$=0.552, p=0.587); altogether, the above tests indicate that Tau-lox/CaMK mice exhibit

elevated anxiety levels compared to Tau-lox mice. (**e-f**) Learned helplessness, an essential characteristic of depressive symptomatology was assessed by FST and TST; Tau-lox/CaMK mice present a depressive-like behavior exhibiting increased immobility time in forced-swim test ($t_{(17)}$=2.890, *p*=0.010) and in tail suspension test ($t_{(23)}$=3.868, *p*=0.0008). (**g-h**) Anhedonic behavior, another core feature of depression, was also found in Tau-lox/CaMK mice as they exhibit decreased preference for sucrose ($t_{(16)}$=3.644, *p*=0.002) as well as for sugar pellet ($t_{(16)}$=2.362, *p*=0.031) in comparison to their littermates controls (Tau-lox) in sucrose preference (**g**) and sweet-drive test (**h**), respectively (**i-n**). Regarding cognitive behavior, Tau-lox/CaMK mice present no differences when compared to control Tau-lox mice in the: (**i**) Novel place recognition (NPR) test ($t_{(32)}$=0.478, *p*=0.635), (**j-k**) long-term ($t_{(31)}$=3.332, *p*=0.192) and short-term ($t_{(17)}$=0.161, *p*=0.873) task of Novel object recognition (NOR) test, (**l**) learning curve of spatial reference memory task of the MWM (*p*=0.08), as well as (m) the probe test ($t_{(21)}$=0.510, *p*=0.615). However, Tau-lox/CaMK mice spend more time to reach the escaping platform located in the opposite quadrant of the MWM pool in first trial of the reversal learning task (**n**) ($t_{(23)}$=3.055, *p*=0.005) while they equally learn in the next trials. (**o**) GFP staining is restricted to the CeA area of Tau-lox mice bilaterally injected with AAV5-CMV-Cre-GFP at CeA for local Tau ablation; magnified view of the region to the right. (**p**) CMV-Cre-GFP-injected Tau-lox mice present an anxious behavior four months after virus injection exhibiting reduced (**p**) time ($t_{(14)}$=3.776, *p*=0.002) and number of entries ($t_{(14)}$=2.912, *p*=0.011) spent in the open arms of EPM compared to CMV-GFP injected mice. (**q**) In addition to EPM, distance and time in the center of OF was also decreased (distance $t_{(14)}$=2.425, *p*=0.029; time $t_{(14)}$=2.519, *p*=0.024); no differences in total distance travelled between two groups was also found ($t_{(14)}$=0.3490, *p*=0.732). All numeric data represent mean±SEM; *p<0.05, **p<0.01 and ***p<0.001.

This study provides the first *in vivo* evidence that Tau protein does impact on neuronal plasticity as its reduction or deletion causes dendritic atrophy in pyramidal neurons of different brain areas. Interestingly, Tau-lox/CaMK neurons with reduced Tau didn't lose their ability to blind new spines, most probably in a potential effort to compensate the dendritic damage. However, reduction of essential scaffold proteins such as Shank and cortactin points towards alterations of synaptic structure and function in line with recently suggested participation of Tau in (normal) synaptic activity and related signaling as Tau moves to synaptic compartment binding to actin (Frandmiche et al. 2014). The above Tau-related neuroplastic alterations affect different brain areas participating in limbic circuitry such as PFC and amygdala leading to clear anxious and depressive-like behavior. In line with previous studies supporting an essential role of amygdala in the establishment of anxiety (Davidson, 2002; Tovote, Fadok, & Lüthi, 2015), specific deletion of Tau in the amygdala or PFC was able to phenocopy the anxious behavior found in animals with forebrain reduction or deletion of

Tau. Collectively, this study demonstrate that Tau protein exhibits an essential role in brain structure and homeostasis of the adult brain highlighting the potential maintaining role of Tau in fully developed and formed circuits and networks of the adult brain. In addition, we hereby present an conditional Tau-KO model that will allow us to further dissect Tau function in the adult as well as aged brain and its relevance for synaptic signaling and function along brain aging.

Material and Methods

Animals and housing conditions

Tau-lox (for details see below) and CaMK-Cre [B6;129S6-Tg(Camk2a-cre/ERT2)1Aibs/J (JAXLaboratory, #012362 stock)] were crossed to generate Tau-lox/CaMK transgenic animals (see also **Supplementary Figure 2**). 2 months old male animals were used in the starting point of this study; mice were group-housed (5 animals per cage) with libitum access to food and water under standard environmental conditions (8a.m.- 8p.m light cycle; 22°C; 55% humidity). All experiments were conducted in accordance with the Portuguese national authority for animal experimentation, Direcção Geral de Veterinária (ID: DGV9457)) and Directive 2010/63/EU of the European Parliament and Council. Experimenters involved in these studies are authorized by the Portuguese national authority for animal experimentation, Direcção Geral de Veterinária

Generation of MAPT^{tm1nsis} transgenic mice

Generation of Tau-lox mouse line (MAPT^{tm1nsis}) was performed at Gene Targeting & Transgenic Facility, University of Connecticut, Health Center (USA). The Mapt targeting vector was prepared by recombination as described by Lee et al (2001). Briefly, 9 kb of Ant1 genomic sequence containing exon 4 including approximately 4.8 Kb and 4.15 Kb of intron 3 and 4 sequence was retrieved from the RP23-344E9 BAC (obtained from the BACPAC Resources Center, Children's Hospital Oakland Research Institute, Oakland, CA) by gap repair. The first loxP site was inserted into intron 3 approximately 0.5 kb upstream of exon 4 and the second loxP site together with the Frt-PGKneo-Frt cassette was inserted approximately 0.35 kb 3' of exon 4. The targeting vector was then linearized by NotI digestion, phenol/chloroform purified, precipitated and then resuspended in PBS at 1g/L. The linearized targeting vector was then electroporated into ES cells derived from F1(129Sv/C57BL6j) blastocyst. ES cell culture and electroporation were performed as described by Wurst and Joyner (1993). Drug (G418 and Ganciclovir) resistant colonies were picked and grown in 96-well plate. Targeted ES clones were identified by long range nested PCR using Platinum HiFi Taq purchased from Invitrogen with condition according to the manufacturer. Chimeric animals were generated by aggregation of ES cells with CD1 morula according to Nagy and Rossant (1993). Chimeric males were bred with ROSA26-Flpe female (Jax stock no: 009086) to remove the PGKneo cassette and generate F1 pups with Mapt floxed allele. Positive pups were identified by PCR genotyping using two different primer pairs, LoxP gtF/R and Frt gtF/R (**See Supplementary Figure 1**).

Tamoxifen Treatment

Tamoxifen (Sigma, #T5648) was dissolved in a corn oil (Sigma, #C8267) with 10% ethanol, at a concentration of 20 mg/ml of tamoxifen. Animals were subjected to a 5 week long injection scheme which includes 3 sets of 5 consecutive i.p. injections of 4mg Tamoxifen (one per day) with 1 week interval between sets (see also, **Supplementary figure 2**).

Immunofluorescence Analysis

Deeply anesthetized animals (pentobarbital 50mg/Kg) were transcardially perfused with 0.9%NaCl. Brains were immersed in O.C.T. reagent and stored at -80°C. 20μm cryostat brain sections were cut. Cryostat sections were fixed in 5%PFA for 30min before immunofluorescence protocol was performed. Slides were exposed to antigen retrieval followed by 0.3% triton X-100 treatment before incubation with antisera against Tau5 (1:500, ABCAM #80579) and Cre-recombinase or GFP antisera (1:500, ABCAM #6673), and the appropriate secondary antibodies, followed by DAPI incubation for nuclear staining. Images were collected and analysed by confocal microscopy (Olympus FluoViewTMFV1000).

Western Blotting

After behavior tests completed animals were decapitated and brains were excised immediately. Prefrontal cortex (PFC), dorsal hippocampus(dHIP), ventral hippocampus (vHIP), amygdala(AMY) and Cerebellum were dissected (on ice) and immediately stored at –80°C. After homogenization in RIPA buffer (50mM TrisHCl, 2mM EDTA, 250mM NaCl, 10% glycerol)) with phosphatase inhibitors (Phosphatase inhibitor cocktail 2, Sigma #5726; Phosphatase inhibitor cocktail 3, Sigma #0044) and protease inhibitors (Roche #11697498001), lysates were centrifuged at 13000rpm for 15min and supernatant was collected. Samples were quantified using Bradford Assay method. After SDS–PAGE electrophoresis of 20ug of sample, and semi-dry transfer using TURBO BioRad System, all membranes were incubated in different antisera (actin (1:2500; ABCAM, #ab8224) and Tau5 (1:2000; ABCAM, #ab80579)) while blots were revealed by enhanced chemiluminescent (ECL, BioRad) using Chemidoc®BioRad detection system.

RNA extraction and qRT-PCR

Total RNA was isolated from macrodissected brain areas of interest using TRIZOL (Invitrogen #15596) according to the manufacturer's protocol. RNA was quantified using NanoDrop apparatus and samples were stored at -80°C. 1μg of RNA sample was treated with DNase and then cDNA was synthesized using the IScript Kit (BioRad, #170-8891). cDNA samples were diluted in 1:10 in RNase-free water, and mRNA quantification by qRT-PCR was performed using EVAGreen (BioRad #172-5202), with the following cycles: 95°C, 10 min; followed by 95°C, 15min, 60°C, 30min, 72°C, 30 min, 40 cycles. The results were then normalized against housekeeping B2m genes and results were presented as $2^{-\Delta\Delta_{CT}}$.

The primers used were for MAPT were F-5'-TGAAGACGTGACTGCGCCCCT-3' and R-5'-GTTTTGCCATCAGCGCCCTTGG-3'; for B2m F- 5'-CCTTCAGCAAGGACTGGTCT-3' and R- 5'-TCTCGATCCCAGTAGACGGT-3'.

Behavior Tests

Elevated-Plus Maze (EPM). This test was used to access anxious behavior as previously described (Lopes et al. 2016). 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 during 5min. 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 EthoVision®XT software (Noldus).

Open Field (OF). This test was conducted in an arena with transparent acrylic walls and white floor (Med Associates Inc., St. Albans, VT, USA). Mice were placed in the center of the arena and movement was monitored over a period of 5 min with the aid of two 16-beam infrared arrays. Time spent in the center of the arena was used as a measure of anxious-like behavior. Total distance traveled was used as an indicator of locomotor activity.

Light-Dark Box (LDB) test. LDB test was conducted in an arena with transparent acrylic walls and white floor (Med Associates Inc., St. Albans, VT, USA), with a black box over one half of the apparatus (Dark). Mice were placed in the center of the light boxand their movement was monitored over a period of 10 min with the aid of two 16-beam infrared arrays. Time spent in the light part of the box was used as a measure of anxious behavior. Total distance traveled was used as an indicator of locomotor activity.

Novelty-Supressed Feeding (NSF). Food deprived mice were gently placed in a corner of the OF apparatus for a maximum time of 10 minutes. In the center of OF, one pellet of food was placed in the center of a rectangle. The time the animal needed to reach the rectangle was manually recorded and determined as latency to center. Experimenter was blind to animals genotype. Immediately after the test, mice were placed individually in a standard cage with food for 20min, and the amount of food consumed was monitored.

Forced Swim Test (FST). This test was used to assess learned helplessness parameter of depressive-like behavior. Briefly, mice were individually placed into transparent cylinders filled with water (24°C; depth 30cm; 7min). During the last 5min of the test was manually scored using Kinoscope software (http://sourceforge.net/projects/kinoscope/). Depression-like behavior was evaluated by immobility time as previously described (Lopes et al. 2016).

Tail suspension Test (TST). This test was used to assess learned helplessness parameter of depressive-like behavior. Briefly, mice were individually placed into transparent cylinders filled with water (24°C; depth 30cm; 5min). During the last 5min of the test scoring was automatically performed using EthoVisionXT software. Depression-like behavior was evaluated by immobility time as previously described (Lopes et al. 2016).

Sucrose-Preference Test (SPT) was tested in all (individually-housed for 48 h) animals before the exposure to any behavioral paradigm started. They received two drinking bottles, one containing water, the other 2% sucrose with food ad libitium. Sucrose preference was calculated according to the formula: sucrose preference (%) = [sucrose intake/total intake] x 100.

Sweet-Drive Test (SDT). As previously described (A.Pinheiro et al 2014), this test assesses anhedonic behavior. Using a two chamber black acrylic enclosed arena, food deprived animal is allowed to freely choose between normal food and sweet pellets (Cheerios®, Nestlé). Three trials were conducted (1 trial every 48 h). Preference for sweet pellets was determined as (%) = Consumption of Sweet Pellets (g)/Total Food Consumption (g) × 100.

Novel Place Recognition (NPR). Briefly, animals were habituated to an open arena for three days. Then, each animal was allowed to explore two identical objects for 10 min. One hour later, mice were returned to the arena, with one of the objects being placed to a new position. Discrimination index was calculated based on the following formula [time spent in (novel place/total)-(familiar/total)x100]. Analysis was automatically performed using EthoVisionXT software.

Novel Object Recognition Test (NOR). For Long term version of the test, animals were allowed to explore two identical (familiar) objects for 10 min. After 24 h, mice were returned to the arena, where one of the familiar objects was replaced with a novel one (different shape, color and texture). For short term NOR tetsing, animals were allowed to explore a second set of two familiar objects (completely different set from the first one) for 10min. After 1h, mice were returned to the arena where one of the familiar objects was replaced with a novel one. Discrimination index for both long and short term versions was calculated based on the following formula [time spent in (novel place/total)-(familiar/total)x100]. Analysis was automatically performed using EthoVisionXT software.

Morris Water Maze (MWM) consists of a cylinder (1m diameter) filled with water (24°C) made opaque with a white bio-safe dye. The cylinder contained a slightly submerged transparent escape platform and placed in a room with landmark (reference) objects. Learning trials start by gently placing mice on the water surface close to the cylinder wall. Animals were tested over 4 consecutive days (4 trials/day; 60-s trial period). On the 5th day, animals were subjected to Probe Test. After probe test, animals preformed the reversal learning task where the platform was changed to the opponent quadrant of the swimming pool. Animals performed 4 trials (60s each) swimming to find the new platform. Swim paths during these tests were monitored and recorded by aCCDcamera, using Image J software (http://rsb.info.nih.gov/nih-image/). Data were subsequently analyzed using customized software based on Matlab (version 7.2, Mathworks Co Ltd, CA), with an image analysis tool box (Mathworks). Learning was assessed by the time por distance that the animals need to reach the escaping platform.

Neuronal Structure Analysis

For 3D morphometric analysis, animals were transcardially perfused with 0.9% saline under deep anesthesia. Brains were immersed in a Golgi-Cox solution for 14 days and then transferred to a 30% sucrose solution. Vibratome coronal sections (200 μm thick) were collected in 6% sucrose and dried onto gelatin-coated microscope slides. Sections were then alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed, dehydrated and mounted. Per experimental group, 25-30 neurons were studied and individual neuron measurements from each animal were averaged. For each selected neuron, all branches of the dendritic tree were reconstructed at 600x (oil) magnification using a motorized Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany) and Neurolucida software (Microbrightfield, Williston, VT) and dendritic length was automatically calculated. Dendritic spine density was also accessed. For Sholl analysis (index of dendritic complexity and degree of arborization), the number of dendritic intersections with concentric spheres positioned at radial intervals of 20 μm from the soma was accessed using NeuroExplorer software (Microbrightfield) as previously described (Cerqueira & Mailliet, et al., 2007; Bessa et al., 2009).

Subcellular fractionation and Western blot analysis

An established protocol was used to obtain subcellular fractions (**Supplementary figure 9**). Briefly, macrodissected brain tissue was homogenized (10x homogenization buffer [sucrose 9%; 5mM DTT; 2mM EDTA; 25mM Tris pH7.4; Complete Protease Inhibitor (Roche), and Phosphatase Inhibitor Cocktails II and III (Sigma)]) and centrifuged (1000 g). The post-nuclear supernatant was subsequently centrifuged (12,500 g) to yield crude synaptosomal and synaptosome-depleted fractions. The latter was ultracentrifuged (176, 000 g) to yield a light membrane/Golgi fraction (P3) and a cytoplasmic fraction (S3). The crude synaptosomal fraction was lysed in a hypo-osmotic solution and centrifuged (25,000 g) to obtain the synaptosomal membrane fraction (LP1). (See **Supplementary Fig. 9**) Lysates were electrophoresed and semi-dry transferred onto nitrocellulose membranes (Trans-Blot® TurboTM Blotting System, BioRad); membranes were blocked in 5% nonfat milk in TBS-T buffer and then incubated with the following antibodies: Tau5 (1:2000, Abcam), PHF1 (1:2000, recognizes p396/404-Tau; kind gift from Dr Peter Davies), PSD95 (1:10000, NeuroMab), Homer (1:1000, SantaCruz Biotech.)Pan-Shank (1:1000, NeuroMab), cortactin (1:10000, Abcam), acetylated-cortactin (1:500, Abcam) and actin (1:2000, abcam). After incubation with appropriate secondary antibody, antigens were revealed by ECL (Clarity, BioRad) and signal quantification was

achieved using a ChemiDoc and ImageLab software from Bio-Rad. All values were normalized and expressed as a percentage of control values.

Virus intracranial injections

Viral constructs were obtained from Gene Therapy Center Vector Core, University of NCxxxxxx, USA. Briefly, mice were anesthetized with 75mg/Kg ketamine (Imalgene, Merial) and 1mg/Kg of medetomidine (Dorbene, Cymedica). Adeno-associated virus type 5 (AAV5-)-cytomegalovirus (CMV)-Cre-green fluorescent protein (GFP) (0.5 uL of $4.6x10^{10}$ viral particles/ml) or its control viral vector (AAV5-CMV-GFP, $3.5x10^{10}$ viral particles/ml) was stereotaxically injected bilaterally into the CeA (coordinates from bregma, according to Paxinos and Franklin: -1.0 mm anteroposterior, 2.4 mm mediolateral, -4.6 mm dorsoventral). After injection, mice were removed from the stereotaxic frame, sutured and let to recover while behavioral analysis was performed 4 months after the viral injections.

Statistical Analysis

Data were analyzed using Student;s t-test or one-way ANOVA using GraphPad 6.0 (GraphPad Software Inc., USA). Differences were considered statistically significant when $p < 0.05$.

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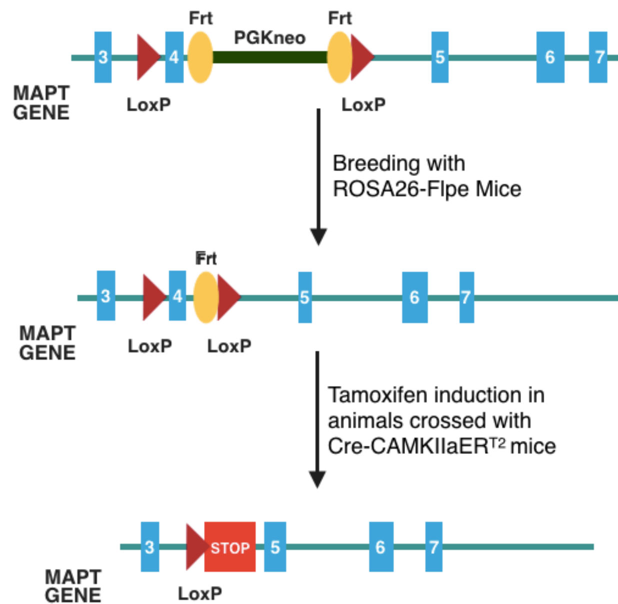
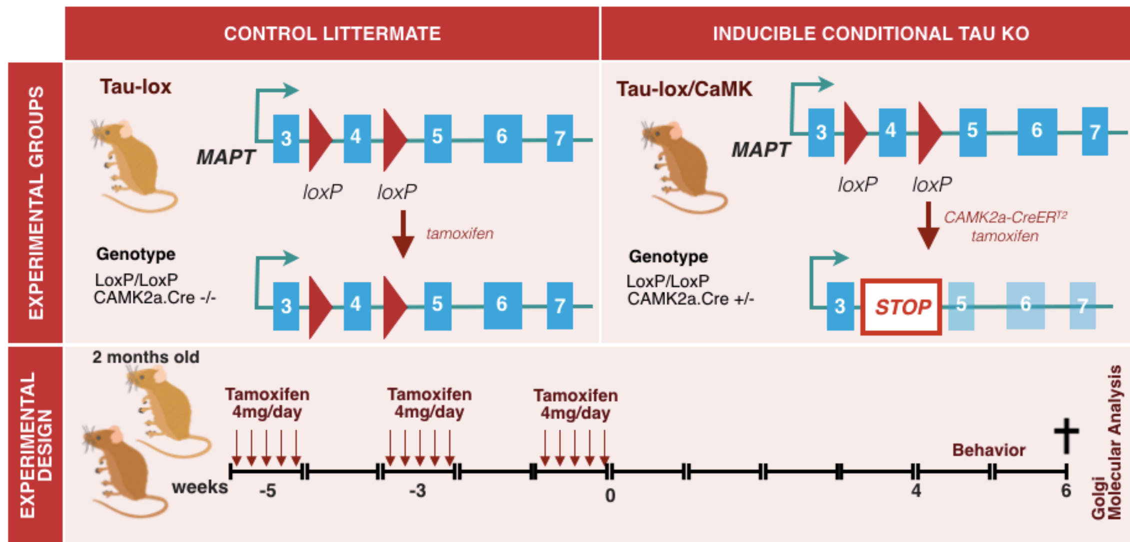
Supplementary Information

Supplementary Figure 1



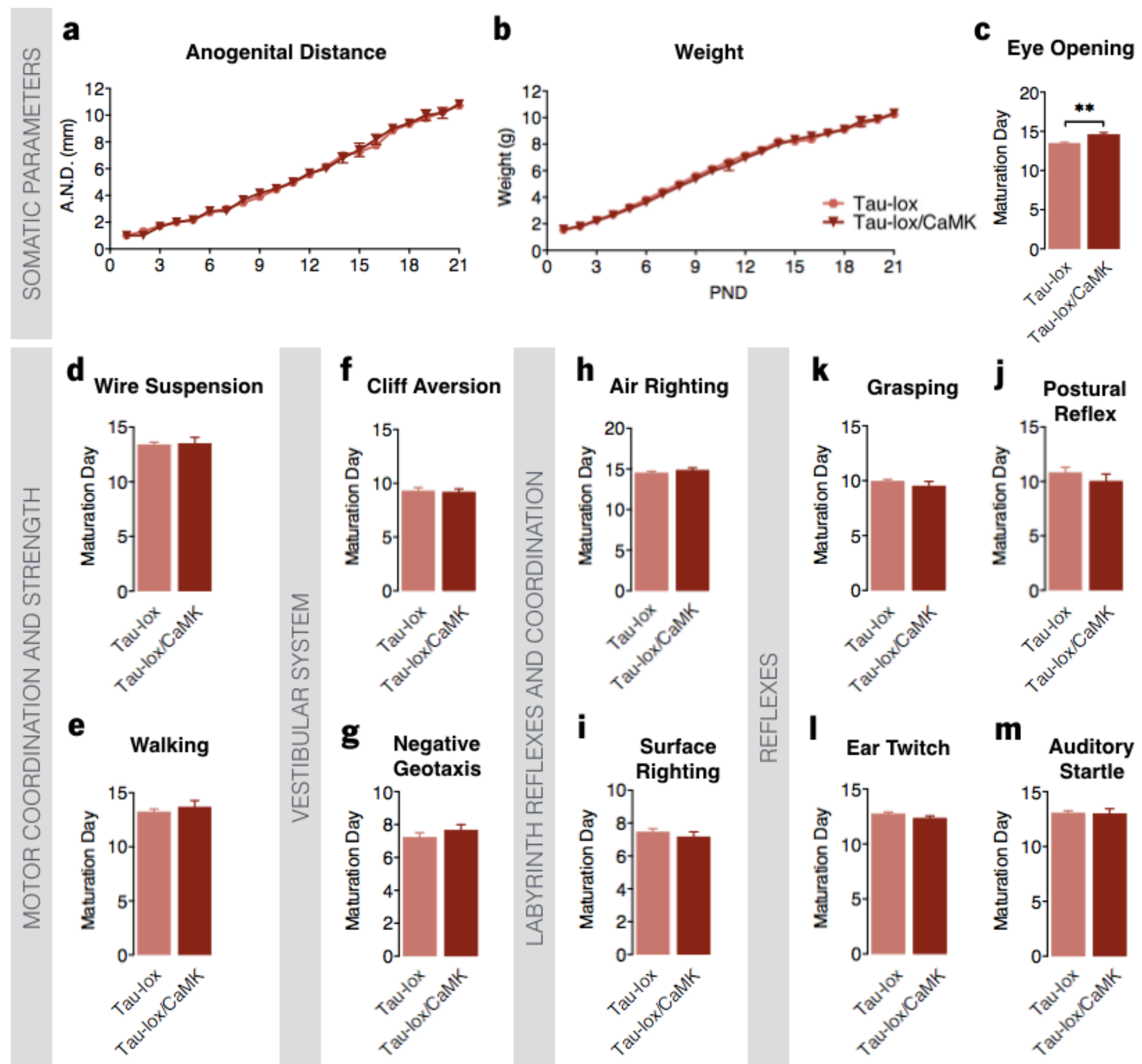
Supplementary Figure 1. Generation of Tau$^{loxP/loxP}$ mice. Chimeric mice with Frt-PGKneo-Frt-LoxP cassette inserted in the intron 4 sequence of *mapt* were crossed with ROSA26-Flpe mice to remove the PGKneo cassette while further backcrossin were made to obtain the Tau$^{loxP/loxP}$ mouse line. After crossing with the Tamoxifen-inducible Cre line B6;129-Tg(Camk2a-cre/ERT2)1Aibs/J, Cre activation leads to the formation of a STOP codon in the *mapt* gene, stopping transcription.

Supplementary Figure 2



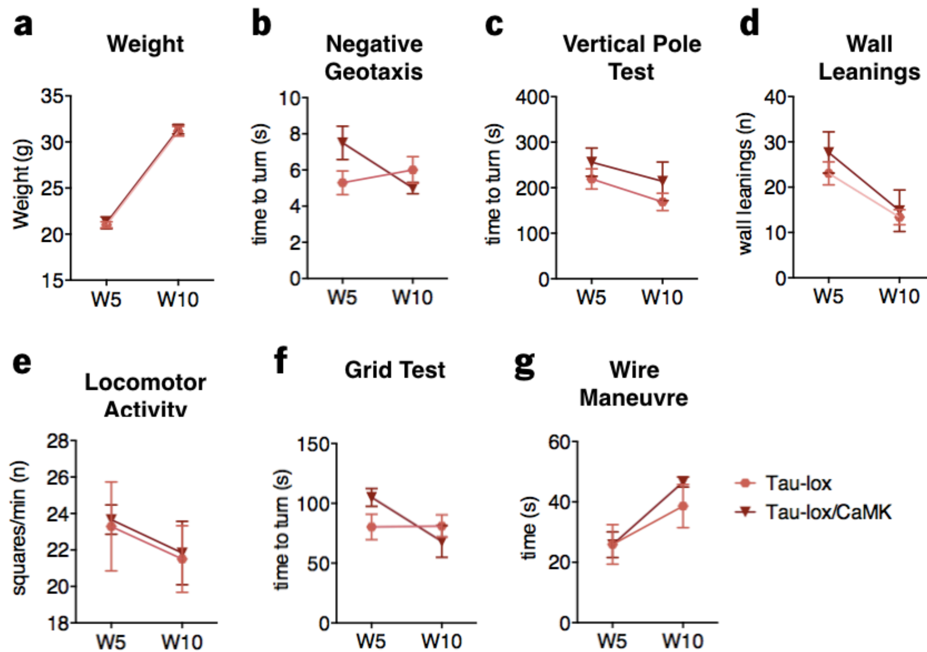
Supplementary Figure 2. Experimental groups and tamoxifen treatment. In contrast to Tau-lox mice (left), the presence of one copy of Camk2a-cre gene in Tau-lox/CaMK (right) mice result in *mapt* deletion after tamoxifen treatment to both groups. 2 months old animals were subjected to a 5 week long tamoxifen injection scheme followed by a 6 weeks post-injection period until further (molecular and Golgi) analysis. The injection scheme includes 3 sets of 5 consecutive i.p. injections of 4mg Tamoxifen (one per day) with 1 week interval between sets.

Supplementary Figure 3



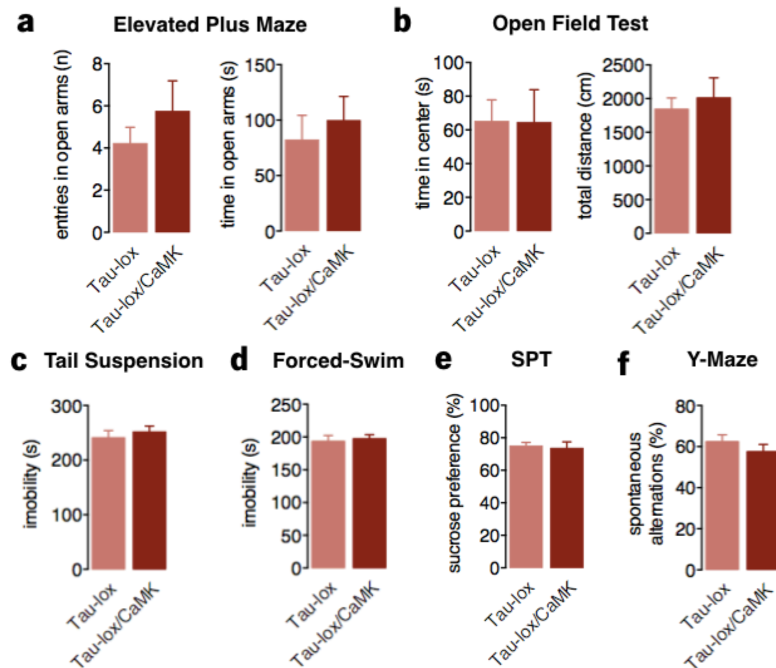
Supplementary Figure 3. Developmental characterization of Tau-lox and Tau-lox/CaMK mice. Milestones protocol was used to access developmental characteristics of both Tau-lox/CaMK and Tau-lox (control) littermates during PND1-21 period (no tamoxifen treatment). With a exception of eye opening parameter ($t_{(21)}$=3.373, p=0.002) (**c**) where Tau-lox/CaMK exhibit a slight delay in their maturation (still in normal range), no differences are observed in other somatic parameters tested such as anogenital distance (**a**) and weight gain (**b**). In addition, no difference between Tau-lox and Tau-lox/CaMK animals were observed in different parameters/tests monitoring motor coordination and strength (**d-e**), vestibular system (f-g), labyrinthic reflexes and coordination (h-i) and reflexes (k-m). All numeric data represent mean±SEM; **p<0.01.

Supplementary Figure 4



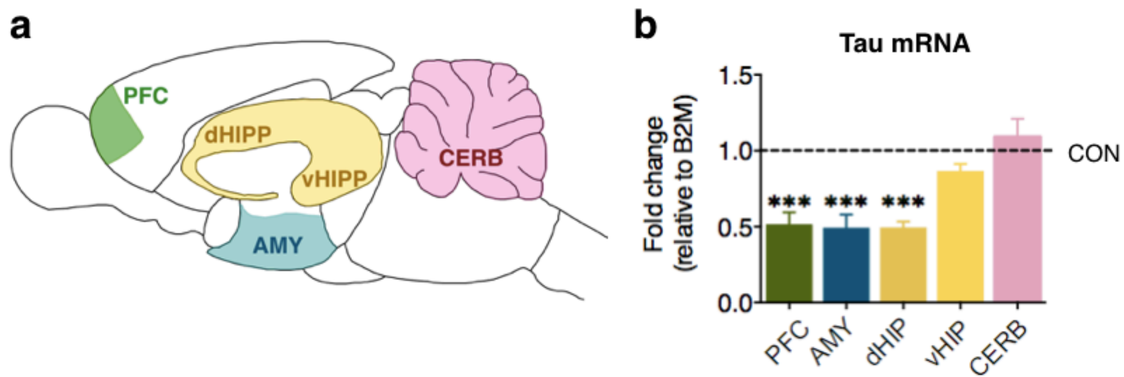
Supplementary Figure 4. SHIRPA characterization of Tau-lox and Tau-lox/CaMK mice at 5 and 10 weeks of age. SHIRPA protocol was used to monitor muscle, cerebellar, sensory and neuropsychiatric function. Tau-lox/CaMK and Tau-lox mice present no significant differences in different parameters tested such as body weight (**a**), negative geotaxis (**b**), vertical pole (**c**), wall leanings in jar test (**d**), locomotor activity (**e**), as well as grid (**f**) and wire maneuver (**g**) tests. All data represented as mean±SEM.

Supplementary Figure 5



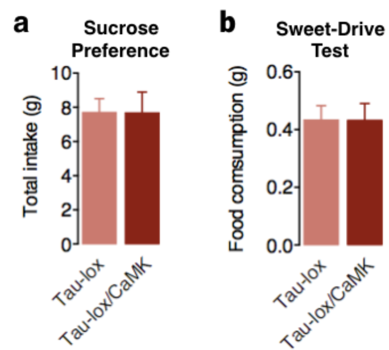
Supplementary Figure 5. Behavior characterization of Tau-lox and Tau-lox/CaMK mice before tamoxifen administration. Adult behavior of Tau-lox and Tau-lox/CaMK mice were monitored using a battery of tests. No difference in anxiety levels were found between two groups as assessed by similar entries and time that animals spend in open arm of Elevated Plus Maze (EPM) apparatus (**a**) and time in center of Open field (OF) apparatus (**b**); note no differences in total distance in OF indicating an absence of locomotor change. (**c-d**) Tau-lox/CaMK mice present no depressive-like behavior when compared to their Tau-lox (control) littermates. Specifically, animals of two genotypes were not different in immobility and climbing time time in forced-swim and tail suspension test, respectively; both tests monitor learned helplessness. (**e**) Anhedonic behavior was accessed by sucrose preference test (SPT) where no differences in sucrose preference was observed between groups. **(l)** Y-maze test was used to access cognitive performance where Tau-lox/CaMK and Tau-lox mice present no differences. All numeric data represent mean±SEM.

Supplementary Figure 6



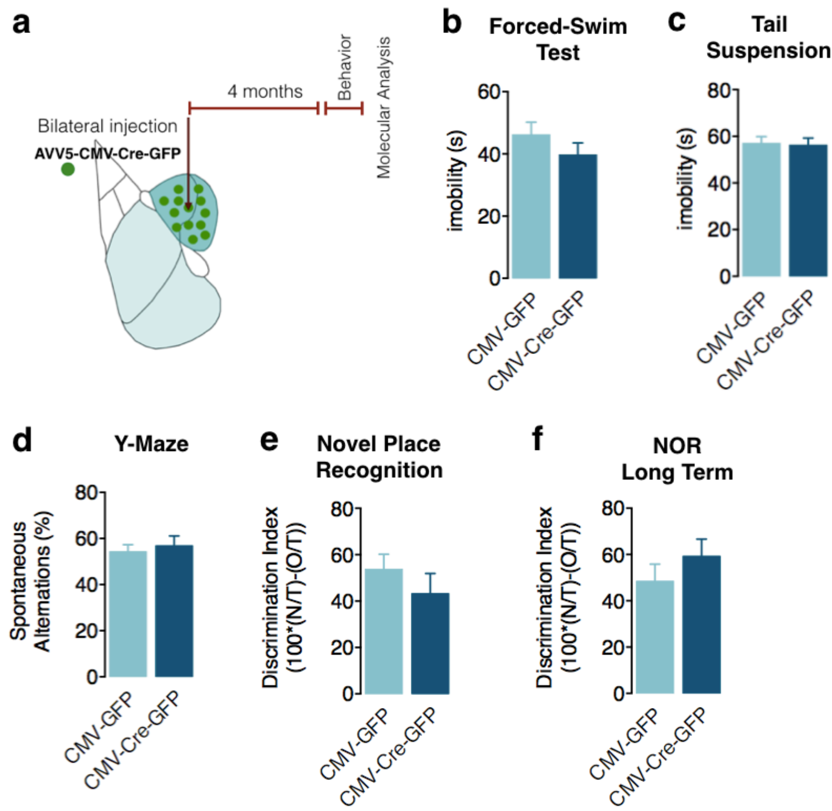
Supplementary Figure 6. Tau mRNA levels were decreased in different brain areas of Tau-lox/CaMK mice after tamoxifen administration (**a**) Tau mRNA levels are decrease in the prefrontal cortex (PFC) ($t_{(20)}$=5.261, p<0.0001), Amygdala (AMY) ($t_{(16)}$=4.908, p=0.0002) and dorsal hippocampus (dHIP) ($t_{(19)}$=7.954, p<0.0001) of Tau-lox/CaMK mice in comparison to Tau-lox; no difference of Tau mRNA levels were found in ventral hippocampus (vHIP) and cerebellum (CERB) 6 weeks after the last tamoxifen injection; mRNA levels of Tau are normalized to B2M levels and represented as % to control (100). All numeric data represent mean±SEM;; ***p<0.001.

Supplementary Figure 7



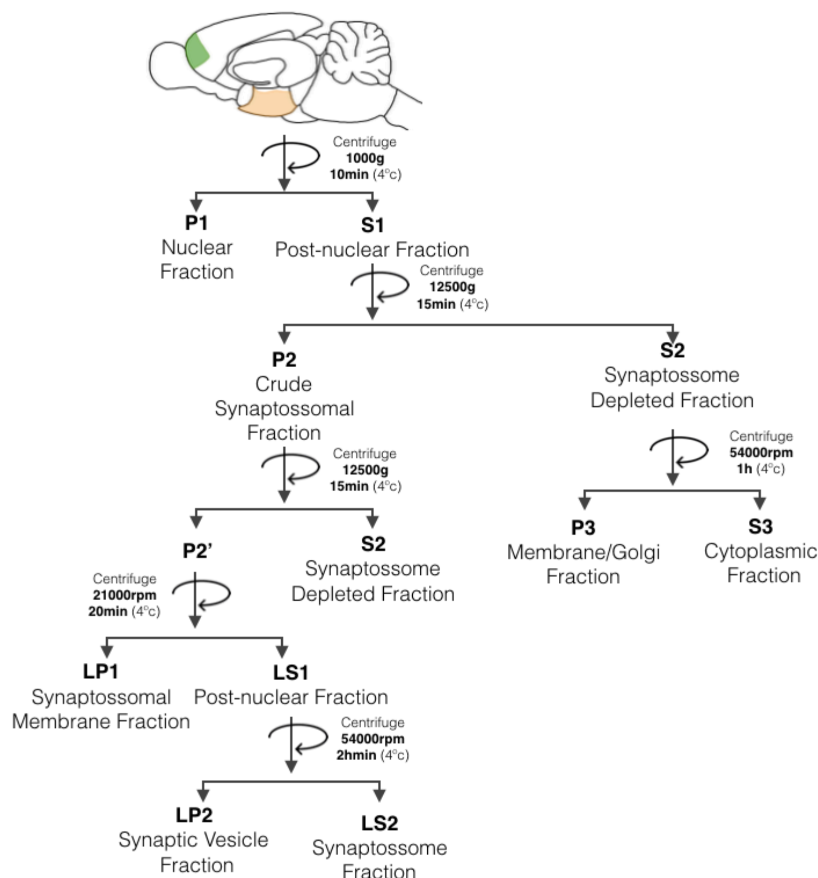
Supplementary Figure 7. No differences in food or liquid consumption in SPT or SDT tests. No differences are found between Tau-lox/CaMK and their Tau-lox (control) littermates in total liquid consumption in sucrose preference test (SPT) (**a**) as well as in food consumption in sweet-drive test (SDT) (**b**). All numeric data represent mean±SEM.

Supplementary Figure 8



Supplementary Figure 8. Absence of depressive-like behavior or cognitive impairments in animals with specific Tau ablation in amygdala. (**a**) Schematic representation of the experimental design used for restricted Tau loss in the central amygdala (CeA). AAV5-CMV-Cre-GFP or AAV5-CMV-GFP (control) virus was injected in CeA followed by behavioral analysis 4 months later. (**b-c**) AAV5-CMV-Cre-GFP injected mice present no depressive-like behavior as assessedd by absence of differences in immobility in FST and TST, respectively, between two groups. (**d-f**) No changes of cognitive performance between two groups as monitored by alterations in Y-maze as well as discrimination index in both Novel place and object recognition (NPL and NOR, respectively). All numeric data represent mean±SEM.

Supplementary Figure 9



Supplementary Figure 9. Fractionation procedure of neurosynaptossomes. As previously described (Lopes et al., PNAS 2016), brain tissue of Tau-lox and Tau-lox/CaMK mice were homogenized and sequentially centrifuged to obtain post-nuclear fraction (S1) which was subsequently centrifuged to yield crude synaptosomal (P2) and synaptosome-depleted fractions (S2). The later was ultracentrifuged to yield a light membrane/Golgi fraction (P3) and a cytoplasmic fraction (S3) while the crude synaptosomal fraction (P2) was lysed in a hypo-osmotic solution and centrifuged to obtain the neurosynaptosome fraction (LP1)- for details, please see materilas and methods.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE PRESPECTIVES.

6. GENERAL DISCUSSION AND MAIN CONCLUSION

6.1 GENERAL DISCUSSION

Although Tau protein was discovered more than 40 years ago, its precise functions in different neuronal compartments under healthy and pathological conditions remain poorly understood or unclear. While some of Tau functions are known in great molecular detail, they are established in rather reductionist paradigms as its *in vivo* significance is still uncertain. For example, despite that many *in vitro* studies support the essential role of Tau in cytoskeletal stability, neuronal structure and intracellular trafficking, the *in vivo* evidence of Tau role in adult brain doesn't confirm them, as the currently available constitutive Tau-knockout (Tau-KO) mouse lines fail to exhibit any behavioral, structural and functional brain anomalies in young/adult animals. The above controversy highlights a significance gap of knowledge about the real role of Tau in neuronal and brain function.

In addition, as Tau was initially identified as a microtubule-associated protein, the predominant view of its function focused on its microtubule (MT)-binding capacity for many years (Drechsel et al. 1992; Brandt and Lee 1993) while novel evidence supports a novel role for Tau in the nucleus being involved in the DNA protection (Sultan et al. 2011; Violet et al. 2014), as well as in synaptic structure, function and plasticity; e.g. log term depression (LTD) (Kimura et al. 2014; Frandemiche et al. 2014). Indeed, Tau is now known to be present at synapses where it acts as a scaffold protein, altering signaling pathways e.g. Tau tether Fyn to PSD-95/NMDA receptor signaling complexes (Ittner et al. 2010; Mondragon-Rodriguez et al. 2012). Moreover, the Tau-Fyn-NMDA receptor cascade is suggested to be involved in early stages of synaptic malfunction in Alzheirmer's Disease (AD) brain (Ittner et al. 2010; Hoover et al. 2010; Ittner et al. 2014) suggesting a novel pathway of Tau malfunction related with AD brain pathology. In addition, recent evidence supports the involvement of Tau and its malfunction in brain pathology beyond AD; for examples, in neuropathologies with different etiology e.g. epilepsy (DeVos et al. 2013), glutamate-driven excitotoxicity (Roberson et al. 2007; Zempel et al. 2010) as well as chronic stress (Sotiropoulos et al. 2011); note that both clinical and animal studies suggest the later as risk factor for AD. However, the precise mechanisms by which Tau contributes to stress-driven brain malfunction and AD pathology are poorly clarified.

Against the above background, this PhD thesis provide novel *in vivo* evidence about the (normal) Tau function in adult brain, the implication of Tau malfunction in stress/glucocorticoid(GC)-driven brain plasticity as well as the molecular mechanisms through which chronic stress precipitates

Tau aggregation and neurotoxicity in AD brain. Further investigation on the Tau biology and pathology will allow us to better understand the molecular events and cascades that precipitate brain pathology providing potential novel targets of delay or cure.

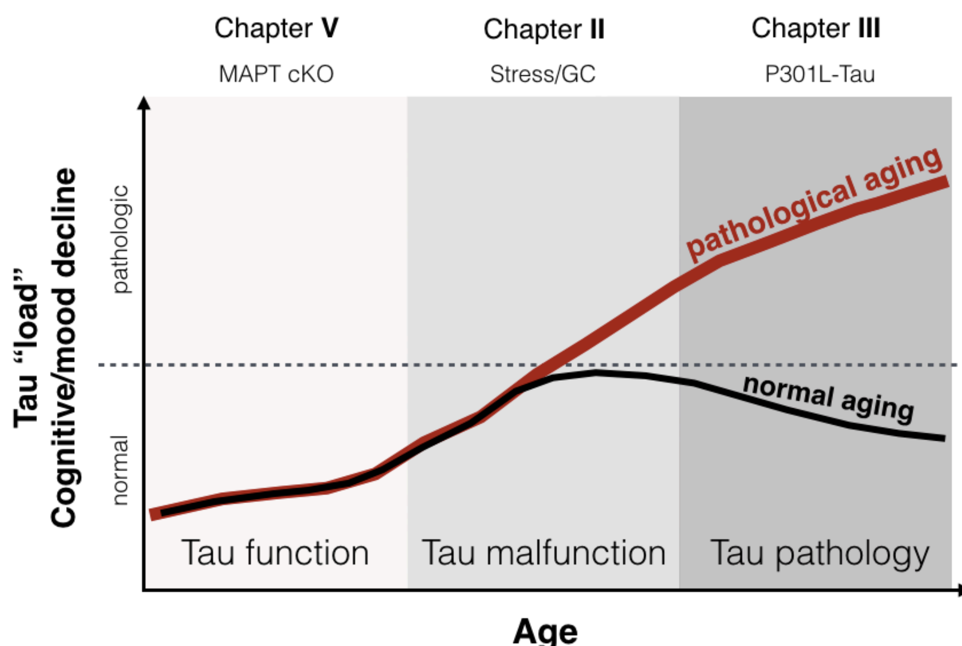


Figure 1. Tau protein in different moments of brain homeostasis and pathology. Schematic representation of Tau implication in different stages of brain function and its decline during aging towards the establishment of (brain) pathology as it is reflected in different result chapters of this PhD thesis. Tau and its dynamic alterations in different intraneuronal compartments are essentially involved in (normal) neuronal function and plasticity (Chapter 5; cTau-KO). Imbalance of Tau dynamics, (hyperphosphorylation and missorting) is involved in neuronal malfunction and behavior deficits in non- or pre-AD conditions e.g. Tau malfunction related to (reversible) stress-related neuronal atrophy and cognitive/mood deficits under prolong exposure to stress/GC (Chapter 2). During aging, the Tau malfunction could be increased and cumulatively lead to non-reversible damage of neurons (neurodegeneration and cell death) precipitating to AD neuropathology (Tau pathology; Chapter 3) (drawn by Silva JM and Sotiropoulos I).

6.1.1 Altered intracellular trafficking and missorting of hyperphosphorylated Tau as part of the stress/GC-driven neuroplastic changes.

Besides its predominant localization in axons, Tau protein is also found in dendrites and spines (Morris et al. 2011). Several observations suggest that the (normal) presence and (pathological) accumulation of Tau at somatodendritic compartment can essentially modulate synaptic function under both normal and pathological conditions (Frandemiche et al. 2014; L. M. Ittner et al. 2010; Hoover et al. 2010). Indeed, most of our knowledge about synaptic role of Tau is

based on pathological conditions (e.g AD brain) where Tau is aberrantly hyperphosphorylated and missorted in dendrites and dendritic spines (Ittner et al. 2010; Hoover et al. 2010).

Exposure to chronic stress and glucocorticoids (GC) is often escorted by cognitive and affective deficits in association with adaptation of neuronal structure. Previous work from our group and others have shown that chronic stress and stress hormones (GC), trigger Tau hyperphosphorylation followed by memory impairments (Sotiropoulos et al. 2008; Sotiropoulos et al. 2011); but the exact mechanisms that relate Tau hyperphosphorylation to stress/GC-driven neuronal and synaptic atrophy and malfunction that underlie cognitive deficits remains unknown. Thus, this thesis findings show for the first time that prolong exposure to high GC levels trigger the accumulation of Tau in both dendritic and synaptic compartments (**Chapter 2**), which is an indicator of neuronal malfunction and pathology.

Moreover, we found no GC-evoked changes in mRNA levels of Tau, which is in line with previous *in vitro* studies showing that Tau accumulation by GC is related with a reduction of Tau turnover (Sotiropoulos et al. 2008). As cellular proteostasis depends on both proteasome and lysosome degradation mechanisms, the above GC-driven Tau accumulation could be attributed to alterations in mechanisms of Tau proteasomal degradation, which goes in line with previous work from the PhD candidate showing that stress leads to alteration in molecular chaperone network, involved in Tau protein ubiquitylation and degradation by the proteasome (Sotiropoulos et al. 2014). In addition, autophagy could be also affected under stress or GC conditions in wildtype animals. Indeed, other studies of this PhD thesis show that chronic stress and GC induced autophagic inhibition in P301L-tau Tg mice exhibiting pathological, insoluble tau aggregates (**Chapter 3**). However, as autophagy is involved in degradation of aggregated (insoluble) Tau rather that soluble Tau, someone would expect that the autophagy implication in wild-type animals under control or stress conditions should be limited as both control or stressed wildtype animals exhibit no/or very little Tau aggregates (Rissman et al. 2012; Sotiropoulos et al. 2014). Future studies should carefully monitor the autophagic role in stress-driven neuronal malfunction in wild-type animals as autophagy was recently shown to be involved in spine loss and dendritic atrophy beyond neurodegenerative disorders (Yang et al. 2013).

Except for a few studies (Cereseto et al. 2006), the effects of prolong stress and GC on cytoskeleton proteins have received little attention, even though the cytoskeleton is important for maintaining neuronal architecture and function (Morris et al. 2011). In line with previous work from our team (I Sotiropoulos et al. 2008; I Sotiropoulos et al. 2011), in this PhD thesis we show that GC

lead to the abnormal hyperphosphorylation of Tau through the induction of two well-known Tau kinases (GSK3β, cdk5). In addition, the current studies demonstrate that chronic stress leads to an accumulation of Tau and different hyperphosphorylated Tau epitopes in the cytosolic and synaptic compartments of hippocampal neurons (**Chapter 2**). Hyperphosphorylation of Tau has been shown to result in destabilization of dendritic cytoskeleton (Zempel et al. 2010) due to the disruption of the microtubule-Tau interactions, and the release of Tau to the dendritic shafts from where it may diffuse to spines and synaptic compartment (**Figure 2c**). Notably, two of the Tau phosphorylated epitopes affected by GC, pThr231 and pSer262, reduce the microtubule-binding capacity of Tau which subsequently results in destabilization of the neuronal cytoskeleton (Lauckner, Frey, and Geula 2003; Luna-Muñoz et al. 2007). Consistently, we observed that GC-evoked Tau hyperphosphorylation occurs in tandem with decreased levels of the stable detyrosinated microtubules, suggestive of reduced microtubule stability (Sengupta et al. 1998; Cho and Johnson 2004), which may be causally related to the dendritic remodeling that occurs after GC and stress (Yoshiyama et al. 2003; Sousa and Almeida 2012). Interestingly, other Tau phosphorylated epitopes, e.g. pSer199/202 and 212/214, which mainly present in later stages of AD pathology (Luna-Muñoz et al. 2007), are not affected by GC reinforcing the idea that our GC-driven findings may be relevant to the earliest stages of cytoskeletal disturbances where Tau is believed to contribute to synaptic dysfunction and atrophy (**Figure 2c**). This notion is further supported by the fact that GC-triggered dendritic remodeling was limited to reduced dendritic branching and spine loss.

Interestingly, GC impact on Tau phosphorylation state exhibited a subcellular and distinct pattern. Normalized pThr231-Tau levels were increased in cytosol, but decreased in synaptosomal fraction, while p262-Tau levels were increased only in cytosol (not in synaptic fraction), suggesting a preferential subcellular localization and/or accumulation of different phosphorylated Tau epitopes in both intracellular compartments after GC treatment. It is suggested that different pools of Tau with different phosphorylation state exist within the neuron exhibiting potentially distinct roles (Billingsley and Kincaid 1997; Lim and Lu 2005). For example, blockage of Tau phosphorylation at Ser396/404 is previously shown to be necessary for Aβ-driven mislocation of Tau at synapses (Frandemiche et al. 2014), while Tau phosphorylation at pThr231, but not at other epitopes, regulates its synaptic binding to PSD-95 (Mondragón-Rodríguez et al. 2012). Interestingly, we found that the levels of PSD95, a crucial protein for synaptic structure and function, were reduced by the exposure to GCs, consistent with our finding of synaptic loss using Golgi-based neuronal structural analysis. Moreover, the reduction of PSD95 levels was accompanied by GC-driven decrease in levels of pThr231-Tau

within synaptosomal fraction, further supporting the previously suggested interrelationship between this phospho-Tau epitope and PSD95 (Frandemiche et al. 2014).

Recent studies in AD models suggest that abnormal Tau hyperphosphorylation and synaptic missorting represent an early event in AD, preceding the manifestation of detectable neurodegenerative processes (Hoover et al. 2010; Ittner et al. 2010; McKinney 2010). Based on this model of AD synaptic toxicity, missorting of Tau depends on its hyperphosphorylation (Hoover, et al. 2010; Mondragon-Rodriguez et al. 2012; Miller et al. 2014) and leads to increased postsynaptic targeting of Fyn (Ittner et al. 2010). Fyn is a Src family kinase which selectively modulates the function of GluN2B-containing NMDARs, by phosphorylation of the GluN2B at the Y1472 epitope (Nong et al. 2003; Ittner et al. 2010). This phosphorylation is known to stabilize GluN2B at postsynaptic density linking NMDARs to downstream excitotoxic signalling due their overexcitation (Nong et al. 2003; Ittner et al. 2010). Accordingly, the current PhD thesis demonstrates that GC triggers synaptic missorting of Tau and thus, leads to elevated forms of active (phosphoTyr1472) GluN2B receptor units. These findings are in line with recent work from our group showing that chronic stress triggers Tau missorting at synapses resulting to increase postsynaptic targeting of Fyn and consequently to increase GluN2B phosphorylation and excitotoxic signaling (Ittner and Gotz 2011; Lopes et al. 2016). Indeed, previous studies have shown that reduction of Tau or Fyn protein as well as disruption of the Fyn/GluN2B interactions prevents excitotoxic damage in AD Transgenic animals as well as in stroke models, suggesting the involvement of the above mechanism in different pathologies (Ittner et al. 2010). Beyond AD, NMDARs are also shown to be involved in stress- and GC-driven neurotoxicity (Yang et al. 2005) as blockage of NMDARs, but not AMPARs, attenuates neuroremodeling actions of stress and/or GC (Magariños et al. 1996). Altogether, these PhD findings provide new insights into the cellular cascades triggered by GC and highlight the important role of Tau hyperphosphorylation in neuronal and synaptic malfunction and atrophy underlying prolong exposure to high GC levels and related hippocampal pathology (**Figure 2**).

The impact of loss of Tau and its function in adult brain: what do we miss?

Accumulating evidence demonstrate that Tau protein exist in different intracellular parts besides of the axon. Based on *in vitro* and *in vivo* models that overexpress Tau, different studies have shown the presence and accumulation of Tau at somatodendritic compartment (e.g. dendritic spines) (Ittner et al. 2010; Hoover et al. 2010 ; Frandemiche et al. 2014). This PhD work confirms the

presence of endogenous Tau in dendrites and synapses of the hippocampus of wild-type, non-transgenic, animals providing both biochemical (WB) and ultrastructural (TEM) proof. These results are in agreement with previous studies (Mondragón-Rodríguez et al. 2012; Kimura et al. 2014). Note that these PhD studies support the use of these complementary approaches (subcellular fractionation-based biochemical analysis and TEM detection) for future detailed "mapping" of the presence of Tau in different intracellular compartments. But what is the function of dendritic Tau? Is Tau part of the structural and functional plasticity in neurons of the adult brain?

Microtubule-associated protein Tau has been described to have various and important function from neuronal homeostasis related to both cell structure as well as function. The creation of different transgenic animals overexpressing various forms of Tau or fragments of it, have been of clear importance to improve our limited understanding about the involvement of Tau protein in brain pathology, in AD or other neurodegenerative disorders called Tauopathies (Wang and Mandelkow 2015). However, the Tau knock-out (KO) mouse lines available don't present major alterations in behavior, neuronal structure or survival (Morris et al. 2011; Ke et al. 2012) - with the exception of very few studies reporting some minor alterations in mechanisms of synaptic plasticity (LTP or LTD) (Kimura et al. 2014; Ahmed et al. 2014). More interestingly is that three out of four available constitutive Tau-KO models don´t exhibit MT alterations (for review see (Morris et al. 2011)), and neither axonal transport abnormalities, highlighting a significance gap of knowledge about the real role of Tau in neuronal function. This paradoxical *in vivo* phenotype of Tau-KO mice is suggested to be partly attributed to compensatory mechanisms during animal (brain) development, mainly associated with other microtubule-associated proteins (MAPs). This notion is supported by studies showing that MAP1a protein expression is increased in the first months of life of Tau-KO animals (Harada et al. 1994; Tucker, Meyer, and Barde 2001; Dawson et al. 2001), and simultaneous KO of *mapt* and *map1b* display severe phenotype and lethality (Takei et al. 2000). Based on this background, these PhD studies designed and presented a conditional Tau-KO mouse line avoiding the developmental compensatory mechanisms suggested to occur in previously generated Tau-KO mouse lines, using the Cre/LoxP system, a site-specific recombinase system widely used to carry out conditional gene deletion in mice.

In contrary to previous constitutive Tau-KO models (Dawson et al. 2001; Rapoport et al. 2002), this PhD thesis shows for the first time, that conditional loss of Tau in the adult brain leads to behavior deficits impacting on neuronal structure of pyramidal neurons of the adult brain. More specifically, conditional deletion of Tau in the adult brain lead to anxious and depressive-like behavior

but no memory deficits, surprisingly, as someone would expect that loss of (normal, wildtype) Tau and its function will impact on memory, as Tau hyperphosphorylation and malfunction in AD conditions impair primarily memory and cognitive performance. A possible explanation could be provided based on the underlying brain areas and circuitries that are affected by Tau loss in these animals. For example, brain areas essentially involved in anxiety and depressive-like pathology, such as PFC cortex and amygdala, exhibit clear dendritic atrophy/simplification in these animals. On the contrary, Tau levels and neuronal (dendritic) structure in hippocampus, a mainly memory-regulating brain area, are not/or very little affected in these animals. In support to this notion, specific deletion of Tau in amygdala of these animals resulted in anxiety, but not depression, supporting a clear involvement of amygdala neuronal malfunction in establishment of anxious pathology. We are currently performing electrophysiological measurements in order to understand the synaptic strength, plasticity and overall communication among the aforementioned brain areas in the adult brain of animals with conditional deletion of Tau. An important question to be also answered in future studies focused on the reason of the reduced efficacy of tau deletion in hippocampus that, even though is part of forebrain, the CaMK-driven Tau deletion was not efficient.

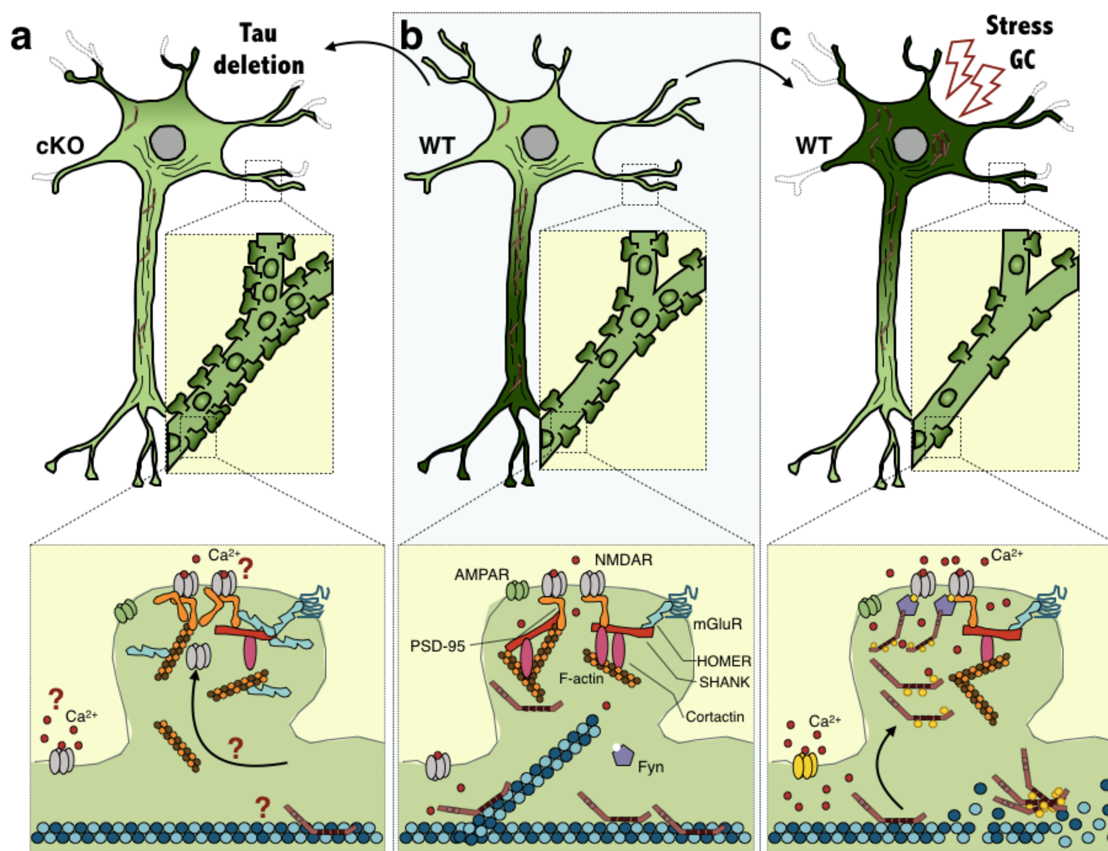


Figure 2. Hypothetical model summarizing the implication of Tau protein in neuronal function and stress-driven malfunction. In healthy neurons (**b**), Tau protein has specific intracellular distribution

as it is mainly found at the axon while it is also present (in small amounts) at dendrites it is suggested to participate in synaptic structure and signaling/palsticity through its interaction with F-actin and Fyn. Under prolong stress or glucocorticoid exposure (**c**), Tau becomes hyperphosphorylated, detaches from microtubules (MT) and it is accumulated into the somatodendritic compartment leading to microtubule destabilization and neurostructural simplification/atrophy. In addition, Tau is mislocalizes (missorted) to the dendritic spines and synapses targeting Fyn to the postsynaptic density (PSD) that in its turn phosphorylated and stabilize NMDA receptors coupling them to downstream excitotoxic signaling. On the other hand, conditional loss of Tau in adult brain (**a**) results in dendritic simplification and atrophy in parallel to reduced PSD levels of scaffold proteins, Shanks and Homer indicating potential synaptic malfunction (drawn by Silva JM).

Interestingly, Tau deletion (even not complete, at least at the time point of 6 weeks after the last tamoxifen injection) trigger clear alterations in neuronal morphology in different brain areas such as prefrontal cortex (both PrL and IL subareas), central amygdala and dorsal hippocampus. These findings provide the first *in vivo* support of the essential cytoskeletal role of Tau related to maintenance of microtubule structure and stability. Ongoing analysis of different cytoskeletal (e.g. microtubule) and other MAPs will provide further support to this notion. However, tamoxifen-treated Tau-lox/CaMK animals exhibit a clear increase in synapses in their dendrites, most probably in a potential cellular effort to compensate the dendritic damage. These findings suggest that, in contrast to dendrites, the dendritic spine structure and overall synaptogenesis capacity of neurons in adult brain does not dependent of Tau protein, in line with previous elegant work from Professor Brandt's lab showing that damage of synaptic loss by Aβ is Tau-independent (Tackenberg and Brandt 2009). Moreover, in agreement with increased spine density, overall protein levels of PSD-95 and HOMER scaffold proteins were increased in neurosynaptosomes of consitional Tau-KO animals. While detailed molecular analysis of different synaptic receptor, their signaling and trafficking is ongoing, it is worth to note that these findings may point to an abnormal regulation and stabilization of both NMDA (Li et al. 2003; Elias et al. 2008; Won et al. 2016; Taft and Turrigiano 2014) and AMPA receptors (Chen et al. 2000; Constals et al. 2015) in the synaptic cleft and alterations in synaptic transmission. Previous studies show that increased PSD-95 levels are reported to selectively enhance AMPAR-mediated synaptic transmission and blocks LTP (Ehrlich and Malinow 2004; Makino and Malinow 2009; Huganir and Nicoll 2013) and affect NMDAR internalization or stability in response to glutamate or glycine (Li et al. 2003; Nong et al. 2003; Lalo et al. 2016). Moreover, HOMER increase can also point to receptor trafficking alterations and consequently disturbed mGluR-related synaptic plasticity (Roche et al. 1999; Tao-Cheng et al. 2014; Ribeiro et al. 2017) (**Figure 2a**).

In contrast to elevated levels of PSD-95 and Homer, tamoxifen-injected Tau-lox/CaMK animals exhibit a reduction in SHANK and cortactin proteins levels, proteins known to be involved in

the formation and maintenance of synapses as well as their excitatory and inhibitory imbalance (Toro et al. 2010). These proteins assemble into large molecular platforms at the PSD, interconnecting proteins from the PSD membrane with actin cytoskeleton (Tomasetti, and De Bartolomeis 2013; Grabrucker et al. 2011; Sala et al. 2015). Previous studies have shown that Shank-KO mouse models present anxious behavior, repetitive grooming and social behavior deficits (Hung et al. 2008; Schmeisser et al. 2012; Yang et al. 2012), while both animals and human studies show that AD neurodegeneration and synaptic malfunction is related to decrease SHANK levels (Roselli et al. 2009; Pham et al. 2010). The reduction that we show (**Figure 2a**) can be indicators that the spines, which are increased, are deficient in some structural proteins of the PSD, pointing again for a possible dysfunction of the synapse. Further studies need to be done to understand the functional impact of these alterations in neuronal excitability and brain circuits, with electrophysiological studies. The on-going and future studies should focus on dissecting the alterations in spine scaffold structure and synaptic signaling induced by conditional deletion of Tau in adult brain.

Several groups have described various approaches of controlling the spatial and/or temporal expression of the Cre recombinase. The one used in the current work is a fusion gene created between Cre and a mutant form of the ligand-binding domain of the estrogen receptor (ER_{TM}). This mutation of ER prevents binding to its natural ligand, estradiol, at normal physiological concentrations, but renders the ER_{TM} domain responsive to tamoxifen (Danielian et al. 1993; Fawell et al. 1990; Littlewood et al. 1995), allowing for temporal control. A current limitation of these PhD studies is the use of male, but not female, mice as female animals were not used in the Tamoxifen experiments in order to avoid potential side effects due to female hormones and female sensitivity to estrogen and tamoxifen. Nevertheless, the use of Cre-Lox system in our animal model allows for deletion of *mapt* gene in region-, cell- and time-specific manner. Despite to the disadvantage related to the fact that the excision reaction is effectively irreversible, this novel Tau-lox mouse line will provide a novel powerful experimental model tool to study the real function of Tau in different brain areas circuits, and specific cell types, allowing also studies in diferent time-windows of animal lifespan much as middle-aged and aged brain.

This opportunity is particularly relevant as Tau-related pathology and neurodegeneration in AD brain is suggested to be attributed to both hyperphosphorylation-evoked loss of (normal) Tau function and aggregation-dependent gain of toxic function for Tau. Still, the impact of loss of normal Tau function in adult or aged brain is not studied properly, probably due to lack on experimental models. Thus, this model offers the possibility to clarify the contribution of loss of normal Tau function

in the (wild-type) aged brain, partly resembling the conditions of sporadic AD where the disease starts in absence of any gene mutation. Based on the current PhD findings, we could speculate that loss of normal tau function in AD brain could contribute to the dendritic simplification and atrophy whereas synaptic loss could be attributed to synaptic missorting of Tau. Future studies should also monitor the deletion of *mapt* in old animals as well as monitor brain and neuronal function for later time-periods after the last tamoxifen injection in order to see the temporal profile of Tau loss in the adult, fully matured brain.

6.1.2 Unraveling the molecular mechanisms of stress-driven tau aggregation and ad neurodegeneration

Prolong stressful life experiences and excessive glucocorticoid (GC) exposure are suggested to increase susceptibility to brain pathology with increasing attention to its implication in AD, as clinical studies report high cortisol levels in AD patients (Hatzinger et al. 1995; Rasmuson et al. 2001), negatively associated with memory scores in AD patients (Csernansky et al. 2006). Moreover, chronic stress has been also suggested to lower the age of onset of the familial form of AD (Simard, Hudon, and van Reekum 2009), further highlighting the potential implication of chronic stress and GC in the pathogenesis and/or progression of AD (Yang et al. 2014). Our and others work supports the neurodegenerative potential of chronic stress, showing that stress and GC trigger several parameters of Tau pathology such as aberrant hyperphosphorylation and somatodendritic accumulation (Green et al. 2006; Sotiropoulos et al. 2011), while findings of this PhD thesis suggest synaptic missorting of Tau as a potential stress/GC-driven mechanism of synaptic malfunction (**Chapter 2**) (Lopes et al. 2016; Pinheiro, Silva et al. 2015). While work during the Master studies of this PhD candidate showed that chronic stress induces truncation and misfolding of Tau leading to the formation of neurotoxic Tau aggregates (Sotiropoulos et al. 2014), little is known about the cellular mechanisms through which chronic stress and GC precipitate Tau accumulation and (insoluble) aggregation in neuronal soma.

The "stressed" autophagy as precipitator of Tau-driven neuropathology

As the guardian of cellular homeostasis, autophagy plays a pivotal role in physiology and in pathology progression of several neurodegenerative disorders (Nixon 2007; Banerjee, Beal, and Thomas 2010). It is responsible for the degradation of long-lived proteins and misfolded proteins,

like Tau, huntingtin and synuclein, and its blockage is known to lead to the accumulation of protein aggregates, a common pathological feature of a range of neurodegenerative disorders (Komatsu et al. 2005; Frake et al. 2015). We hereby demonstrate for the first time that chronic stress as well as GC inhibit autophagic process, providing an explanation for the accumulation and aggregation of Tau under stressful conditions. Indeed, this notion is in line with previous studies showing that stress/GC reduced Tau turnover (I Sotiropoulos et al. 2008) and deregulates molecular chaperones responsible for Tau degradation (e.g. Hsp90 and Hsp70) (Sotiropoulos et al. 2014). Moreover, activity of autophagy is also affected in human and animal models of Tauopathies highlighting a specific relationship between autophagy deficits and Tau pathology (Ambegaokar and Jackson 2012). Autophagy has been indicated as the main degradation pathway in AD brain (Hamano et al. 2008; Wang et al. 2010; Inoue et al. 2012) as numerous reports have suggested that, although a proteasomal substrates (Brown et al. 2005; Feuillette et al. 2005), Tau inclusions and aggregates may not be accessible to the ubiquitin-proteasome system (Hara et al. 2006; Boland et al. 2008). Accordingly, our current findings demonstrate that chronic stress and GC increase mTOR signaling, that together with the reduced LC3II/LC3I ratio and accumulation of p62, suggests that chronic stress inhibits autophagic process by activating mTOR pathway; in line with previous findings where chronic stress is shown to trigger mTOR in hippocampus (Polman et al. 2012). As mTOR activation is associated with increased total Tau levels in AD brains (An et al. 2003; Pei and Hugon 2008), it is highly plausible that part of the stress/GC-driven Tau accumulation maybe attributed to mTOR. Moreover, and in line with previous studies showing that decrease in mTOR signaling can revert Tau pathology (Menzies et al. 2010; Jiang et al. 2014), we also demonstrate mTOR inhibition blocks the GC-driven Tau-related neurotoxicity and induction of aggregation-related cascades (**Chapter 3**). Altogether, this studies point towards the involvement of mTOR and autophagy in the cellular mechanisms through which GC may trigger accumulation of Tau and its aggregates. Future studies should use mTOR inhibition in Tau Tg models under stress conditions, in order to monitor the role of mTOR signaling inhibition in blocking or reverting the deleterious impact of chronic stress on brain Tau aggregation and consequent behavior deficits.

As of any other protein that should be targeted for protein degradation by autophagy or proteasome, Tau needs to be ubiquitylated. However, it is described that the rate of abnormal Tau ubiquitylation can be greater than the rate of its degradation, leading to an increase in Tau aggregation. In AD brains, NFTs are also responsive to ubiquitin immunoreactivity (Bancher et al. 1991; Cripps et al. 2006) while the p62 presence in NFTs starts early in the disease, indicating that

Tau ubiquitylation occurs early in the disease (Kuusisto, Salminen, and Alafuzoff 2002). Future studies should analyze further the impact of stress on molecular chaperones machinery responsible for Tau ubiquitylation and degradation as, in parallel to protein degradation machinery, Hsp90 and Hsp70 are essentially involved in GC receptor (GR) signaling offering a clear cross-point between GC/GR cellular signaling and Tau degradation machinery. It is particularly relevant that stress/GC-driven hyperphosphorylation of Tau could reduce its ubiquitylation (Sahara et al. 2005) and it is plausible that neurons under prolong exposure to stress/GC face an abnormal chaperone response driven by Hsp90/Hsp70/CHIP direct interaction with GR, altering the normal protein degradation signaling (**Figure 3**).

Stress granules as partners of stress-induced Tau pathology

Recent work in AD and FTDP human brains, as well as in Tau Tg mice has causally implicated the formation of stress granule (SGs) in the development and progression of Tau pathology (Wolozin 2012). Eukaryotic stress response involves translational suppression of non-housekeeping proteins and the sequestration of unnecessary mRNA transcripts into SGs, in a RNA binding proteins (RBPs) dependent manner. While our understanding about SG formation is limited, SGs constitute a protective mechanism against cellular stress allowing the protection of mRNA and the fast production of cytoprotective proteins (Wolozin 2012). The SGs formation is known to be a cell survival and protection mechanism (Arimoto et al. 2008; Arimoto-Matsuzaki, Saito, and Takekawa 2016); however, prolonged SG induction can become pathological and neurotoxic, as it is related with several neurodegenerative diseases including AD and Tau aggregation in NFTs (Kampers et al. 1996; Liu-Yesucevitz et al. 2010; Vanderweyde et al. 2012; Liu-Yesucevitz et al. 2014). Recently, SGs are suggested to accelerate Tau aggregation while, in a vicious cycle, Tau stimulates SG formation with TIA1 exhibiting a leading role in Tau misfolding and aggregation (Vanderweyde et al. 2016). Note that both hyperphosphorylation and aggregation-prone mutation of Tau can enhance, but are not required for, SG formation (Vanderweyde et al. 2016). In this thesis, we show for the first time that chronic stress and GC increased the levels of different SG markers in soluble and insoluble fractions of P301L-Tau animals or cells. As shown to directly interact with Tau and stimulate its aggregations (Vanderweyde et al. 2016), TIA-1 aggregation is regulated by molecular chaperones and it is blocked by Hsp70 overexpression resulting to inhibition of SG formation (Mazroui et al. 2007). Note that chronic stress reduces Hsp70 levels (Sotiropoulos et al. 2014) providing a potential cascade through

which TIA-1 is increased under stressful conditions. Our data are in agreement with previously published data in AD and other neurodegenerative disorders suggesting that prolong environmental stress and GC stimulate SG formation, probably by affecting the equilibrium between dispersed and aggregated RBPs, leading to persistent pathological SGs; this (persistent) SGs could serve as niches for further aggregation of other aggregation-prone proteins or even sequester functional mRNAs, causing their silencing (**Figure 3**). As SG formation is induced by the translocation to the cytoplasm (Kedersha et al. 1999) and the increase expression of different SGs proteins (Tourrière et al. 2003; Gilkes et al. 2004; Ohn et al. 2008), our findings suggest a novel role for SG biology in the stress/GC-driven neuronal pathology opening a wide range of avenues for research and therapeutic exploration focusing on RNA-protein intraneuronal trafficking and function in stress-related pathologies. Future studies should also monitor the impact of stress/GC on intracellular trafficking of Tau protein as well as its mRNA in order to provide further details of the temporal profile of stress/GC-driven interplay between Tau and SG proteins in both soluble and insoluble states.

Furthermore, similarly to AD brain, studies of this PhD thesis and recently published work from the group, demonstrate that Tau missorting and dendritic accumulation is part of chronic stress/GC hippocampal pathology (Lopes et al. 2016; Pinheiro, Silva et al. 2015). Dendritic and synaptic missorting of Tau is recently suggested to facilitate formation of SGs as part of the translational stress response (Vanderweyde et al. 2016) providing a possible explanation for this intracellular missorting of Tau under AD and stress pathological conditions. Future studies should monitor the contribution of SGs proteins as well as SG formation is the establishment and manifestation of stress-driven Tau pathology. Indeed, this is particularly relevant as *in vitro* results of these PhD studies show that autophagy induction, through mTOR inhibition, can block the GC-driven increase of SGs and Tau accumulation. Ongoing studies aim to clarify the etiopathogenic relationship and temporal profile between SGs and Tau pathologies under stress/GC conditions especially as very recent work suggest that inhibition of autophagy impact on SGs.

Although inhibition of autophagy is shown to impair the degradation and dynamics of SGs (Buchan et al. 2013; Seguin et al. 2014), the existence of a direct interaction between autophagy and SGs is still debatable. However, cytoskeletal machinery facilitates the aggregation of RBPs to form SGs and have an important role in autophagy maturation and autophagosome/lysosome fusion, as most of the processes in the autophagic pathway, especially autophagosomes and lysosome transport, depend on their movement along microtubules (Aplin et al. 1992). Furthermore, it has been shown that Tau phosphorylation alters autophagy processing while Tau reduction can decrease

and impair autophagic flux and impair autophagosomes-lysosome fusion in cell cultures (Pacheco, Elrick, and Lieberman 2009b; Pacheco, Elrick, and Lieberman 2009a). The above findings suggest that Tau could regulate both autophagic and SG formation processes through its role on regulating MT dynamics and overall cytoskeleton, in line with these PhD findings showing that stress and GC trigger microtubule instability decreasing levels of acetylated tubulin as well as acetylated cortactin (**Figure 3**).

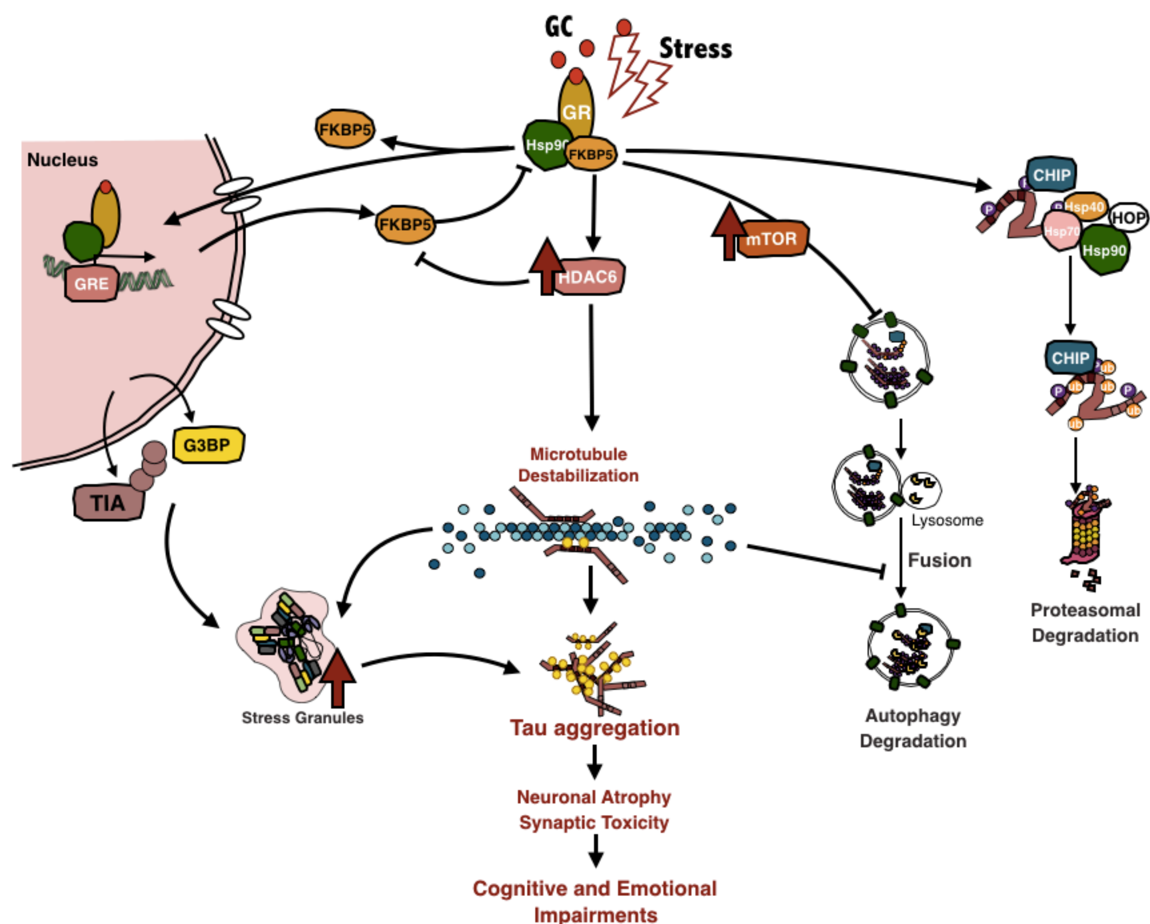


Figure 3. Chronic stress and GC trigger different molecular mechanisms precipitating Tau aggregation and AD neurodegeneration. This schemes summarizes the potential mechanisms that chronic stress and GC trigger in neuronal cells that result in Tau accumulation and neurotoxic aggregation. Upon stress stimulation, neurons are exposed to high GC levels that bind to their receptors (GR) while GR translocation to the nucleus is mediated by several molecular chaperones such as Hsp90, Hsp70, FKBP51 while other proteins such as HDAC6 are also involved in this cytoplasmic GR signaling. Importantly, stress/GC-driven induction of HDAC6 also may lead to cytoskeletal instability (through reduced acetylation of tubulin and cortactin). In addition, stress/GC induce hyperphosphorylation of Tau and its consequent detachment from microtubules, leading to microtubule destabilization and cytoskeletal disturbances that, together with HDAC6, may contribute to: i) the formation of Stress granules (SGs), known to be causally involved in Tau aggregation and ii) the inhibition of autophagic process that can also contribute to Tau accumulation and aggregation. Note that Stress/GC is also found to inhibit autophagy at the initial steps by increases mTOR levels. Previous

work has also implicated deficits in molecular chaperones and the related proteasome degradation. Altogether, the stress-driven cellular cascades could cumulative facilitate tau aggregation and precipitate AD pathology (drawn by Silva JM).

The multiple roles of Histone Deacetylase 6 in stress and AD pathologies

One of the molecules involved in both autophagic and SGs formation processes is histone deacetylase 6 (HDAC6), which, through the deacetylation of tubulin, reduces microtubule-dependent motility and thereby promotes the consolidation of cellular complexes such as SGs and autophagosomes (J Y Lee et al. 2010; Joo Yong Lee et al. 2010; Hoover et al. 2010). But what is HDAC6 and which is its role in neuronal function and AD pathology?

HDAC6 is a histone deacetylase that seems to possess special and extending role(s) as it is the only HDAC containing two functional N-terminally located catalytic sites in combination with a ubiquitin-binding domain at the C-terminal part of the protein. Thus HDAC6 was the first actively HDAC identified in the cytoplasm suggesting that, besides acting as an epigenetic regulator on histones, it may also catalyze non-histone reactions in the cytosol related to cellular processes and functions that involve lysine acetylation and ubiquitylation (Verdel and Khochbin 1999; Grozinger, Hassig, and Schreiber 1999; Verdel et al. 2000; Seigneurin-Berny et al. 2001; Kawaguchi et al. 2003; Boyault et al. 2006). Recent work implicates HDAC6 in the formation of SG in AD brain as HDAC6 seems to localize and interact with SG proteins under cellular stress; interestingly, HDAC6 is a SGs component interacting with G3BP (Seigneurin-Berny et al. 2001; Kwon 2007). Additionally, pharmacological inhibition or genetic ablation of HDAC6 abolished SG formation (d'Ydewalle, Bogaert, and Van Den Bosch 2012) while the expression of HDAC6 significantly increases in the hippocampus and other brain regions of AD patients and animal models of the disease(Ding, Dolan, and Johnson 2008; Perez et al. 2009; Zhang, Sheng, and Qin 2013).

Some cellular targets of HDAC6 include cytoskeletal elements (e.g. α-tubulin and cortactin), antioxidant enzymes (e.g. peroxiredoxin) as well as molecular chaperones involved in signal transduction and protein homeostasis (e.g. Hsp90). As both *in vivo* and *in vitro* findings of this PhD thesis support a stress/GC-driven increase of HDAC6 accompanied by its increase cytoplasmatic action (deacetylation of tubulin and cortractin), this stress/GC action could involve many cellular alterations on e.g. microtubule dynamics (Noack, Leyk, and Richter-Landsberg 2014) as well as impairment of ubiquitylated protein degradation by proteasome/autophagy (Iwata et al. 2005; Lee et al. 2010a; Guthrie and Kraemer 2011). Indeed, this thesis findings show that stress/GC reduces

the levels of detyrosylated tubulin, acetylated tubulin and acetylated cortactin pointing towards microtubule instability which is also suggested to cause enlargement of SGs (Chernov et al. 2009) (see **Chapter 2** and **3**) as well as inhibition of autophagic process (**Chapter 3**). Furthermore, as recent work supports the importance of Tau acetylation in its aggregation process implicating HDAC6 (Ding, Dolan, and Johnson 2008), future studies should also monitor the potential impact of stress and GC on Tau acetylation levels and its role in stress-induced Tau pathology. Furthermore, HDAC6 is also involved in Hsp90 acetylation and dysregulation of GR signaling (Kovacs et al. 2005; Espallergues et al. 2012) (**Figure 3**) while recent reports in mice show that HDAC6 inhibition increases resilience to stress through Hsp90 hyperacetylation, disabling GR translocation from the cytoplasm into the nucleus (Espallergues et al. 2012; Jochems et al. 2014); suggesting HDAC6 as potential link between stress/GC signaling, molecular chaperones and Tau aggregation pathology. While future studies that will monitor the multi-target role of HDAC6, are necessary, these PhD studies add novel evidence to our limited understanding of stress-triggered cellular cascades and molecular pathways that precipitate Tau-related AD pathology supporting further investigation of HDAC6 neuroprotective role in stress-related brain pathologies beyond AD e.g. depression and anxiety.

6.2 MAIN CONCLUSIONS

Tau is described as a microtubule-associated protein more than forty years ago with the predominant view about its cellular function focusing on MT stabilization. During more recent years, Tau is shown to be implicated in many and different cellular processes through its interaction with different structural and non-structural proteins such as microtubules, actin, Fyn, HDAC6, TIA-1. Many studies support the critical role of Tau on neuronal cell morphology during division, growth and differentiation, axonal cargo transport while recently, Tau is suggested to be implicated in structure and function related with synaptic plasticity. In addition, Tau malfunction is related to AD pathology while recent evidence support the involvement of Tau in neurological pathologies with reverse etiology e.g. epilepsy, excitotoxicity and chronic stress. However, critical questions about the real role of Tau in adult brain as well as its implication in brain pathology in and beyond AD remain unclear.

Thus, this PhD thesis characterize and validate a novel conditional KO model for *mapt* in adult, fully matured brain, providing a powerful tool for unrevealing the real and direct function of Tau protein. Using this conditional Tau-KO model, these PhD studies demonstrate for the first time that loss/reduction of Tau in the adult brain triggers dendritic atrophy supporting a role for Tau in

dendritic maintenance. In addition, tau deletion resulted in alterations of synaptic scaffold assembly that cumulatively lead to anxious and depressive behavior. Thus, these findings consist the first report about the impact of Tau loss in adult brain while it opens a new window of research opportunities to further understand and clarify the role of Tau in synaptic structure and functions beyond AD pathology, where animal models are based on Tau overexpression. Monitoring the impact of Tau and its malfunction/pathology in the mechanisms through which chronic stress and prolong exposure to high GCs precipitates brain pathology, these PhD studies suggest that chronic stress/GC evoke Tau-related cytoskeletal disturbances that maybe related to the inhibition of autophagic process and induction of SGs, precipitating Tau accumulation, aggregation and related neurotoxicity. Furthermore, our findings show that stress/GC trigger Tau missorting in synapses triggering NMDA receptor-related excitotoxicity signaling and synaptic malfunction/atrophy. Conclusively, these PhD studies provide a new analytical "tool" and many insights adding to our limited knowledge about the *in vivo* role of Tau protein in brain homeostasis and pathology beyond AD.

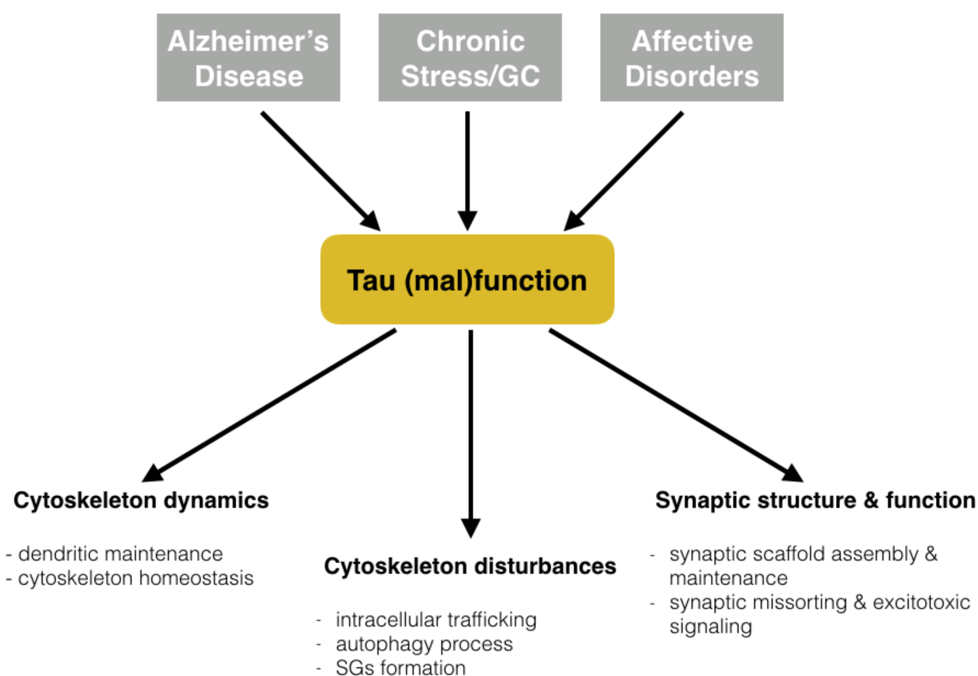


Figure 4: Tau (mal)function in brain homeostasis and pathology. The above scheme summarizes some of findings of this PhD thesis related with the function of Tau and its implication in different pathological conditions such as AD and chronic stress/GC describing both cytoskeletal and synaptic functions of Tau protein (drawn by Silva JM and Sotiropoulos I).

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APENDICES

APPENDIX I

CHRONIC STRESS AND GLUCOCORTICOIDS: FROM NEURONAL PLASTICITY TO NEURODEGENERATION

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Review Article

Chronic Stress and Glucocorticoids: From Neuronal Plasticity to Neurodegeneration

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Stress and stress hormones, glucocorticoids (GCs), exert widespread actions in central nervous system, ranging from the regulation of gene transcription, cellular signaling, modulation of synaptic structure, and transmission and glial function to behavior. Their actions are mediated by glucocorticoid and mineralocorticoid receptors which are nuclear receptors/transcription factors. While GCs primarily act to maintain homeostasis by inducing physiological and behavioral adaptation, prolonged exposure to stress and elevated GC levels may result in neuro- and psychopathology. There is now ample evidence for cause-effect relationships between prolonged stress, elevated GC levels, and cognitive and mood disorders while the evidence for a link between chronic stress/GC and neurodegenerative disorders such as Alzheimer's (AD) and Parkinson's (PD) diseases is growing. This brief review considers some of the cellular mechanisms through which stress and GC may contribute to the pathogenesis of AD and PD.

1. Introduction

Stress is broadly defined as an actual or anticipated threat of well-being or disruption of organism homeostasis [1]. Although the sensing and reaction to stress evolved to promote adaptation, modern workstyles and lifestyles represent challenges that render individuals susceptible to physical and mental disorders [2–5]. Multiple factors influence an individual's ability to cope with stress, for example, early life experiences, gender, or personality traits. Both vulnerability and resilience may be determined by genetic and epigenetic (gene environmental interactions) background [5–9].

Since the discovery of the communication between hypothalamus and pituitary in early 70s that opens a new window in our understanding of the brain-body communication, there are plethora of studies describing the high biological significance of stress and its responses which enables various adaptive processes to changing conditions. The most easily measureable and critical physiological response to stress involves the release of glucocorticoids (glucocorticoids, GCs). These hormones are synthesized and secreted into systemic circulation from the adrenal glands following stimulation by the anterior pituitary hormone adrenocorticotropic hormone (ACTH) [1]. The release of ACTH itself is increased in response to the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from neurons in the hypothalamic paraventricular nucleus (PVN). Together, the hypothalamus, pituitary, and adrenal glands constitute the so-called hypothalamo-pituitary-adrenal (HPA) axis, which plays an essential role in the adaptive response to psychogenic (e.g., fear) and physical (e.g., cellular lesion or pathogen invasion) stressors. The adaptive responses that are initiated by GCs occur in multiple tissues and involve alterations in numerous

physiological (e.g., metabolic, cardiovascular, and immune) as well as behavioral (e.g., emotion, cognition, and motor) processes [1, 10–12]. Normally, GC-driven negative feedback mechanisms at the different levels of the HPA axis serve to normalize GC secretion and restore homeostasis; however, and depending on the type, duration, and intensity of the stressful stimulus, GC hypersecretion may persist and become a potential threat for health [1].

There is now abundant evidence that GCs can exert profound modulatory effects on a variety of brain functions from early development through to late life [12]. Their actions are mediated by two receptors: the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), which belong to the superfamily of nuclear receptors that act as transcription modulators [13, 14]. In the brain, GR is ubiquitously expressed, whereas MR expression is more restricted to just a few structures (hippocampus, locus coeruleus, amygdala, prefrontal cortex, and nucleus of the solitary tract, as well as PVN neurons). MR is also present in nonneuronal cells, namely, in glia and epithelial cells of the choroid plexus and ependyma [15].

Binding assays using ^{3}[H] corticosterone have shown the MR has a 10-fold higher affinity ($K_d = 0.5$ nM) for GC compared to GR ($K_d = 5$ nM), which means that, at basal GC levels, MR is occupied and activated [16] whereas GR is only activated when GC levels reach a certain level, for example, during the circadian peak of GC secretion and during stress [17]. Importantly, brain MR and GR both respond to the same endogenous ligand (cortisol in humans and larger mammals, corticosterone in rodents); further, MR and GR were reported to colocalize in the same pyramidal and granular neurons of the hippocampus [17]. Given the GR and MR colocalization and relatively small difference in their affinity for endogenous GCs, the question arises as to whether they regulate distinct genes and/or coregulate transcription by heterodimerization. Heterodimerization of GR and MR was shown with high concentration of GC (stress level) in the nuclei of cultured hippocampal neurons. Moreover, evidence suggests that their cellular responses through regulation of distinct gene expression (as homodimers) depend strongly upon specific recruitment of coregulators [18, 19].

Synthetic GCs (e.g., dexamethasone, methylprednisolone) are routinely used in clinical situations due to their powerful anti-inflammatory and immunosuppressive actions. However, a growing body of evidence suggests that high GC exposure in early life can adversely program the HPA axis and increase the susceptibility to develop metabolic, neuropsychiatric, and neurodegenerative disorders [5, 20, 21]. In addition, there is now ample experimental evidence where elevated GC levels and prolonged exposure to stressful conditions induce structural remodeling of neurons with synaptic loss as well as alterations in glial functions, which are frequently maladaptive [22]; see also Figure 1. In this brief review we discuss some of current knowledge about cellular targets and mechanisms through which stress and altered GC levels trigger changes in the brain that may lead towards the development and progression of neurodegenerative pathologies such as Alzheimer's (AD) and Parkinson (PD) disease.

2. From Stress-Driven Brain Programming to Neurodegenerative Pathologies

In addition to nongenomic mechanisms that are still incompletely identified [23], chronic stress and GC levels most likely influence neuronal function and connectivity by activating GR-mediated transcription. GRs are normally located in the cytoplasm in association with chaperone proteins such as the heat shock proteins Hsp90 and 70 and the immunophilins FKBP51 and FKBP52. Upon GC binding, conformational change of the GR-chaperone complex results in nuclear translocation of the GR [24, 25]. In the nucleus, GR binds to specific regions of DNA, which possess glucocorticoid response elements (GRE) within the promoters of target genes, leading to cell-type and context-dependent gene expression [26–28]. Transcriptional regulation by GR may occur by (a) direct binding of GR homodimers to GRE within DNA sequences to stimulate transcription, for example, *mitogen-activated protein kinase phosphatase-1* gene; (b) direct binding to negative GRE elements to repress transcription; the gene encoding the prohormone from which ACTH is derived (proopiomelanocortin, *POMC*), *CRH,* and the *CRH receptor* genes are examples of negatively regulated genes; and (c) trans-repression or "tethering," that is, association with other transcriptional factors that inhibit the transcriptional activity of GR. In the brain, identification of GR-modulated genes is difficult due to the anatomical complexity and cellular heterogeneity. Nevertheless, transcriptomic studies in the hippocampus have identified functional classes of GR target genes which include genes coding for neurotransmitter catabolism, neurotrophic factors and their receptors, signal transduction, energy metabolism, and cell adhesion [29].

In addition to altering gene expression, growing evidence suggests that epigenetic mechanisms represent a means through which stress and GCs can leave long-lasting "memories" of past experiences which, in turn, contributes to shaping the organism's physical and mental health trajectory [21, 30, 31]; see Figure 1. Broadly, epigenetics refers to stable changes in the regulation and/or function of DNA, RNA, and/or proteins that do not involve alterations of their primary sequences. Two well-known examples of epigenetic marks induced by environmental stimuli (e.g., stress) are DNA methylation and histone modification. The first evidence of epigenetic programing in the brain by early life adversity showed that poor maternal care in rats leads to methylation of exon 1_7 in the *GR* promoter, being accompanied by aberrant behaviors and altered HPA axis responses during adulthood [32, 33]. Subsequently, similar mechanisms were reported in humans who had experienced childhood adversity [34] and in infants born to depressed mothers [35]. The earlier studies in rats were replicated in mice in paradigms of prenatal GC exposure and early postnatal stress; we showed that these pre- and postnatal manipulations resulted in epigenetic modifications of the promoters of neurotransmitter (*dopamine receptor 2*) [36], *GR*, and various GR target genes [37, 38] with long-lasting maladaptive behavioral consequences.

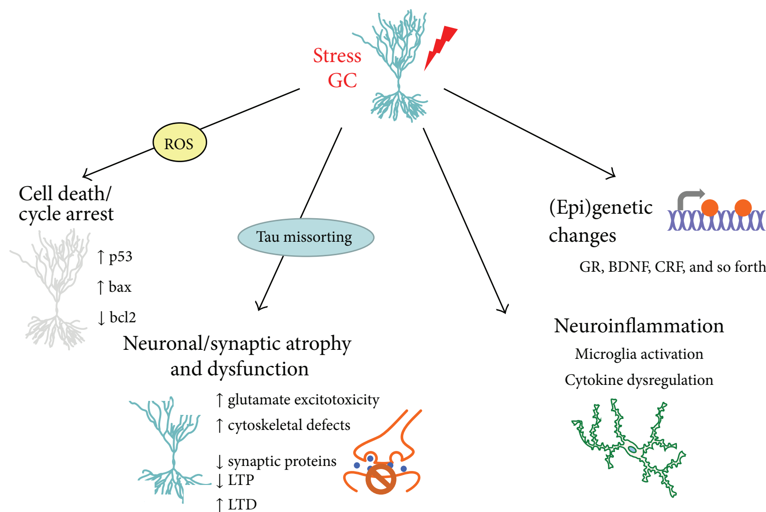

Figure 1: Cellular targets and actions of chronic stress mediated by glucocorticoid receptors. This schema depicts some cellular targets and mechanisms that are targeted by glucocorticoids (GCs), whose actions are mediated by glucocorticoid receptors (GR). GCs are secreted under conditions of stress; neuronal damage and brain pathologies are a common consequence of persistently elevated GC secretion. GC can trigger mitochondrial dysfunction and the apoptotic machinery, as well as cell cycle arrest and cell death. In addition, stress/GC may induce neuronal atrophy and synaptic dysfunction/loss by stimulating hyperphosphorylation of the cytoskeletal protein Tau, thus disturbing the integrity of the cytoskeleton and missorting Tau at synapses. Together, these latter events may eventually result in the degradation of synaptic proteins and receptors and consequently, synaptic plasticity. Stress and GC are also established as modulators of microglial activation and neuroinflammatory processes. Lastly, accumulating evidence indicates that stress and GC can influence neuronal structure and function through epigenetic mechanisms.

Recent studies also suggest that early life events (e.g., intrauterine infections, maternal stress, and poor maternal and perinatal nutrition) may play a role in the onset of Alzheimer's disease (AD), an age-related neurodegenerative disorder characterized progressive memory and cognitive deficits [39]. From this perspective, AD is probably not determined by a single etiologic factor but results from the interplay between genetic and environmental factors throughout life, possibly explaining why monozygous twins can be discordant for AD. Albeit this is still controversial and the literature is sparse, it has been suggested that adverse events in early life, for example, maternal stress and poor maternal and perinatal nutrition, can potentially predispose eventually to AD through epigenetic programing of specific genes/pathways related to AD neurodegeneration. For example, maternal separation for the first 3 weeks of rodent life is shown to result in increase of AD cellular pathways (e.g., APP misprocessing and Tau hyperphosphorylation; see below) followed by synaptic and neuronal damage as well as cognitive deficits in adulthood [40] suggesting the potential impact of early-life stress exposure to the precipitation of AD neurodegeneration later in life. While most current research on epigenetic mechanisms focuses on DNA methylation, one recent study demonstrated that GC, acting via GR, increase the levels of histone deacetylase 2 (HDAC2), an enzyme regulating DNA expression, in the CK-p25 mouse [41]. In general, how early life stressors reprogram the fetal brain and contribute to late-life development of neurodegenerative disorders (e.g., AD) is emerging as an exciting, new research field [42].

Experimental evidence in animal studies indicates that stressful events in early life can impact the etiopathogenesis of another neurodegenerative disorder, Parkinson's disease (PD), which is characterized by both motor and nonmotor symptoms. Depression, anxiety, apathy and interestingly fatigue are common nonmotor features occurring in around 30 to 58% of patients before the onset of motor symptoms in PD patients. In addition, the prevalence of cognitive impairment in PD ranges from 19 to 36% [43]. The cellular mechanisms underlying these nonmotor symptoms in PD may share similarities to AD, particularly with respect to the molecular pathways activated by stress.

Maternal separation was reported to exacerbate motor deficits and nigrostriatal lesion in an experimental model of PD [44]. In an interesting study, pups of female animals, exposed to the bacterial endotoxin lipopolysaccharide (LPS) during pregnancy, showed loss of dopaminergic (DA) neurons. Since loss of dopaminergic neurons as well as related motor deficits is a characteristic feature of PD pathology, the above findings suggest that high LPS levels in mothers might interfere with the development of DA neurons in the fetus, thus enhancing susceptibility to PD [45]. Accordingly, developmental stress may represent the first imprint in the brain and accumulatively with later stressful stimuli to affect nigrostriatal neurochemical reserve and precipitate the PD phenotype [46].

3. Chronic Stress and GC as a Risk Factor for AD

AD is a multifactorial neurodegenerative disorder with complex etiopathology. Besides early life stress (see above), accumulating clinical evidence strongly suggests that chronic

stress in adulthood as well as elevated GC levels may have a role in the development of AD pathology and related dementia [47, 48]. In fact, high levels of cortisol are commonly found in AD patients' plasma, saliva, and/or CSF [49–53]; AD patients also show higher total daily secretion of cortisol [54]. The potential link between stress/GC and AD described above is strengthened by emerging evidence that stress may advance the age of onset of the familial form of AD [47, 48, 55] and that cortisol levels in AD patients correlate with their memory deficits [56, 57] suggesting a role for GC on AD. Nevertheless, in the absence of longitudinal studies it is not clear from the available evidence as to whether elevated GC secretion is a cause or a consequence of AD disease.

An important brain area in unraveling the interrelationship between stress, elevated GC, and AD pathology is the hippocampus, which is among the first areas affected in AD patients. Hippocampal lesions in AD brain are not only associated with the deficits in declarative, spatial, and contextual memory but could also be responsible for the suggested HPA axis dysregulation and the subsequent overproduction of GC found in AD patients due to the inhibitory role that hippocampus exhibits on HPA axis. Indeed, previous studies from our laboratories (and others) have shown that hippocampal neurons are particularly vulnerable to the adverse effects of stress and GC, their effects being manifested as dendritic atrophy and apoptotic cell death [22, 58]. Moreover, a large number of studies have shown that stress and elevated GC levels affect neurogenesis in adult brain with subsequent impairments of mood and cognitive behavior [59, 60]. More specifically, both acute and chronic exposure stress reduces adult neurogenesis, affecting hippocampal cell proliferation and, in certain studies, survival of newborns [61, 62]. In addition, administration of corticosterone showed the ability of glucocorticoids to damage neurogenesis in adult brain by inhibiting cell proliferation, differentiation and survival [63] while the deleterious effect of stress and/or corticosterone on neurogenesis is GC-dependent [64]. In a vicious cycle, alteration in neurogenesis of adult brain is recently shown to impact on GC negative feedback on the central elements regulating HPA axis activity [65, 66]. Moreover, perturbations in adult neurogenesis may also be related to the cognitive deficits associated with AD whereas contradictory findings support both increases and decreases of neurogenesis in brain of AD patients and Tg animal models [67]. Here, it is also worthwhile noting that stress and GC interfere with hippocampal-prefrontal cortex (PFC) connectivity [68] and dendritic and synaptic plasticity in the PFC, thus disrupting executive functions [58]. These PFC structural deficits are also likely to have consequences for central regulation of the HPA axis providing another neuroanatomical link between HPA axis dysregulation and subsequent GC hypersecretion and AD pathology.

4. Impact of Stress and GC on Neurodegenerative Mechanisms of AD

At the molecular level, AD pathology is characterized by amyloid beta (Aβ) that forms deposits (senile plaques) and hyperphosphorylated forms of the cytoskeletal protein Tau that aggregate into neurofibrillary tangles (NFT) [69–71]. Aβ is the proteolytic product of a large transmembrane protein called amyloid precursor protein (APP), which is sequentially cleaved by β-secretase (BACE-1) and γ-secretase (a complex of enzymes) to generate the production of Aβ; this cellular pathway is often called APP misprocessing. Many studies have demonstrated that the products of APP misprocessing trigger neuropathological processes associated with AD such as synaptic malfunction (including impairment of long-term potentiation), neuronal atrophy and synaptic disintegration and loss [72] as well as mitochondrial dysfunction, oxidative stress, and glial activation [73].

Although still a subject of debate, several studies suggest that Aβ also triggers the abnormal hyperphosphorylation of Tau, NFT formation, and neuronal loss. Moreover, cumulative evidence suggests that the detrimental effects of Aβ are abolished in Tau-KO mice, highlighting the essential mediatory role of Tau protein in the neuro- and synaptotoxic effects of Aβ [73–77]. Further support for an essential role of Tau in the establishment of AD pathology derives from clinical findings that have consistently shown that the cognitive deficits in AD patients correlate better with NFT rather with Aβ deposition *per se*. Moreover, Gómez-Isla et al. [78] demonstrated a strong correlation between neuronal loss in the cerebral cortex and increased NFT burden with disease progression; no such correlation was found with Aβ. In addition, the reduction of hippocampal volume in AD patients correlates better with CSF levels of phosphorylated Tau than with those of Aβ [79].

The evidence of a causal relationship between stress/GC and AD includes that from studies showing that either high GC levels and/or stress increase the production of Aβ and exacerbate memory deficits in transgenic mouse models of AD [80, 81]. Specifically, chronic immobilization stress in transgenic mice expressing the amyloid precursor protein (APP) V717ICT-100 (a mutation which results in aggressive early onset AD) accelerates the appearance of extracellular Aβ deposits and worsens memory deficits. Similar findings were obtained *in vivo* when young (prodromal) 3XTg-AD mice were treated with the synthetic GC, dexamethasone [80]; the same authors also reported dexamethasone-induced APP misprocessing in the N2A cell line, a finding matched by our own observations in PC12 cells [82]. Further, Green et al. demonstrated that GCs upregulate the transcription of *APP* and *β-secretase*, whose promoters contain a glucocorticoid response element (GRE) [80]. Consistent with the above, our studies in middle aged rats showed that stress and chronic GC drive APP processing towards the generation of Aβ and its precursor molecule (C99), both of which have neurotoxic and cognition-impairing properties [83] (see also Figure 1). The latter changes were accompanied by increases in the levels of β-secretase (BACE-1) and Nicastrin, a protein found in the γ-secretase complex. Further experiments that attempted to mimic intermittent stressful events that may exert cumulative effects over the lifetime indicated that GC potentiate the APP misprocessing pathway in previously stressed rats receiving Aβ-infusions [83] (see Figure 2).

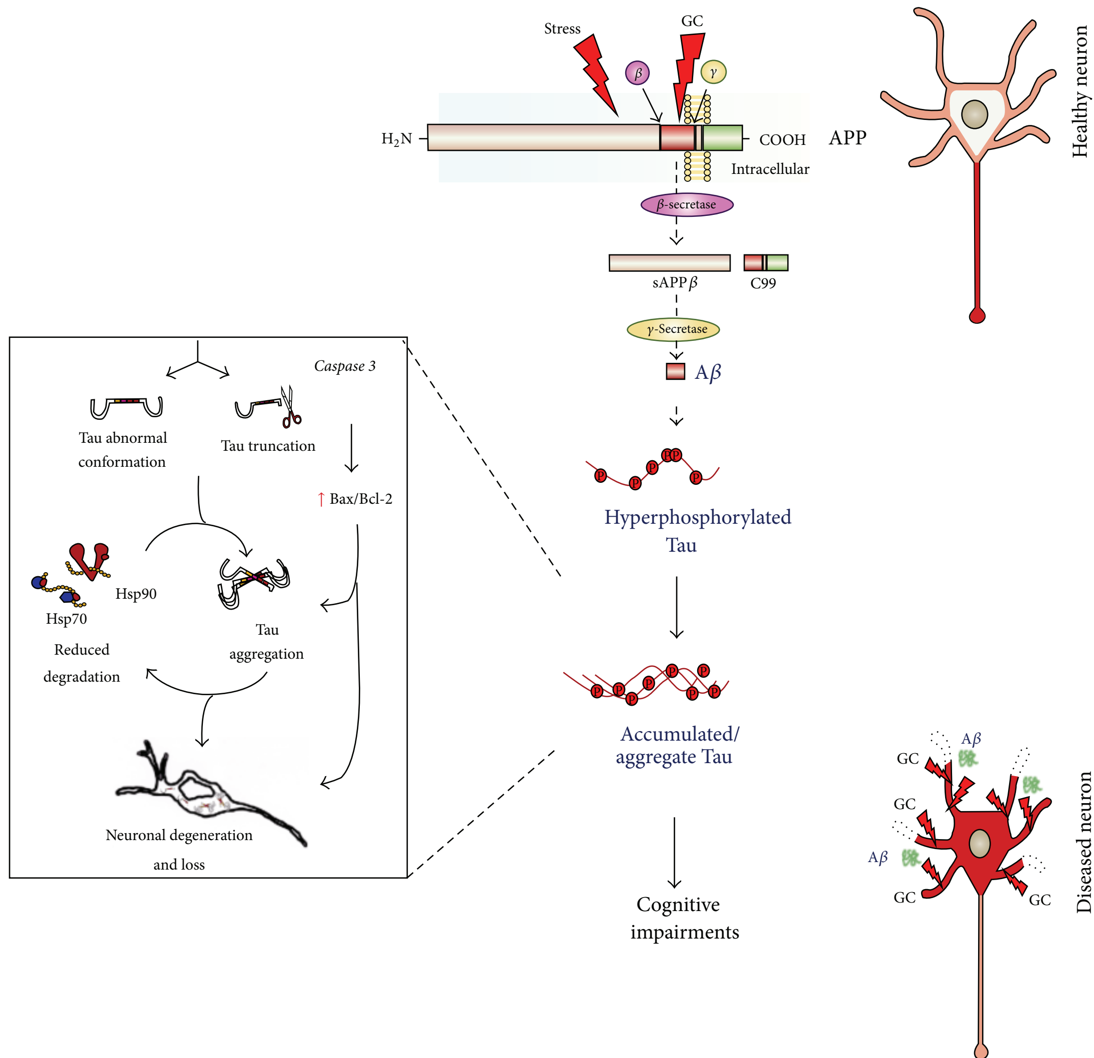

Figure 2: Proposed model through which chronic stress and glucocorticoids (GCs) may contribute to Alzheimer disease (AD) pathology. The model illustrates how chronic stress and high GC levels can trigger AD pathology; the figure is based on experimental evidence obtained in cellular and animal models of AD. Extended exposure to stress/high GC levels activates the amyloidogenic pathway of amyloid precursor protein (APP). This so-called misprocessing of APP involves the sequential cleavage of APP by β- and γ-secretases, resulting in the generation of toxic amyloid β (Aβ). Subsequently, the cytoskeletal protein Tau, which is mainly localized in axons (red in the representation of a healthy neuron), becomes aberrantly hyperphosphorylated, catalyzed by glycogen synthase kinase (GSK3β) and/or cyclin-dependent kinase 5 (CDK5). Hyperphosphorylated Tau is trafficked to, and accumulates in, the somatodendritic compartment, where it oligomerizes and forms insoluble aggregates (red in the diseased neuron). In addition, the abnormal conformation adopted by Tau and caspase 3-mediated truncation of Tau is accompanied by dysregulation of the molecular chaperones Hsp90 and Hsp70, which normally serve to promote Tau degradation (left panel). This cascade of events causes neuronal atrophy and loss, followed by cognitive impairments.

In addition to triggering the amyloidogenic pathway, high levels of GC and stress can also instigate the aberrant hyperphosphorylation of Tau protein that also characterized AD brain. Among the first reports suggesting a potential connection between GCs and Tau was that from Stein-Behrens et al. [84] who demonstrated that GC exacerbate kainic acid-induced hippocampal neuronal loss with a contemporaneous increase in Tau immunoreactivity. A later study showed that chronic treatment of 3xTg AD mice with dexamethasone leads to the somatodendritic accumulation of Tau in the hippocampus, amygdala and cortex [80].

Supporting those earlier studies, we showed that chronic stress or GC increase the levels of aberrantly hyperphosphorylated Tau in the rat hippocampus and PFC [85] (see Figure 2). Importantly, the hyperphosphorylation occurred at certain Tau epitopes that are strongly implicated in cytoskeletal dysfunction and synaptic loss (e.g., pSer262) [86, 87] and hippocampal atrophy (e.g., pThr231) [88] in AD patients. Here, it is pertinent to note that the extent of phosphorylation at Thr231- and Ser262-Tau correlates strongly with severity of memory impairment, speed of mental processing, and executive functioning in AD patients [89–91].

Although chronic stress and GC treatment exert similar, but not identical, effects on individual Tau phosphoepitopes *in vivo* and *in vitro* [82], the overall evidence points to GC as the key mediator of the AD-like pathology induced by stress. On the other hand, some studies have suggested a role for at least one other stress-related molecule, namely, corticotrophin-releasing hormone (CRH), as deletion of the *CRH receptor 1* gene in mice was found to block the detrimental effects of stress on Tau phosphorylation [92, 93].

As shown at Figure 2, information on the mechanisms underlying stress/GC-induced hyperphosphorylation of Tau is only just beginning to emerge. For example, *in vitro* experiments indicate that the effects of stress/GC are mediated by glycogen synthase kinase 3 (GSK3) and cyclin-dependent kinase 5 (CDK5), both of which have well-established roles in Tau hyperphosphorylation and the subsequent disruption of microtubules, features seen in the AD brain [82]. We now also know that GC exposure increases Tau accumulation by affecting turnover of the protein by reducing its degradation [82]; the latter appears to result from dysregulation of molecular chaperones (e.g., Hsp90 and Hsp70) that are responsible for Tau proteostasis [94] (see Figure 2). Interestingly, both these heat shock proteins also serve to maintain GR in a high affinity state, suggesting that these proteins may be the point at which GC/GR signaling intersects with the cellular machinery that regulates Tau degradation. Using a transgenic mouse that expresses human P301L-Tau (the most common Tau mutation), we recently showed that chronic stress triggers different aspects of Tau pathology in addition to inducing, its aberrant hyperphosphorylation and aggregation of Tau into insoluble forms [94]. Adding to the mechanistic understanding of stress-driven aggregation of Tau, we also showed that chronic stress enhances caspase 3-mediated truncation of Tau at its C-terminal, leading to an abnormal conformation of Tau in the hippocampus (Figure 2). This truncation-dependent misfolding of Tau into an abnormal conformation is known to facilitate nucleation and recruitment of other Tau molecules into neurotoxic aggregates [95, 96] before NFT are formed [95, 97, 98].

It is interesting to note that chronically elevated GC secretion, usually in response to stress, is a major cause of major depressive illness [99]. In light of the increasing volume of data implicating high GC levels in AD, it is important to consider that epidemiological studies implicate depression as a risk factor for the development of AD; this is supported by the observation that previously depressed subjects have increased amyloid plaque and NFT loads [100]. Different studies have in fact sought to discriminate between subjects undergoing normal aging from those suffering from depression or AD through the measurement of the various APP cleavage products [101–104]. While much remains to be discovered about the potentially important role of depression in AD pathology, it is interesting to note that antidepressant drugs, whose actions often involve reductions in GC secretion, inhibit the proteolytic cleavage of APP into amyloidogenic products [104, 105].

Lastly, it deserves mentioning that a recent epidemiological study found that the prevalence and incidence of dementia in war veterans suffering from posttraumatic depression (PTSD) is twice as high as that in age-matched PTSD-free subjects [106]. While PTSD is a condition quite distinct from major depression, these findings hint at the important influence lifetime stressful experiences can have on mental health, possibly through epigenetic mechanisms. The findings are also interesting since PTSD patients usually show hypoactivity of the HPA axis (versus hyperactivity in depression), suggesting that just a single—but major stressful—event involving transient GC hypersecretion can have long-lasting neuropathological consequences.

4.1. Inflammation and AD: Role of GCs? Chronic inflammation is one of the central pathological features of AD with reactive microglia and astrocytes surrounding senile β-amyloid plaques observed in both postmortem AD brain and animal models [107, 108]. Evidence from human studies suggests that glial activation is an early event; thus inflammatory markers are present in mild cognitive impairment cases that eventually progress to AD [109]. Thus proinflammatory cytokines produced by activated glia in response to amyloid fibrils would be expected to activate HPA axis and increase GC levels. *In vitro* studies clearly show that Aβ can be taken up through phagocytosis in microglia and thereafter degraded [110, 111]; thus, in AD setting, microglial likely have a beneficial role early in pathology. However, elevation of proinflammatory cytokines such as IL-1β may also participate in mood disorders such as depression [112] in AD.

The importance of immune-related responses in the emergence of Aβ burden, tau pathology, and dementia is gaining momentum as molecular comprehension of their actions is increasingly unraveled by human genetic and animal studies. Recent genome-wide association studies have identified variants in at least 16 genes involved in microglia/macrophage functions as risks for developing AD [113]. Among them, *ε4* allele of *APOE* gene is a known strong risk factor, accelerating the age of onset of AD. APOE is produced by both microglia and astrocytes; it regulates not only lipid and Aβ metabolism but also microglial chemotaxis and proinflammatory cytokine expression [114]. Recently, another strong link was found between variants in *TREM2* gene and AD. TREM2 is specifically expressed in myeloid cells where it promotes phagocytosis whilst inhibiting cytokine production [115]. These and most other GWAS genes identified [113] are involved in aberrant microglial/macrophage responses with regard to Aβ clearance and spread of Tau pathology.

In addition to genetic susceptibility, prolonged exposure of Aβ affects microglial functions. Thus, crucial microglial functions such as motility and phagocytosis were impaired in APP/PS1 mice [116]; also in these mice the levels of Aβ receptors (SRA, CD36, RAGE) and Aβ degrading enzymes (neprilysin, MMP9) were decreased with concomitant increase in proinflammatory cytokines TNF-α and IL-1β [117]. Age, a primary risk factor for AD, is also an important contributor to dysfunction of innate immune responses. Microglial dystrophy and fragmentation observed in aging brain [118] occur before the appearance of abnormal Tau suggesting dysfunctional microglia could contribute to appearance of Tau pathology.

Chronic stress through GCs is known to prime and augment neuroinflammatory processes in the cortex and hippocampus upon subsequent proinflammatory challenges such as LPS [119, 120]. Peripheral infections and stress are both known to affect the activation state of microglia and in AD pathology both could have detrimental effects on the functions of microglia. There is little known on how glucocorticoids influence glial functions during prodromal to emergence and progression of AD pathology. It would be important to understand whether GC through GR has any role in Aβ degradation in astrocytes or myeloid cells.

5. Role of Glucocorticoids in Onset and Progression of Parkinson's Disease

Parkinson's disease (PD) is a complex systemic and progressive neurodegenerative disease associated with both motor and nonmotor symptoms. The cardinal motor symptoms such as akinesia, resting tremor and rigidity mostly arise from preferential and substantial loss of dopaminergic neurons (50–60%) in the substantia nigra pars compacta (SNpc) with significant dopamine depletion in the sensorimotor striatum. The nonmotor symptoms include olfactory dysfunction and sleep behavior disorder as well as mood changes and cognitive impairment as discussed above. One principle histopathological feature is the presence of Lewy bodies (LBs), which are proteinaceous inclusions containing mainly structurally altered presynaptic protein, alpha-synuclein, which, as recent evidence shows, plays a central role in PD pathology. Alpha-synuclein LB deposition was used by Braak et al. [121] as a principle pathological marker to monitor the progression and severity of PD. PD is believed to originate from olfactory nucleus and autonomic nervous system progressing in an ascending manner to many brain regions such as substantia nigra, striatum, raphe, locus coeruleus, hypothalamic nuclei, hippocampus, amygdala, and cerebral cortex accounting for both motor and nonmotor symptoms [121–123]. Thus, for example, PD patients with cortical LBs also suffer from dementia and visual hallucinations [124].

While several gene mutations have been identified in familial forms of PD, the majority of PD cases are sporadic and of unknown etiology. Nevertheless, significant advances in the last decade on PD genetics, particularly genome-wide association, as well as pathophysiological mechanisms in various PD model systems, have contributed much to our comprehension of PD. Cellular processes such as oxidative and nitrative stress, mitochondrial dysfunction, and deregulated intracellular calcium levels as well as damaged proteostasis related to alpha-synuclein aggregation are the most studied and relate to dopamine neurodegeneration [125].

As in AD patients, the HPA axis is likely dysregulated in PD patients. Specifically, previous studies [54, 126–128] including our own work [129] show that plasma cortisol levels are significantly higher in idiopathic PD patients compared to control subjects; however, these high levels do not correlate to disease duration or to L-3,4-dihydroxyphenylalanine (L-DOPA) treatment. Interestingly, the diurnal pattern of cortisol secretion in PD patients, in particular the normally quiescent nocturnal cortisol secretory pattern, is affected [54].

6. The Neurodegenerative Potential of Altered GC Levels in PD Pathology

Chronically elevated GC levels in PD patients suggest that HPA regulated-stress responses may impact PD pathology. Indeed, the role of stress was proposed as one of the underlying causes of PD as clinical reports show that stress triggers the appearance of PD symptoms or exacerbates the motor symptoms [130–132]. The role of stress in PD is supported by few experimental studies such as food deprivation and tail-shock and maternal separation aggravate motor deficits in the 6-hydroxydopamine (6-OHDA) PD model (6-hydroxydopamine local injections lesions the nigrostriatal pathway) [133]. In combined chronic stress exposure with 6-OHDA lesion, stress was shown to worsen the 6-OHDA-driven motor deficits, aggravate the neurodegeneration of nigrostriatal system, and completely block compensatory recovery of motor tasks [131, 134]. The precise actions of high GC levels in motor control following nigrostriatal lesions are yet not known. Analysis of GR expression in PD brains revealed that GR levels were reduced in the SNpc and augmented in the putamen, compared to age-matched control subjects; similar results were found in MPTP- (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine-) treated mice [129]. GCs are known to profoundly modulate dopaminergic neurotransmission. The role of GC on the limbic arm of the dopaminergic circuitry related to reward and motivation as well as neuropsychiatric diseases has been extensively investigated (see below). Thus, from its known roles in mesolimbic circuitry, it has been postulated that GR also likely affect motor automated or habitual skills of the sensorimotor circuitry in the striatum by influencing NMDA/AMPA receptor functions in D1 and D2 receptor-medium spiny neurons (Figure 3). Indeed, it has been shown that chronic stress leads to opposing structural changes in the limbic/associative and sensorimotor striatal circuitry with atrophy in the former and hypertrophy of sensorimotor striatum, leading to habit behavior [135]. In addition, the roles of both glucocorticoids and noradrenaline were recently reported in habit memory [136]. It is possible that GR-mediated changes in the putamen during the prodromal stage of PD play a role in preventing the appearance of motor symptoms, culminating in dopamine depletion and death of dopaminergic neurons in the substantia nigra.

Altered stress responses most likely play an important role in nonmotor PD symptoms, particularly anxiety, depression, and mild cognitive impairment, which often precede motor symptoms. Interestingly, there is also evidence in PD for lower novelty-seeking and high harm avoidance personality traits with anxiety-associated symptoms [43, 137]. These observations suggest that, in the initial disease stage, stress-related alterations in GC-GR activity could impact both the motivation/cognitive-associated dopaminergic as well as nondopaminergic (serotonergic and noradrenergic)

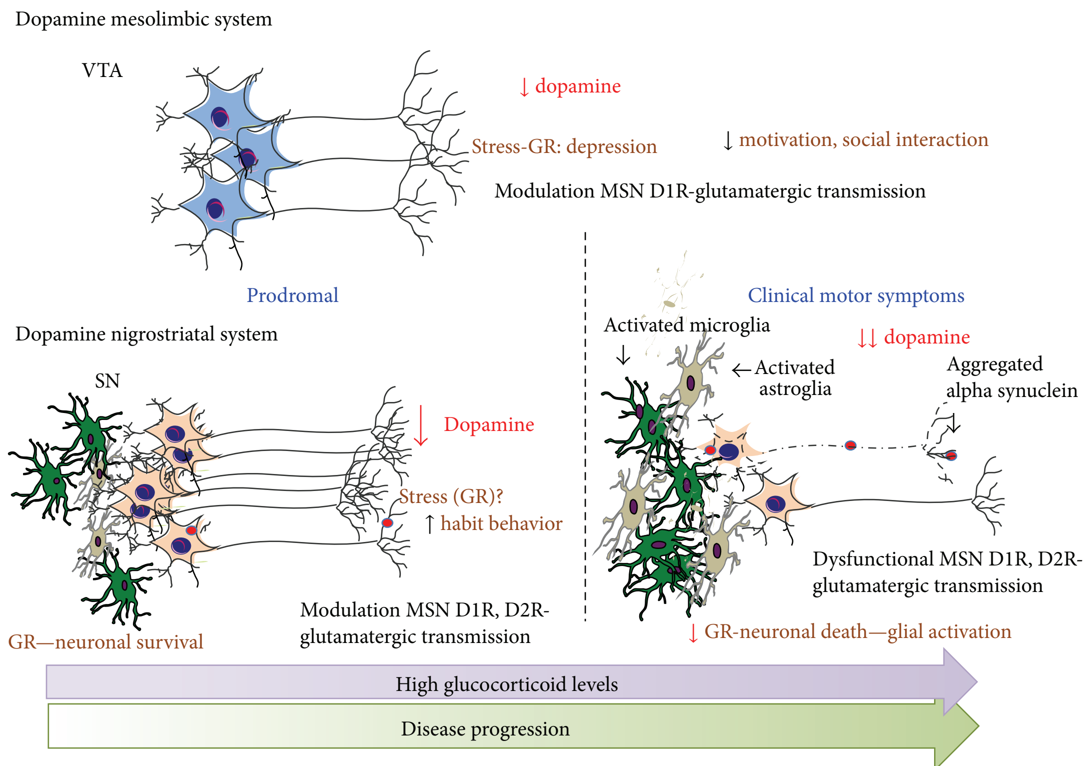

Figure 3: Putative impact of elevated GC levels on GR function in nigrostriatal and mesolimbic dopaminergic systems in PD. Stress-level elevation of GCs may be an early feature of PD, potentially impacting both motor and nonmotor dopaminergic systems. Mesolimbic dopaminergic circuitry is likely affected through structural and functional changes occurring in D1R MSNs. These changes lead to depression and reduced motivation and social interaction which are key prodromal features of PD. Dopaminergic neurons in VTA are relatively spared in PD. In the nigrostriatal system, high levels of GCs initially protect dopaminergic neurons of substantia nigra through dampening the immune responses, namely, mediated by activated microglia and astrocytes. In the putamen, high stress levels of GCs through GR augment habit learning and may act to prevent the appearance of motor symptoms. With disease progression, GR function is affected, leading to chronic glial and immune activation, which exacerbates dopamine neurodegeneration with significant dopamine depletion in the striatum. Changes in GR activity may also affect striatal D1 and D2R MSNs further participating in the appearance of clinical motor symptoms.

neuronal circuitry. This would also implicate dopaminergic neurons in the ventral tegmentum area (VTA), which although relatively spared in PD are well-known to regulate reward and aversion by stress and have been implicated not only in addiction but also depression involving the transcriptional factor CREB and BDNF [138–141]. On the other hand, dorsolateral dopamine neurons in the SN (vulnerable in PD) were shown to respond to tasks involving working memory [142]; thus, their demise could explain, in part, the cognitive deficits observed in PD. Studies on the dopaminergic transmission during stress have revealed the complexity of the system. In fact, firing patterns of dopamine neurons in VTA correlated with depressive-like behaviors in mice, although the effect appears to depend on the stress paradigm used to induce the depressive-like behavior [139, 143]. Electrophysiological evidence implicates changes in both D1R and D2R-medium spiny neurons (MSNs) in the ventral striatum [144], but the depressive-behaviors seems to preferentially affect D1R MSNs [145] (Figure 3). Glutamatergic receptors, NMDA and AMPA receptor functions were shown to be also altered in the D1R MSNs, notably NMDAR-dependent LTD, reduced AMPA/NMDA receptor ratio and increased endocytosis of AMPA receptors [146].

7. Role of Glucocorticoid Receptors in Inflammation-Induced Neurodegenerative Processes and Nonmotor Symptoms in Parkinson's Disease

Accumulating evidence points to inflammation resulting from chronic activation of innate and adaptive immune cells as playing an important role in both neurodegenerative processes and in nonmotor symptoms of PD. Using radiolabeled ligand ^{11}C-PK-11195 for translocator protein, Positron Emission Tomography (PET) studies in PD patients revealed an early activation of microglia in many brain regions including basal ganglia and midbrain [147, 148]. Furthermore, postmortem studies as well as analyses of serum and cerebrospinal fluid from PD showed high levels of proinflammatory mediators such as TNF-α, IL-1β, iNOS, IFN-γ, and COX-2 [149]. In line with observations in PD patients, presence of inflammatory mediators and glial reactivity in striatum and substantia nigra is a key feature in many of the experimental animal models of PD [150]. Evidence from recent genome-wide studies points to involvement of the immune system in the etiology of idiopathic PD. A number of susceptibility loci identified relate to genes

expressed in immune cells such as HLA-DQB1, LRRK2 or BST-1 [151, 152]. In addition, identified PD risk factors [such as age, environmental toxins (e.g., heavy metals or pesticides,) traumatic brain injury, and bacterial or viral infections] activate immune responses in periphery and brain.

7.1. GR Regulation of Inflammation Important for Dopamine Neuronal Survival. Activated microglia functioning as innate-immune competent cells are likely involved in releasing the above inflammatory molecules, thereby inducing dopamine neurodegeneration. Indeed, the important role of these proinflammatory mediators in promoting degeneration of dopaminergic neurons of substantia nigra was demonstrated using mice with specific knockout of these genes [153–156]. Many of the proinflammatory mediators found in PD patients are transcriptional targets of GR. The synthetic analogue of GCs, dexamethasone, was shown to attenuate dopamine neuronal loss by precluding activated microglia from releasing toxic inflammatory molecules [157, 158]. In adrenalectomized mice (lacking endogenous production of GCs), dopamine neuronal loss was augmented following MPTP intoxication indicating that endogenous GCs do play a role in protecting dopamine neurons [159]. Examination of GR in microglia revealed an increase in nuclear localization of GR following MPTP treatment in mice, which coincided with a rise in systemic corticosterone levels, indicating that GR is activated in microglia during the degeneration of dopamine neurons [129]. The unequivocal evidence that GR in microglia normally protects dopamine neurons was provided by experiments with mice in which the GR gene was selectively deleted in microglia/macrophages. MPTP treatment in these mice resulted in increased dopamine neuronal loss as well as increased microglial activation and expression of proinflammatory mediators [129]. Indeed, the absence of GR in microglia resulted in sustained activation of NF-κB as was shown in these microglial GR mutants. The above findings have a significant relevance for PD pathogenesis as nuclear expression of p65 subunit of NF-κB, indicative of transcriptional activity, was found in the substantia nigra microglia of PD postmortem [160].

Inflammatory reaction mediated by immune-competent cells such as microglia is normally a very tightly regulated process of limited duration. It is very likely that the processes involved in the regulation of glial immune responses including the expression and secretion of inflammatory mediators are compromised in PD and also AD resulting in a chronic inflammatory state with sustained activation of glia spanning many years. One likely factor contributing to dysfunction of glial immune responses is aging. Immune-regulatory processes are compromised in aging (immunosenescence) and also during chronic stress [161] where there is an increased susceptibility to infections as well as proinflammatory cytokine production [162]. In aging, microglia show enhanced sensitivity to inflammatory stimuli, a process called "priming" which could be also induced by chronic stress and a dysregulated HPA axis. In this regard, there are several studies showing that chronically elevated GCs levels in response to different stressors cause proinflammatory cytokine production and sensitization or "priming" of microglia. Importantly, subsequent inflammatory or toxic stimuli result in aggravation of neuronal injury [119, 120, 163]. Moreover high and sustained GCs can exacerbate inflammation because of GC resistance whereby GR activity is affected. Thus it is plausible that GR transcriptional activity regulating inflammatory response of microglia is compromised in AD and PD patients who display persistently high GC levels.

7.2. GR, Inflammation and Nonmotor PD Symptoms. Recent experimental evidence shows that glia and peripheral immune cells are activated upon chronic psychogenic stress and that their actions are important in mood and behavior [164–167]. Glial production of potent proinflammatory cytokines such as TNF-α, IL-6, and INF-γ are implicated in depression through stimulation of the kynurenine pathway (shift of serotonin synthesis from tryptophan to kyneurin) in activated astroglia, microglia, and infiltrating peripheral immune cells. Kynurenine, produced from tryptophan by activation of indoleamine 2,3-dioxygenase (IDO), can be further converted to kynurenic acid or quinolinic acid, the latter affecting the function of both monoaminergic and glutamatergic neurons. Quinolinic acid toxicity with increased glutamate release results in lipid peroxidation and nitrative stress [168, 169] Evidence shows that the kynurenic acid/tryptophan ratio is altered in CSF and serum in PD patients [170].

Another means by which glial activation and proinflammatory cytokines promote mood anomalies in PD is through reducing neurogenesis in hippocampal subgranular zone, thus affecting hippocampus-mediated regulation of mood and cognition [171].

8. Conclusion

Clinical and preclinical studies suggest that chronic stress/elevated GC levels may be an etiological factor in the development and progression of both AD and PD pathologies. Growing evidence indicates that the pathological manifestations of chronic stress include neuronal and synaptic atrophy/malfunction as well as immunosuppression, but our understanding of the underpinning mechanisms is still poor and calls for more research not only to identify therapeutic inroads but, also, preventative measures or ways to delay onset of disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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APPENDIX II

GLUCOCORTICOIDS AND NEURODEGENERATION

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Chapter 15

GLUCOCORTICOIDS AND NEURODEGENERATION

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ABSTRACT

Glucocorticoids (GCs) exert wide-spread actions in central nervous system ranging from gene transcription, cellular signaling, modulation of synaptic structure and transmission, glial responses to altered neuronal circuitry and behavior through the activation of two steroid hormone receptors, glucocorticoid receptor (*NR3C1*, GR) and mineralocorticoid receptor (*NR3C2*, MR). These highly-related receptors exert both genomic and non-genomic actions in the brain, which are context-dependent and essential for adaptive responses to stress resulting in modulations of behavior, learning and memory processes. Thus, GCs through their receptors are implicated in neural plasticity as they modulate the dendritic and synaptic structure of neurons as well as the survival and fate of newly-generated cells (neuro- and glio-genesis) in adult brain. GCs are also important in fetal brain programming as inappropriate variations in their levels during critical developmental periods are suggested to be casually related to the development of brain pathologies and maladaptive responses of hypothalamic-pituitary adrenal (HPA) axis to stress during adulthood. They regulate immune responses in brain, which have important consequences for neuronal survival. In situations of chronic stress and HPA axis dysfunction resulting in chronically high or low GCs levels, a multitude of molecular, structural and functional changes occur in the brain, eventually leading to

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maladaptive behavior. In fact, clinical studies suggest a causal relation of deregulated GC responses with development of neurodegenerative disorders such as Alzheimer´s (AD) and Parkinson's (PD) diseases. AD and PD patients have high levels of circulating cortisol while animal studies suggest that this chronic GC elevation participates in neurodegenerative processes in both AD and PD pathologies. This chapter will focus on the role of HPA axis and GCs on neurodegenerative processes involved in AD and PD pathogenesis.

Keywords: glucocorticoids, neurodegeneration, Alzheimer's disease, Parkinson's disease, epigenetics

INTRODUCTION

Glucocorticoid (GC) hormone is synthesized and released into systemic circulation from adrenal glands following activation of hypothalamic-pituitary-adrenal (HPA) axis, which entails synthesis of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) by paraventricular neurons (PVN) of hypothalamus and their release from median eminence into portal blood. These hormones stimulate the synthesis of adrenocorticotropic hormone (ACTH) in the anterior pituitary, which when released into general circulation binds to ACTH receptor (melanocortin type II receptor) in adrenal glands promoting GC synthesis from cholesterol. GC release by HPA axis is under circadian control and occurs in an oscillatory pattern or ultradian rhythm that varies in amplitude according to the time of day (peak in the morning and trough in the evening/night in diurnal animals including humans and vice versa in nocturnal animals, e.g., rodents). In addition, there is a surge of GC release in response to a stress stimulus, which can be either psychogenic (e.g., fear) or physical (e.g., cellular lesion or pathogen invasion). In response to stress, GCs exert critical adaptive functions by modulating most biological processes (e.g., metabolism, cardiovascular and immune systems as well as behavior); and through feedback inhibition of HPA axis they play a role in terminating the stress response as well as facilitating the restoration of physiological homeostasis [1]. In addition to their role in stress response, appropriate GCs levels are important during development, for example in cell maturation, and in the differentiation of lungs, kidneys and brain [2-4].

It is now thoroughly established that GCs have the capacity to profoundly modulate different brain functions, as well, increasing evidence points to their role in brain development. The appreciation that brain is a key target of this circulating adrenal steroid hormone emerged from the pioneering work, principally by the laboratories of McEwen and de Kloet, on identification and biochemical characterization of two receptors in the hippocampus to which GCs bind - the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) [5, 6]. Since then, GR presence in brain was observed to be widespread with every cell type expressing this receptor in contrast to MR expression, which is more restricted. MR is expressed by the neurons of the limbic system, i.e., hippocampus, locus coeruleus, amygdala, prefrontal cortex and nucleus of the solitary tract, as well as neurons of hypothalamus. MR is also present in non-neuronal cells, namely in glia and in epithelial cells of choroid plexus and ependyma [7]. In brain, ^{3}[H] corticosterone binding assays showed that MR has 10-fold higher affinity (K_d= 0.5 nM) for GCs compared to GR (K_d= 5 nM), which

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means that at basal GC levels, MR is occupied and activated [8] whereas GR is only activated when GC levels reach a certain level as it happens in circadian peak and during stress [9]. GC actions are pleiotropic, the principle factors determining their functions are: a) circulating levels with accessibility to each cell type and b) context in which the receptors are activated. GC levels are tightly regulated at each level of HPA axis and this is important in ensuring that stress response is correctly executed. Deregulated HPA axis resulting in sustained high or low GC levels are implicated in different diseases, for example disorders of metabolism (e.g., diabetes, obesity), immune (e.g., rheumatoid arthritis) and nervous systems (e.g., depression) [10-12].

Synthetic GCs (e.g., dexamethasone, methylprednisolone) are routinely used in clinical situations, particularly in disorders with an inflammatory component such as rheumatoid arthritis or brain edema as they exert powerful anti-inflammatory and immunosuppressive actions. However, prolonged GC use suppresses HPA axis resulting in harmful side effects such as increased risk of infection, hyperglycemia, weight gain, behavioral or cognitive problems. Interestingly, GCs are now also used clinically in neonates, as endogenous GCs are required for fetal lung maturation as they promote the production of lung surfactant. This could affect the programming or subsequent responsiveness of HPA axis particularly with regards to stress responses in adults [13]. Thus prolonged GC exposure or exposure to high levels of GC in specific developmental windows such as the prenatal and perinatal period can impair the HPA axis negative feedback, increasing the propensity for developing neuropsychiatric and metabolic disorders [14].

Glucocorticoid actions through MR and GR in brain have been particularly studied in relation to glutamatergic as well as monoaminergic (e.g., dopaminergic and serotonergic) systems, which have wide-range consequences from mood behaviors to cognition. Several excellent reviews already exist on our current understanding of neuronal functions of GCsin brain via these two receptors [15-19]. Our aim in this chapter is to describe how their actions in neurons and glia impact the neurodegenerative processes, emphasizing on Alzheimer (AD) and Parkinson diseases (PD). One of the arguments for their implication relates to GC functions being exquisitely dependent on environmental changes, and in this regard, both genetic susceptibility and environmental factors are believed to play key roles in the etiology of these neurodegenerative diseases. Most of our current understanding of GCs involvement in brain disorders relates to the functions of GR as this receptor plays a major role in stress responses. Thus, before describing our current knowledge of GCs in neurodegeneration, we reiterate the regulation of GC release by HPA axis and functional activity of GR as both are likely affected in AD and PD as discussed below.

REGULATION OF GC RELEASE AND AVAILABILITY

Paraventricular nucleus (PVN) of hypothalamus receives integrated information from suprachiasmatic nucleus for circadian control of GCs and from the limbic system for psychogenic stress-induced GC release [20, 21]. In stress-induced GC release, limbic structures such as amygdala are involved in stimulating PVN neurons to synthesize CRH whilst hippocampus plays a crucial role in negative feedback inhibition of the HPA axis [22]. The fast feedback inhibition of HPA axis following acute stress is important to prevent

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depletion of GC needed for both successive stress and ultradian release, which interestingly is impaired in aging as well as in patients suffering from depression. Both MR and GR at hypothalamic and hippocampal levels play an important role in regulating the activity of PVN neurons. In addition GR in anterior pituitary was found to regulate pulsatile ACTH release [23]. HPA axis is also activated in response to cellular lesion or pathogen invasion by pro-inflammatory cytokines such as IL-1β, IL-6 or TNF-α released by either peripheral immune cells or microglia [24]. IL-6 through activation of its receptor can also stimulate ACTH release from anterior pituitary and GC from adrenal glands [25].

The availability of GCs to neurons and non-neuronal cells in brain is controlled in two ways. Firstly, in the blood, most GCc are bound to corticosteroid binding globulin (CBG) whose levels are down regulated by stress thereby increasing free-circulating GC levels [26]. Secondly, once inside the cells, the availability of GC for GR activation is controlled by GC-metabolizing enzymes: 11-β-hydroxysteroid dehydrogenase type I (HSD11β1), which regenerates active glucocorticoids (e.g., cortisol from cortisone) thus amplifying GR activation. In addition, 11-β-hydroxysteroid dehydrogenase type II (HSD11β2) has an opposite function, i.e., increasing the inactive form of GC. Using mice deficient for HSD11β1, previous studies have shown that these mice are protected from hippocampal memory impairments associated with aging. However, cognitive problems arise normally because GR activity predominates due to high GC levels catalyzed by this enzyme [27, 28].

GENOMIC AND NON-GENOMIC ACTIONS OF GLUCOCORTICOID RECEPTOR (GR)

GR exerts both genomic and non-genomic actions in brain. The genomic actions of GR pertain to its ligand-activated transcriptional activity. Non-liganded GR in the cytoplasm is normally in complex with chaperone proteins such as heat shock proteins 90, 70, 40, 23 as well as immunophilins such as FKBP51 and 52. Upon GC binding, the conformational change of the complex results in exposure of nuclear localization signal of GR, which allows importin-mediated translocation of GR into the nucleus. Recent studies highlight the importance of correlation between GR transcriptional activity and ultradian pulsatile nature of GCs for generation of appropriate response to stress stimulus [29, 30].

GR protein is comprised of N-terminal transactivation domain which is important site for GR co-regulatory binding proteins such as cAMP-response-element binding protein binding protein (CBP), it also contains phosphorylation sites, e.g., serine 203, serine221 and serine 226. The central zinc-finger DNA-binding domain is important for the GR binding to the so-called Glucocorticoid Response Elements (GREs), which are present in promoters of GR target genes. The carboxy-terminal domain is the site of GC binding to GR as well as co-activators such as histone acetylases or co-repressors. The transcriptional regulation by GR is both cell-type and context-dependent. GR can regulate transcription by: a) direct binding as homo-dimers to GRE DNA sequences to stimulate transcription, e.g., *mitogen-activated protein kinase phosphatase-1* gene; b) direct binding to negative GRE elements to repress transcription, e.g., *CRH* or *ACTH receptor* genes; c) trans-repression or "tethering" i.e., association with other transcriptional factors to inhibit their transcriptional activity. This mechanism is by far the most notable in immune cells where GR regulates transcription of

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nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1) and members of interferon regulatory transcription factors (IRFs). In brain, identification of GR-modulated genes is difficult due to anatomical complexity and cellular heterogeneity. Nevertheless, transcriptomic studies in the hippocampus have identified functional classes of genes modulated by GR which include genes coding for neurotransmitter catabolism, neurotrophic factors and their receptors, signal transduction, energy metabolism and cell adhesion [31].

The genomic actions of GR are slow in onset and long lasting. In contrast, GR exerts non-genomic actions at plasma membrane of neurons, which are rapid (seconds to minutes), involve alterations in neuronal excitability and are dependent on the context of the signal. The non-genomic actions of GR at the membranes also involve activation of down-stream signaling pathways involving kinases such as ERK, AKT, PKC and PKA [32]. Altogether, this provides a surprising diversity and complexity of GC modulation of gene expression and cellular signaling.

EPIGENETIC REGULATION OF GR

Animal studies confirm earlier anecdotal observations in humans indicating that early life adverse experience has a profound impact on adult behavior. Early life stress or exposure to GC (endogenous or exogenous) may induce neuroendocrine programming, subsequently altering offspring's growth, metabolism, immune system and even the stress response as previously mentioned. These observations derive from both animal and human studies, where an alteration in the activity of the HPA axis was found [14, 33, 34]. Such prenatal *programing* may be an evolutionary mode of shaping internal characteristics of the developing organisms in order to adapt to the environment. However, such modifications might ultimately result in the development of long-term diseases, from metabolic syndromes to psychiatric disorders [35-39].

This long-lasting effect of early life experiences in brain function and behavior appears to be mediated (at least partially) by epigenetic mechanisms [14, 34, 40]. In the last years, considerable progress has been made in untangling the epigenetic alterations induced by stress/GC. However, most of the studies are merely correlative and the mechanism through which stress/GC induce epigenetic programing remains completely unkown.

One way of buffering the impact of maternal GC exposure in the developing fetus is by converting cortisol/corticosterone into inactive metabolites through the action of placental HSD11β2. However, some studies indicate that maternal adversity can increase the methylation at specific CpG sites within the HSD11β2 gene promoter and lead to a down-regulation of this enzyme [41, 42], which may allow excessive levels of GC to reach the fetus and program different organs and systems. The first evidence of brain epigenetic programing induced by early life adversity was reported by Meaney and colleagues, which showed that natural variations in maternal behavior were correlated with DNA methylation levels of a neuron-specific exon 1_7 promoter of the GR gene.

Briefly, male rats reared by “good dams” (i.e., those that presented high pup licking and grooming) demonstrated lower levels of stress response, greater performance on cognitive tasks and larger exploratory activity in a novel environment, compared to the offspring of

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"bad dams"; this was associated with a differential methylation of this specific region of the GR promoter [43, 44]. Importantly, these results were later replicated in humans showing individuals with childhood stressful experiences (abuse during childhood), presented hypermethylation of this region, in comparison to non-abused individuals [45].

Later studies revealed an increased methylation of a CpG-rich region in the promoter and exon1F of the GR gene in the cord blood of newborns of mothers with depressed mood during the third trimester of gestation [46]. Importantly, this pattern on methylation of the GR gene occurred only in the offspring (and not the mothers), correlated with levels of response to stress in infants at 3 months of age, and persisted beyond infancy. Similarly, pregnancy-related anxiety is associated with the methylation state of the GR gene in the child [47]. These findings suggest a common effect of parental care in both rodents and humans on the epigenetic regulation of hippocampal GR expression. One question that still remains is whether these epigenetic changes are the cause of maladaptive behaviors or a mere adaptation, in the light of evidence showing that healthy individuals with a history of childhood adversity can also present increased GR methylation and an attenuated cortisol response to the dexamethasone test [48]. In this perspective, such adversity-induced epigenetic changes may predispose the individual to disease (in combination with other genetic or extrinsic factors) but are not the cause *per se*.

In addition, other pivotal stress players are also affected by early life stress/GC exposure. For example, mice, in a model of early-life stress present hypersecretion of corticosterone, alterations in passive stress coping and memory followed by a persistent increase in arginine vasopressin expression in neurons of the hypothalamic PVN due to sustained DNA hypomethylation of CpG residues that serve as DNA-binding sites for the methyl CpG-binding protein 2 (MeCP2) [49]. In addition, stress/GC exposure early in life may induce long-lasting epigenetic changes in neurotransmission-related genes. For example, animal studies demonstrated that prenatal GC exposure leads to differential methylation of dopamine receptor D2 [50]. In humans, depressed mood during pregnancy leads to decreased levels of methylation in the promoter of the *SLC6A4* gene, encoding the serotonin transporter, in maternal peripheral leukocytes and in umbilical cord leukocytes collected from their infants at birth [51]. Such changes may affect how the individual senses/processes/responds to environmental stimuli and may explain, in part, the increased vulnerability for neuropsychiatric disorders later in life.

In addition to particular gene epigenetic changes, stress/GC have a strong impact in the epigenome (elegantly reviewed in [52]. Human studies on different cohorts have shown that early life maltreatment induces long-lasting methylation changes in the genome [53-55] while recent animal-based evidence suggest that the epigenomic landscape is also strongly correlated with gestational maternal adversity [56] and even with natural variations in maternal care [57]. In addition to methylation, gene expression can be further controlled by hydroxymethylation and diverse histone modifications, adding additional layers of complexity to the GC-driven changes that may predispose individuals to the development of brain pathologies.

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SUSTAINED GR ACTIVATION AND NEURODEGENERATION IN AD

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by slow and progressive dementia while the major histopathological hallmarks are senile plaques containing amyloid beta (Aβ) deposits and intracellular neurofibrillary tangles (NFT) made of hyperphosphorylated forms of the cytoskeletal protein Tau [58-60]. Aβ is the proteolytic product of the bigger transmembrane protein called amyloid precursor protein (APP), which is sequentially cleaved by β-secretase (BACE-1) and γ-secretase (enzymatic complex of proteins) resulting in the production of Aβ; this cellular pathway is often called APP misprocessing. Many studies have demonstrated that APP misprocessing and Aβ trigger AD neuropathological processes such as synaptic malfunction (impairing mechanisms of synaptic plasticity, e.g., LTP), neuronal atrophy and synaptic loss as well as mitochondrial dysfunction, oxidative stress and glial activation.

While still debated, it is suggested that Aβ also triggers abnormal Tau hyperphosphorylation leading to the formation of NFTs and neuronal loss in AD brain. Indeed, accumulating data suggest the involvement of Tau protein in the detrimental effects of Aβ as use of Tau-KO blocked the Aβ neurotoxic effects [61-64]. Further support of the essential role of Tau in the establishment of AD pathology is based on the clinical findings that have consistently shown that the cognitive deficits in AD patients correlate with NFTs rather with Aβ deposition. Indeed, hyperphosphorylated and aggregated Tau resulting in NFTs is associated with neuronal loss. Gomez-Isla et al. [65] demonstrated that strong correlation of neuronal loss in cerebral cortex and increased NFT burden with disease progression; no such correlation was found with Aβ. Furthermore, reduction of hippocampal volume in AD patients was associated with phosphorylated Tau, but not Aβ levels in cerebral spinal fluid (CSF) [66].

Several risk factors have been suggested for AD while recent evidence supports an etiopathogenic role of chronic stress and glucocorticoid hormones in the establishment and development of AD pathology [67, 68]. Clinical studies report high cortisol levels, measured in plasma, saliva or CSF, of AD patients indicative of altered HPA axis [69-73] while the increase of cortisol levels is negatively associated with memory scores in AD patients [74, 75]. Furthermore, Hartman et al. [76] monitored the 24hr secretory pattern of plasma cortisol in AD patients finding a higher mass of cortisol release; however, the diurnal changes in cortisol levels were not altered. Since chronic elevation of GC levels is known to impair memory and cognitive performance, it is speculated that GCs play a role in progressive cognitive decline in AD. Indeed, it is unclear whether high GCs are a cause or a consequence of the disease as one of the explanations of high GC levels in AD patients is the deregulation of feedback inhibition of the HPA axis, particularly in relation to psychogenic stressors, occurring at the level of the hippocampus, a region significantly damaged in AD brains.

It is noteworthy that many clinical and experimental reports suggest a reduction of adult neurogenesis in AD hippocampus while the same is true for chronic stress conditions [77-79]. Reduction of hippocampal adult neurogenesis was shown to increase HPA activity implying that this region is involved in hippocampal feedback regulation of HPA axis during stress [80]. Thus, high GCs can aggravate hippocampal memory processes in AD by having a

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negative effect on hippocampal neurogenesis, which may, in turn, contribute to maintenance of deregulated HPA axis.

GC Impact on AD Neurodegenerative Mechanisms

Clinical studies show that chronic stress is a risk factor in AD pathogenesis and it also lowers the age of onset of the familial form of AD [67, 68]. Indeed, it has been evoked that chronic stress is among the principal factors that contributes to development of AD [77]. A principal target of GCs is hippocampus, which is a main target area for AD pathology and chronic stress (Figure 1). The hippocampal dysfunction in AD has significant detrimental consequences on declarative, spatial and contextual memory processes. As hippocampal neurons have very strong GR expression and are intimately involved in regulation of HPA axis, there has been a great deal of interest in how high cortisol levels and stress impact the deterioration of hippocampal functions caused by toxic Aβ and Tau hyperphosphorylation in AD.

Previous studies show that elevated GC levels and exposure to chronic stress increase Aβ production in AD transgenic mouse models exacerbating their memory deficits [81, 82], Specifically, chronic immobilization stress in amyloid precursor protein (APP)V717ICT-100 transgenic mice (this APP mutation is known for aggressive early onset AD) evoked acceleration and greater severity of memory deficits and increased extracellular Aβ deposits. Similarly, Green at al [81] showed that prolonged treatment with the synthetic GC, dexamethasone, triggers APP misprocessing resulting in increased Aβ levels using both *in vitro* and *in vivo* approaches (neuronal N2A cell line and pre-pathological 3xTg-AD young mice). In addition, the same study also demonstrated transcriptional up-regulation of APP and β-secretase expression by GR (both contain GRE in their promoter region).

Similarly, other *in vitro* studies have confirmed that GCs trigger APP misprocessing without influencing the non-amyloidogenic pathway, i.e., the other cellular cascade of APP cleavage/processing [83]. Similar observations were made in middle-aged rats in which the amyloidogenic potential of chronic stress (chronic unpredictable stress paradigm) and prolonged GC treatment was demonstrated insofar that both treatments were found to drive APP processing towards the generation of Aβ and its precursor molecule (C99), both of which have neurotoxic and cognition-impairing properties [84]. This study also showed that GC/stress increased β-secretase (BACE-1) levels as well members of γ-secretase complex (Nicastrin). Given that stressful stimuli occur intermittently over the lifetime, and that their effects may be cumulative, an important finding by Catania et al., [84] was that GC potentiate the APP misprocessing pathway in previously stressed animals of AD model (Aβ-infused rats).

Interestingly, clinical studies suggested that the stress-related neuropsychiatric disorder, depression, is a risk factor for the development of AD pathology as the history of depression is correlated with increases of amyloid plaques and NFT [85]. In addition, other studies suggested the utility of measurements of the various APP cleavage products as biomarkers to discriminate between subjects undergoing normal aging from those suffering from depression or AD [86-89]. Interestingly, more recently, some studies report the influence of anti-depressant drugs on the proteolytic cleavage of APP suggesting its anti-amyloidogenic role

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[89, 90] while many antidepressants are shown to normalize the HPA axis and the resulting GC levels which are increased in many depressed patients and models of stress-driven depression.

Besides APP misprocessing, high levels of GC trigger the other main AD neurodegenerative pathway, the aberrant hyperphosphorylation of Tau protein. Among the first reports suggesting a potential connection between GC and Tau was the study by Stein-Behrens et al. which demostrated high GC levels exacerbated neuronal loss induced by kainic acid injection in hippocampus while in parallel increased Tau immunoreactivity. Later on, it was shown that treatment with synthetic dexamethasone for 7 days in 3xTg AD mouse model resulted in Tau accumulation in somatodendritic compartment of neurons in hippocampus, amygdala and cortex [81].

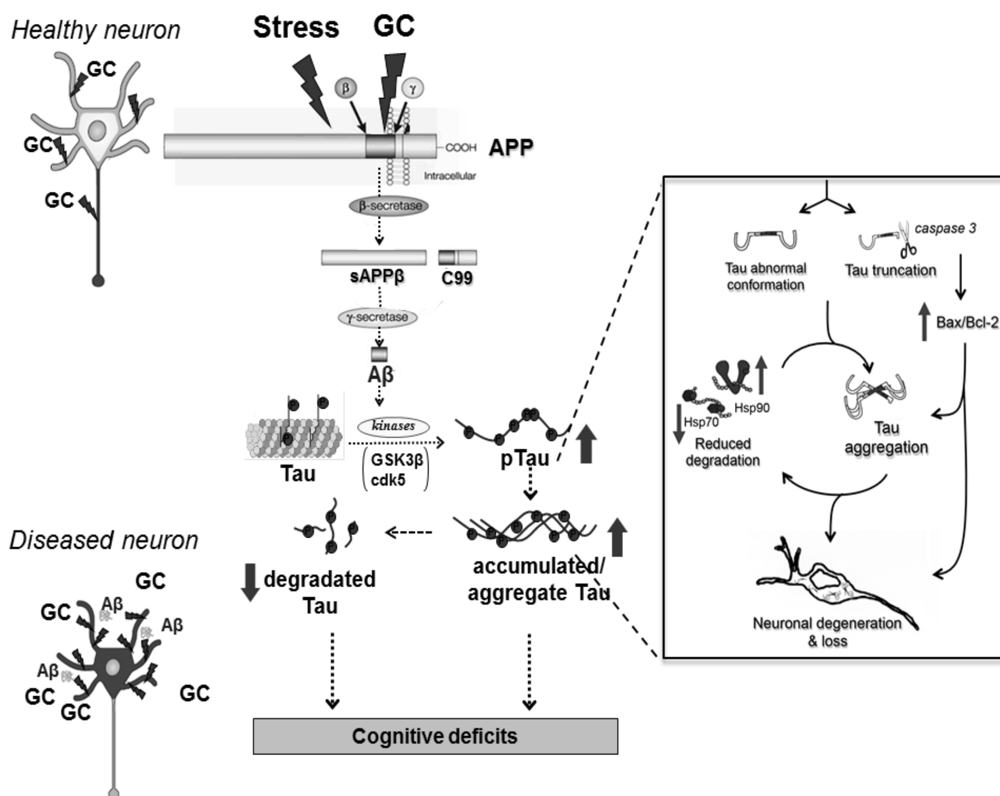

Figure 1. Glucocorticoids (GCs) and Stress impact on AD neurodegenerative mechanisms. The schematic presentation reflects the triggering role of high GC levels and chronic stress on AD cellular mechanisms based on experimental evidence using animal and cellular AD models. Prolong exposure to GC and/or stress activates amyloidogenic cellular pathway resulting in the sequential cleavage of APP by β- and γ-secretase which produces Aβ. Next, the cytoskeletal protein Tau, mainly found at neuronal axon (rdown (dark) part in the healthy neuron scheme), is aberrantly hypersphosphorylated through the activation of different kinases (e.g., GSK3-β and cdk5) which results in Tau somatodendritic accumulation (upper (dark) part in in diseased neuron scheme). In addition, abnormal conformation and caspase 3-mediated truncation of Tau occurs together with a parallel dysregulation of the molecular chaperones (e.g., Hsp90 and Hsp70) facilitating reduced Tau degradation and increased Tau oligomerization and ultimately, aggregation (see panel on the right). The above cellular cascades result in neuronal atrophy and loss leading to the establishment of cognitive impairment.

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In addition, Sotiropoulos et al., [91] showed that chronic stress or GC treatment triggers Tau hyperphosphorylation in different epitopes implicated in cytoskeletal pathology and synaptic loss in AD patients (e.g., pSer262) [92, 93]; note that these epitopes are correlated with hippocampal atrophy in AD patients (e.g., pThr231) [94]. Indeed, clinical studies report a strong correlation between the extent of Tau hyperphosphorylation (e.g., Thr231 and Ser262 residues) and severity of impairments of memory, speed of mental processing, and executive functions [95-97].. Futhermore Tau hyperphosphorylation is associated with synaptic loss and memory impairment in experimental animals [98] that could be also related with the stress-induced synaptic and memory loss.

Albeit specific Tau phosphoepitopes maybe differentially regulated by chronic stress and prolonged GC treatment, the overall *in vitro* and *in vivo* evidence [83] clearly implicates GCs as a key mediator of the cellular response to stress. Nevertheless, other studies have also suggested the contribution of other stress-related molecules, e.g., corticotrophin-releasing hormone [99, 100]. Furthermore, *in vitro* studies suggest the mediation of glycogen synthase kinase 3 (GSK3) or CDK5 in the above GC- and stress-triggered Tau hyperphosphorylation, both known to lead to microtubule disruption as well as formation of NFTs [83]. In parallel, GC were also shown to increased Tau accumulation by affecting turnover of the protein [83], which may involve reduced degradation through dysregulation of molecular chaperones responsible for Tau proteostasis (e.g., Hsp90, Hsp70 [101]). Interestingly, Hsp90 and Hsp70 serve to maintain the glucocorticoid receptor (GR) in a high affinity state (as previously discussed) and thus, offering a clear cross-point between GC/GR cellular signaling and Tau degradation machinery. This reduced degradation could facilitate the increased aggregation of Tau into insoluble forms triggered by stress in P301L-Tau Tg mice [mice expressing human Tau carrying the most common Tau mutation (P301L-Tau)]. In addition, chronic stress also promotes C-terminal truncation of Tau by caspase-3 and, abnormal conformation of Tau in the hippocampus of the same animals. Indeed, both truncation and abnormal conformation of Tau precede its aggregation and formation of neurofibrillary tangles [99, 102, 103] thus serving as early markers of disease. The Tau-C3 species have been suggested to contribute to misfolding of Tau into a conformation that can nucleate and recruit other Tau molecules into aggregates [99, 103, 104], which are shown to be neurotoxic and related to neuronal loss [105].

GLUCOCORTICOID ROLE IN THE ONSET AND PROGRESSION OF PARKINSON'S DISEASE

Parkinson's disease (PD), the most common neurodegenerative movement disorder, is characterized by preferential loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and dopamine depletion in striatum that underlies the appearance of motor symptoms such as akinesia, resting tremor, rigidity and postural instability. The main histopathological characteristic in PD brain is Lewy bodies (LBs), which are proteinaceous inclusions containing the presynaptic protein, alpha-synuclein, and are found in many different brain regions far beyond SN and striatum; e.g., cerebral cortex, limbic system, hypothalamus as well as the autonomic nervous system that are also affected in PD brain [106-108]. Thus, in addition to motor symptoms due to SN and striatum neurodegeneration

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and lessions, PD patients with cortical LBs also suffer from dementia and visual hallucinations [109].

While several gene mutations have been identified in the familial forms of PD, the majority of PD cases are sporadic with unknown etiology. Different cellular mechanisms have been suggested to be involved in PD neurodegeneration and dopaminergic neuronal loss such as oxidative and nitrative stress, mitochondrial dysfunction and deregulated intracellular calcium levels, damaged proteostasis related to alpha-synuclein aggregation [110]. Like in AD, deregulated HPA activity is also reported in PD patients. Specifically, previous studies [76, 111-113] including our work [114] show that plasma cortisol levels are significantly higher in idiopathic PD patients compared to control subjects; however the cortisol levels are not related to disease duration or to L-3,4-dihydroxyphenylalanine (L-DOPA) treatment. Interestingly, the diurnal mode of cortisol secretion in PD patients, in particular the normally quiescent nocturnal cortisol secretory pattern, is affected [76].

Furthermore, monoaminergic neurotransmission in hypothalamus, the first compartment of HPA axis, is also affected in PD patients who exhibit reduced levels of dopamine, serotonin and noradrenaline in this brain area [115, 116] followed by reduced density of dopamine receptors [117]. Notably, this reduction was not altered by dopamine medication, which is often used in PD patients. Future studies are necessary to clarify whether the deregulation of HPA axis in PD patients is situated at the hypothalamic and/or the adrenal level as Lewy body pathology is observed in both regions.

THE NEURODEGENERATIVE POTENTIAL OF GC IN PD PATHOLOGY

The deregulated HPA axis and the subsequent elevated GC levels in PD patients reflects the role of stress which was suggested as one of the earliest proposed causes of PD. Although it may not be a major etiological factor, there are clinical reports showing that chronic stress triggers the appearance of PD symptoms or exacerbates the motor symptoms [118, 119]. Furthermore, experimental studies demonstrate that stressors such as food deprivation or tailshock aggravate motor deficits in the 6-hydroxydopamine (6-OHDA) PD model (6-hydroxydopamine local injections lesions the nigrostriatal pathway) [120]. Using the same model, Smith et al. [118] showed that chronic stress exposure (restraint) before the 6-OHDA injection worsened the 6-OHDA-driven motor deficits, aggravated the neurodegeneration of nigrostriatal system and completely blocked compensatory recovery of motor tasks.

How does high stress level of GC-GR exacerbate motor impairments following nigrostriatal lesions? GCs are known to profoundly shape the dopaminergic neurotransmitter system, exerting differential or heterogeneous effects depending on whether the dopaminergic projections arise from the ventral tegmentum area (VTA) or the SNpc. While plethora of studies have monitored the impact of GC on the limbic arm of dopamine neuronal circuitry related to behavioral changes as well as neuropsychiatric diseases, our knowledge about the exact GC influence on motor-related dopamine neuronal networks is very limited. There is lack of evidence about the impact of chronic GC elevation on nigral and striatal neurons or glia and how this contributes to nigrostriatal degeneration and motor impairments. Analysis of GR in PD brain revealed that global GR levels were lower in SNpc and higher in putamen

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compared to control subjects and these results were recapitulated in MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine)-treated mice [114].

However, the cell types in which GR changes occur have not been identified. Interestingly, high GR levels in putamen of PD patients raises the possibility that dopaminergic nerve terminal degeneration induces upregulation of GR in striatal neurons and/or glia. In a study by Barrot et al. [121], GCs in SNpc or in dorsolateral striatum were found not to modify either tyrosine hydroxylase levels or dopamine transporter activity. On the contrary, adrenalectomy and the subsequent loss of corticosterone resulted in reduced D1 dopamine receptor in dorsolateral striatum suggesting that neurons expressing dopamine receptors may represent a target of GC-GR actions for basal ganglion regulation of movement. While the molecular mechanisms by which high GC through GR activity exacerbate motor deficits are not well understood, it is possible that they alter glutamatergic synapses in striatum that are under dopamine regulation.

ROLE OF GLUCOCORTICOID RECEPTOR IN REGULATION OF INFLAMMATION IN PARKINSON'S DISEASE

Chronic inflammation mediated principally by activated microglia, astrocytes and infiltrating T cells is a major neuropathological characteristic of PD. Evidence from recent genome-wide studies point to involvement of the immune system in the etiology of idiopathic PD. A number of susceptibility loci identified relate to genes expressed in immune cells such as HLA-DQB1, LRRK2 or BST-1 [122, 123]. In addition, identified PD risk factors [such as age, environmental toxins (e.g., heavy metals or pesticides,) traumatic brain injury, bacterial or viral infections] activate immune responses in periphery and brain.

Using radiolabelled ligand ^{11}C-PK-11195 for translocator protein, Positron Emission Tomography (PET) studies in PD patients revealed an early activation of microglia in many brain regions including basal ganglia and substantia nigra [124, 125]. Furthermore, post-mortem studies as well as analyses of serum and cerebrospinal fluid from PD showed high levels of pro-inflammatory mediators such as TNF-α, IL-1β, iNOS, IFN-γ and COX-2 [126]. In line with observations in PD patients, presence of inflammatory mediators and glial reactivity in striatum and substantia nigra is a key feature in many of the experimental animal models of PD. For example, treatment of mice or monkeys with neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which selectively induces degeneration of nigrostriatal pathway, 6-hydroxydopamine lesion of nigro-striatal pathway in rodents or toxicity induced by alpha-synuclein injection [126, 127].

Among all the brain regions, substantia nigra has one of highest density of microglia. Activated microglia functioning as innate-immune competent cells are likely involved in releasing the above inflammatory molecules, thereby inducing dopamine neurodegeneration. Indeed the important role of these pro-inflammatory mediators in promoting degeneration of dopaminergic neurons of substantia nigra was demonstrated using mice with specific knockout of these genes [128-131]. Many of the pro-inflammatory mediators found in PD patients are transcriptional targets of GR. The synthetic analogue of GCs, dexamethasone, was shown to attenuate dopamine neuronal loss by precluding activated microglia from releasing toxic inflammatory molecules [132, 133]. In adrenalectomized mice (lacking

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endogenous production of GCs), dopamine neuronal loss was augmented following MPTP intoxication indicating that endogenous GCs do play a role in protecting dopamine neurons [134] Examination of GR in microglia revealed an increase in nuclear localization of GR following MPTP treatment in mice, which coincided with rise in systemic corticosterone levels indicating that GR is activated in microglia during degeneration of dopamine neurons [114]. The unequivocal evidence that GR in microglia normally protects dopamine neurons appeared in a study using mice in which GR gene is deleted in microglia/macrophages. MPTP treatment in these mice resulted in increased dopamine neuronal loss as well as increased microglial activation and expression of pro-inflammatory mediators [114]. Indeed, the absence of GR in microglia resulted in sustained activation of NF-κB as was shown in these microglial GR mutants. The above finding has a significant relevance for PD pathogenesis as nuclear expression of p65 subunit of NF-κB, indicative of transcriptional activity, was found in substantia nigra microglia of PD post-mortem [135].

Chronic inflammation and sustained activation of glia in PD suggests that processes involved in regulation of glial activation and expression/secretion of inflammatory mediators are likely compromised. Chronic inflammation, an important component of pathology in neurodegenerative diseases, is suggested to be a maladaptive response of homeostasis as successful inflammatory response has a resolution phase which is an active process that enables restoration of homeostatic set points [136, 137]. Inflammation mediated by immune-competent cells including microglia is normally a very tightly regulated process. The immune-regulatory processes are affected in aging leading to increased susceptibility to infections and immune activation. Thus in aging, microglia show enhanced sensitivity to inflammatory stimuli - a process called “priming” which could be also induced by chronic stress and deregulated HPA axis. In this regard, there are several studies showing that chronically elevated GC levels in response to different stressors cause pro-inflammatory cytokine production and sensitization or “priming” of microglia. Importantly, subsequent inflammatory or toxic stimulus results in aggravatation of neuronal injury [138-140]. Aging is associated with chronically high GC levels and immuno-senescence exemplified by a sustained low production of pro-inflammatory molecules [141]. Thus, in contrast to their well-known anti-inflammatory actions, in fact high and sustained GCs can exacerbate inflammation. However, it is currently not known whether GR transcriptional activity regulating inflammatory response of microglia is compromised in AD and PD pathological conditions where deregulated HPA axis and sustained high GC levels of are found.

GC-DRIVEN BRAIN PROGRAMMING AND NEURODEGENERATIVE PATHOLOGIES

Although Alzheimer’s disease (AD) is often seen as an age-related neurodegenerative disorder, recent evidence suggests that early life events may play a role in the onset of the disorder (Borenstein, A.R.; Early-life risk factors for Alzheimer disease. Alzheimer Dis. Assoc. Disord., 2006). In this perspective, AD is probably not determined by a single etiologic factor, but results from the interplay between genetic and environmental factors throughout life, being a possible explanation why monozygous twins can be discordant for AD.

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Albeit there is still controversy and the literature is sparse, it has been suggested that early life adverse events such as maternal stress, intrauterine infections, poor maternal and perinatal nutrition can potentially predispose to AD eventually by epigenetic programing of specific genes/pathways related to AD neurodegeneration. For example, early-life lead exposure of older rats and primates induces overexpression of the amyloid precursor protein and its amyloid beta (Aβ) product, both characteristically found in AD brain as will be discussed later in this chapter. One interesting finding was that cognitive impairment was only observed in mice exposed to lead [142], highlighting the relevance of the "window of opportunity" for some environmental factors to trigger the disease.

Similarly, Tau hyperphosphorylation and accumulation, the other main histopathological characteristic of AD pathology) was elevated in both aging rodents and primates previously exposed to lead at younger age, [143] suggesting the potential impact of early-life stress exposure to the precipitation of AD neurodegeneration later in life. Interestingly, a recent study has also highlighted the GC-related epigenetic drive in the establishment of AD pathology in the brain of CK-p25 AD mouse model (exhibiting Tau pathology). These Tg mice exhibit increased levels of HDAC2 associated with cognitive impairment, which seems to be mediated through glucocorticoid receptor induced HDAC2 transcription [144].

Furthermore, the role of early life stressful events in the etiopathogenesis of another neurodegenerative disorder, Parkinson's disease (PD) has emerged in the last years. In an interesting study, pups of female animals exposed to lipopolysaccharide (LPS), a bacterial endotoxin, during pregnancy, showed loss of dopaminergic neurons. This suggests that high LPS levels in mothers might interfere with the dopaminergic neurons in the fetus enhancing the susceptibility to PD [145].

Accordingly, different stressful stimuli could act cumulatively with the developmental stress exposure representing the first imprint in the developing brain, determining the PD phenotype characterized at the pathological level by a deficient substantia nigra with a low burden of DA neurons at birth corresponding to a limited nigro-striatal neurochemical reserve [146]. The low number of DA neurons in the substantia nigra reflecting the developmental damage may remain subclinical during life. Thus, later exposure to the same or other DA neuron-targeted toxicants might attack the few residual neurons leading to insurgence of PD.

CONCLUSION

Accumulating evidence suggests the neurodegenerative potential of chronic stress and elevated GC levels in triggering clinical symptoms and participating in neuropathological mechanisms and processes in AD and PD, two devastating age-related neurodegenerative disorders. High circulating GC (cortisol) levels and deregulated HPA axis observed in patients of both disorders imply that GR activity in the affected regions is most likely compromised but the cause-consequence interrelationship between elevated GC levels and development of neurodegenerative pathology remains unclear. While the ramifications of prolonged exposure to GC stress are many, being causally implicated in immunosuppression, metabolic syndrome, diabetes and others, our current understanding of the exact actions of GC on these neurodegenerative diseases, although limited, opens a window of opportunities to identify the various parameters that contributes to stress/GC-driven brain pathology. As

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both context and cell type determine GR functions, future works using, e.g., cell-specific mouse models of GR activation/inactivation should shed light on their roles in pathological brain aging and onset of neurodegenerative disorders such as AD and PD.

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