Universidade do Minho
Escola de Ciências da Saúde

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Stress-triggered synaptic malfunction: a gate along the path from depression to dementia

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Trabalho efetuado sob a orientação do
Doutor Ioannis Sotiropoulos
e coorientação do
Professor Doutor Nuno Sousa

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DECLARAÇÃO DE INTEGRIDADE

Declaro ter atuado com integridade na elaboração da presente tese. Confirmo que em todo o trabalho conducente à sua elaboração não recorri à prática de plágio ou a qualquer forma de falsificação de resultados.

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Universidade do Minho, 28 de Outubro de 2015

Nome completo: Ana Sofia Fernandes Lopes

Assinatura: Ana Sofia Fernandes Lopes

À minha família

Posso ter defeitos, viver ansioso e ficar irritado algumas vezes,
Mas não esqueço de que minha vida
É a maior empresa do mundo...
E que posso evitar que ela vá à falência.
Ser feliz é reconhecer que vale a pena viver
Apesar de todos os desafios, incompreensões e períodos de crise.
Ser feliz é deixar de ser vítima dos problemas e
Se tornar um autor da própria história...
É atravessar desertos fora de si, mas ser capaz de encontrar
Um oásis no recôndito da sua alma...
É agradecer a Deus a cada manhã pelo milagre da vida.
Ser feliz é não ter medo dos próprios sentimentos.
É saber falar de si mesmo.
É ter coragem para ouvir um "Não"!!!
É ter segurança para receber uma crítica,
Mesmo que injusta...

Pedras no caminho?
Guardo todas, um dia vou construir um castelo...

(Fernando Pessoa)

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Stress-triggered synaptic malfunction: a gate along the path from depression to dementia

Abstract

Clinical and preclinical studies have shown that depression results from an interaction between genetic and environmental factors. In fact, stressful life events are known to play a major role in triggering episodes of depression, while chronic stress is recently suggested as an environmental risk for developing Alzheimer's disease (AD). Importantly, neuronal and synaptic malfunction are key underlying pathomechanisms in both depression and AD, while stress is also causally associated with neuronal and synaptic atrophy/loss resulting in impaired mood and/or cognition. Previous studies in AD animal models suggest that stress and stress hormones, glucocorticoids (GC), trigger the two major AD pathological pathways, Tau hyperphosphorylation and APP misprocessing. Importantly, these AD mechanisms have been reported to be affected in depression, suggesting a pathological link between the two disorders. In the light of the casual role of chronic stress for both diseases, this PhD thesis monitors the role of APP misprocessing and Tau hyperphosphorylation as critical mechanisms through which stress and GC trigger neuronal atrophy and synaptic deficits related to mood and cognitive impairments.

The findings of this thesis demonstrate that stress-driven depressive behavior and neuronal atrophy are accompanied by APP misprocessing and increased expression of BACE-1, the first enzyme responsible for this neuronal pathway in hippocampus and prefrontal cortex (PFC). Fluoxetine and imipramine, 2 different types of antidepressants, reverse the above cellular pathway resulting in neuronal and behavioral recovery. Furthermore, genetic deletion of *BACE-1* blocks the stress-driven depressive pathology providing neuronal protection against chronic stress, confirming further the involvement of BACE-1 and APP misprocessing in the establishment of stress-driven brain pathology. In line with the recently suggested role of Tau and its synaptic missorting in the detrimental effects of APP misprocessing and Aβ in AD, stress-driven hippocampal deficits in wild-type mice are accompanied by hyperphosphorylation and synaptic missorting of Tau, along with enhanced Fyn/GluN2B-related downstream excitotoxic signaling. In contrast, mice lacking *Tau* (Tau-KO) do not exhibit stress-induced pathological behaviors, e.g. depressive behavior and cognitive deficits, neither atrophy of hippocampal dendrites or deficits in hippocampal connectivity. Furthermore, Tau ablation is shown to block the stress-evoked damage of mitochondria localization at dendritic synapses of PFC neurons

providing protection against the neuronal atrophy and synaptic loss that occurs after exposure to chronic stress. The above findings identify Tau protein and its hyperphosphorylation as an essential mediator of stress-driven synaptic pathology underlying depressive state and cognitive impairment. Moreover, while Tau hyperphosphorylation and malfunction are well-established key events in AD neuropathology, the impact normal Tau function loss in neuronal degeneration and subsequent behavioral deficits are still under intense debate. This PhD thesis shows that loss of Tau precipitates an age-dependent motor impairment that is followed by progressive hypomyelination, increased degenerating myelinated fibers and diminished conduction properties of their efferent fibers conveying motor-related information. These findings suggest that loss of Tau protein may progressively impact on peripheral motor system adding to our understanding of peripheral neurological deficits in AD.

Conclusively, this PhD thesis provides novel insights about the mechanistic involvement of AD mechanisms, APP misprocessing and Tau hyperphosphorylation, in the manifestation of cognitive deficits and depressive pathology driven by exposure to chronic stress. In addition, these findings add to our limited knowledge about the role of Tau and its malfunction/loss of function in the underlying mechanisms of pathological aging beyond AD.

Disrupção sináptica induzia pelo stress: da depressão à demência

Resumo

Estudos clínicos e pré-clínicos demonstraram que a depressão resulta de uma interação entre factores genéticos e ambientais. Com efeito, os eventos indutores de stress desempenham um papel determinante no aparecimento de episódios depressivos, contribuindo de igual modo para um maior risco de desenvolver Alzheimer (AD). A disfunção neuronal e sináptica constituem dois importantes mecanismos patológicos comuns nas duas doenças, sendo que o stress crónico, por sua vez, está altamente associado a ambas resultando, por fim, em défices cognitivos e emocionais. Estudos nesta área de investigação têm demonstrado que o stress e as suas hormonas estão implicados em duas vias patogénicas de AD: a híper-fosforilação da proteína Tau e o processamento inadequado da proteína APP no sentido da produção de Aβ. É importante notar, ainda, que a Tau e a Aβ estão igualmente envolvidas na depressão, o que sugere que o stress é um mediador comum para ambas as doenças. Baseados nestes factos, colocamos como hipótese a possibilidade de a híper-fosforilação da Tau e um processamento inadequado da APP serem também críticos como mediadores dos efeitos do stress na cognição e memória.

Os resultados principais desta tese demonstram que o comportamento depressivo apresentado pelos animais foi também acompanhado por um processamento inadequado da APP, bem como por atrofia neuronal no hipocampo e córtex pré-frontal. Curiosamente, o tratamento com antidepressivos reverteu tanto as alterações bioquímicas como as morfométricas nos neurónios destes animais. Para além destes resultados, mostrámos também que, bloqueando o processamento inadequado da APP e consequente geração de Aβ (Bace-KO), impedimos que comportamento depressivo fosse estabelecido, corroborando assim os resultados anteriores.

Considerando o envolvimento da Tau nos efeitos nefastos do incorreto processamento da APP e da Aβ, verificamos que défices no hipocampo induzidos pelo stress em animais WT são acompanhados pela híper-fosforilação da Tau e um aumento na excitotoxicidade associada à posterior via de sinalização Fyn/GluN2B. Em contraste, ratinhos que não possuem a proteína Tau (Tau-KO) não apresentam défices típicos induzidos pelo stress, bem como défices cognitivos e emocionais. Corroborando os resultados anteriores, animais Tau-KO expostos a um protocolo de stress também não exibiam sinais de atrofia neuronal, nem de atividade neuronal reduzida no hipocampo. Para além dos resultados anteriores, enquanto a exposição ao stress crónico resultou em atrofia das dendrites apicais de neurónios do córtex pré-frontal e défices cognitivos

em ratinhos WT, tais alterações não se verificaram nos animais Tau-KO. Além disso, uma análise de proteómica quantitativa sugeriu um papel proeminente para vias mitocondriais na mediação dos efeitos do stress, sendo que a ablação da Tau previne os efeitos do stress nas mitocôndrias ao nível das dendrites sinápticas dos neurónios do córtex pré-frontal, conferindo proteção contra a atrofia neuronal e perda sináptica após exposição crónica ao stress. Em conjunto, estes resultados permitem identificar a proteína Tau como um mediador essencial na patologia cerebral induzida pelo stress.

Enquanto a híper-fosforilação da proteína Tau e o seu funcionamento anormal são eventos de destaque e bem estabelecidos na patologia de AD, o impacto da perda da função normal da Tau na degeneração neuronal e subsequentes défices comportamentais estão ainda sob intenso debate. Nesta tese, mostrámos que os animais Tau-KO apresentam uma deficiência motora, que é dependente da idade dos animais, e que é acompanhada por défices ultra-estruturais e funcionais das fibras eferentes. Estes resultados sugerem que a perda da proteína Tau pode ter progressivamente impacto no sistema motor periférico.

Em conclusão, esta tese apresenta resultados relativamente ao envolvimento das vias patológicas da AD, incorreto processamento da APP e híper-fosforilação da Tau, na manifestação dos défices cognitivos e depressão induzidos pela exposição a stress. Adicionalmente, estes resultados providenciam conhecimento relativamente ao papel essencial da Tau e sua disfunção ou perda de função nos mecanismos que regulam o envelhecimento patológico para além da relação ansiedade/depressão e a AD.

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Abbreviation list

Aβ - Amyloid βeta

ACTH - Adrenocorticotropic hormone

AD - Alzheimer's disease-

ADs - Antidepressants

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

ANOVA - Analysis of variance

APP - Amyloid precursor protein

AVP - Arginine vasopressin

ATP - Adenosine triphosphate

BACE1 - β site APP cleaving enzyme 1

BDNF - Brain-derived neurotrophic factor

BNST - Bed Nucleus of Stria Terminalis

CA1 - Cornu Ammonis 1

CA3 - Cornu Ammonis 3

CORT - Corticosterone

Cdk5 - Cyclin-dependent kinase 5

CNS - Central Nervous System

CREB - cAMP response element binding

CRH - Corticotropin-releasing hormone

CUS - Chronic Unpredictable Stress

DA - Dopamine

DG - Dentate gyrus

EPM - Elevated plus maze

ERK - Extracellular signal regulated kinase

FST - Forced swim test

GC - Glucocorticoids

GR - Glucocorticoid receptor

Gsk3β - Glycogen synthase kinase 3 βeta

HAA -

HMW - High molecular weight

HPA - Hypothalamic-pituitary-adrenal

HPLC - High Performance Liquide Chromatography

KO - Knockout

LMW - Low molecular weight

LTD - Long-term depression

LTP - Long-term potentiation

MAO - Monoamine oxidase

MAOI - Monoamine oxidase inhibitor

MAP - Mitogen activated protein

MAPs - Microtubule associated proteins

MBP - Microtubules binding domain

MCI - Mild cognitive impairments

mPFC - medial Prefrontal Cortex

MR - Mineralocorticoid receptor

mRNA - Messenger Ribonucleic acid

MT - Microtubules

NE - Norepinephrine

NFTs - Neurofibrillary tangles

NMDA - N-methyl-d-aspartate

NMDAR - N-methyl-d-aspartate receptor

NOR - Novel object recognition

OF - Open field

PHF - Paired helical filaments

PFC - Prefrontal Cortex

PNS - Peripheral Nervous System

PS1 - Presenilin 1

PS2 - Presenilin 2

PSD - Postsynaptic density

PVN - Paraventricular nucleus

SN - Substantia Nigra

SPT - Sucrose preference test

SSRI - Selective serotonin reuptake inhibitors

TCA - Tricyclic antidepressant

TEM - Transmission electron microscopy

TST - Tail suspension test

TGN - Trans Golgi Network

WT - Wildtype

3R Tau - three repeats Tau

4R Tau - four repeats Tau

5 HT - Serotonin

Chapter 1

1.1 General Introduction

During the last century, the world population has been continuously increasing with the population over 65 years old being double. However, improvements in lifespan have, unfortunately, not been matched by improvements in the mental health span with aging being the highest risk factor for the appearance of different diseases. The development of age-related diseases occurs at different rates in individuals, and exposure to long-lasting stress diminishes health and appears to be an important factor promoting earlier onset of age-related diseases (Bosma et al., 1998; Chrousos and Gold, 1992). In fact, psychological stress appears to be increasingly present in our modern, and demanding industrialized society while plethora of clinical and experimental studies have associated chronic stress with appearance of cardiovascular, immunological as well as neurological diseases (Lederbogen et al., 2011). Therefore, it seems that stress exerts an important burden on individual's health with various social and financial implications. For instance, the World Health Organization expects that mental diseases, including stress-related disorders, are among the leading causes of disability worldwide and will be the second leading cause of disabilities by 2020.

As it will be discussed later in this thesis, our knowledge about how our organism perceives stressful stimuli has being increased during the last decades and we now know that the mechanisms that are triggered by stress are primarily adaptive, facilitating the restoration of physiological and behavioral homeostasis. Although our body and brain can be highly influenced by stress, relatively little is known about when, and how, the effects of stress shift from beneficial and protective to deleterious and damaging.

1.2 Stress: the good and the bad sides of it

1.2.1 The concept of stress

Stress is defined as a challenge to homeostatic equilibrium by physical or psychological events (McEwen, 2003). All living organisms strive towards a dynamic equilibrium and whenever an endogenous or exogenous challenge is perceived as unpleasant, aversive or threatening, a coordinated response to that stressor is activated by a series of systems and processes (de Kloet et al., 2005).

Stress is not a single entity and therefore several different types of stressors can be

distinguished. Stressful challenges can be acute or of a chronic nature and may occur only once, or rather take place in a repetitive manner that can be anticipated. Contrariwise, stress can be unpredictable and uncontrollable, mild or severe, and occurring in or out of a context. Furthermore, the perception of the stress stimulus by an individual varies greatly, and so does the persistence of its consequences (Joels et al., 2012; Koolhaas et al., 2011).

In addition, stress generates a cascade of signals that converge to orchestrate an integrated response that 'resets' many peripheral and central processes and allows an individual to adapt to the changes in its environment thus restoring homeostasis. Effective coping implies that a stress response is effectively triggered when needed and terminated afterwards (de Kloet et al., 2005). However, if the stress response is inadequate and/or prolonged in time, a series of pathophysiological changes occur in the brain, the immune system and viscera that may ultimately lead to several disorders (Sapolsky, 2000; Sorrells and Sapolsky, 2007).

1.2.2 Stress action in the brain: towards adaptation

The physiological stress response can be divided into two different time domains with a very quick response and a delayed response. The first phase of the stress response is considered the "alarm reaction" or the "fight-fright-or-flight" response, which involves the rapid activation of the autonomic nervous system that causes the release of epinephrine and norepinephrine from the adrenal medulla. These hormones will rapidly act through elevation of basal metabolic rate, blood pressure and respiration, as well increasing the blood flow to the most vital organs that are important for the "fight-or-flight" response. Following this, the hypothalamic–pituitary–adrenal (HPA) axis is also activated. In this neuroendocrine circuit, limbic and hypothalamic brain structures coordinate emotional, cognitive, neuroendocrine and autonomic inputs. The cooperation between systems determines the magnitude and specificity of an individual's behavioral, neural and hormonal responses to stress (Lucassen et al., 2014).

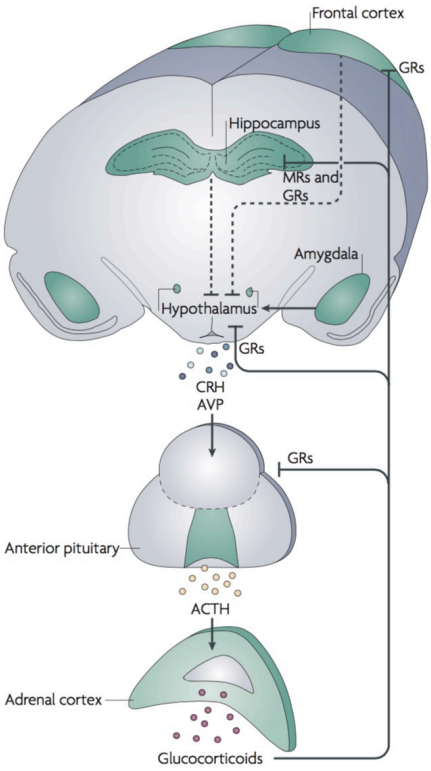

Figure 1. Schematic representation of the HPA axis and its regulation. Stressful stimuli trigger the hypothalamic release of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) activating the synthesis of adrenocorticotropin hormone (ACTH) at pituitary. ACTH secretes into the bloodstream stimulating corticosteroids secretion from adrenal cortex into the blood, which serve as a feedback inhibitory signal to hypothalamus and pituitary (*Adapted from Lupien 2009*).

The core component of the stress response is the activation of the HPA axis (Sapolsky et al., 1986). Upon stimulation, corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) will induce adrenocorticotropic hormone (ACTH) release from the pituitary, which in turn releases glucocorticoids (GC) from the adrenal glands (Figure 1). Regulation occurs through negative feedback after GC binding to high-affinity mineralocorticoid (MR) and lower affinity glucocorticoid receptors (GR) (de Kloet et al., 2005).

Stressful events can activate the PVN by distinct neuronal pathways (Herman and Cullinan, 1997). In addition, this brain region plays a pivotal role regulating the HPA stress response (Whitnall, 1993), by integrating the output of different stress-sensitive brain circuitries (Herman and Cullinan, 1997). More precisely, the excitatory ascending pathways originating in brainstem nuclei that convey noradrenergic inputs from the nucleus of tractus solitaries (Abercrombie and Jacobs, 1987), the serotonergic inputs from the raphe nuclei (Feldman et al., 1987) or from adjacent hypothalamic nuclei are in a privileged situation to receive visceral and autonomic inputs and to mount a rapid “reactive” neuroendocrine response (Figure 1).

Corticosteroids (corticosterone in rodents and cortisol in humans) reach every organ through circulatory system to initiate stress-induced adaptations. The underlying mechanism involves an integrated response, which starts with rapid hormone-induced changes in receptor conformation, referred previously, that leads to slower modulation of gene expression (Figure 2). In the second phase of stress response (the slower mode of operation), GC can bind, with different affinities, to GR and MR, which are co-expressed abundantly in the neurons of limbic structures and seem to exist in both membrane-bound form and nuclear form. MR affinity seems sufficiently high to maintain receptor activation throughout 1-h intervals between hormone secretory bursts of 20-min duration. By contrast, the lower affinity GR seems to respond largely in phase with the ultradian rhythm. This receptor becomes progressively activated during stress- and circadian-induced increases in the frequency and amplitude of corticosteroid secretory bursts (Young et al., 2004). Both receptors can have rapid and delayed effects, which can result from non-genomic mechanisms (mediated by membrane receptors), indirect genomic mechanisms (mediated by membrane receptors and second messengers) and genomic mechanisms (mediated by cytoplasmic receptors that move to the nucleus and act as transcription factors) (Popoli et al., 2012).

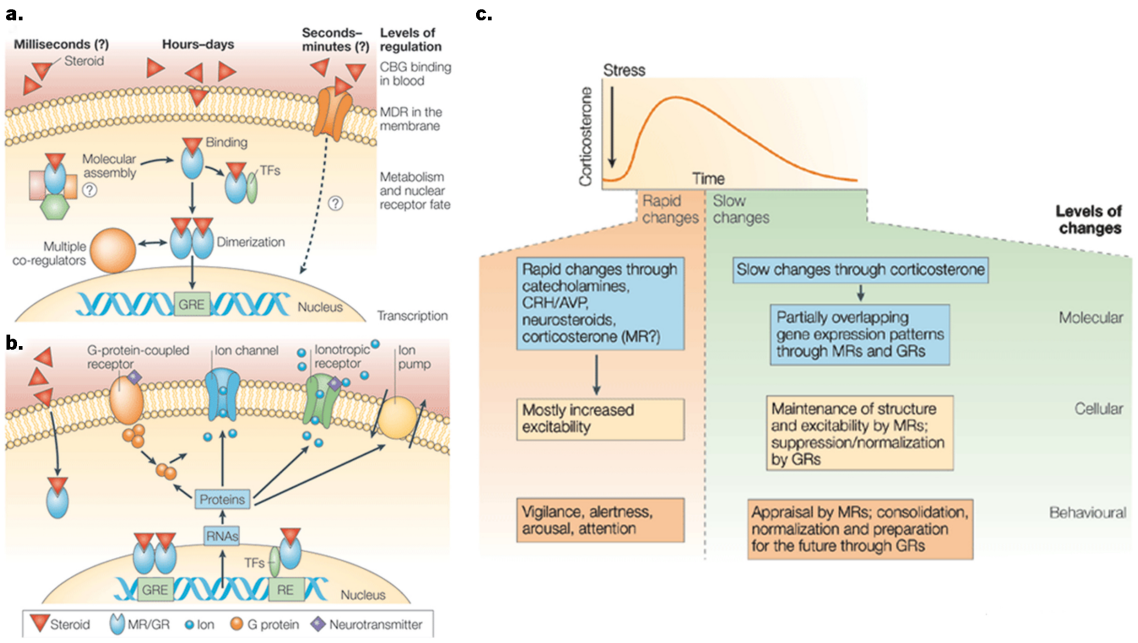

Figure 2. Diversity of mineralocorticoid and glucocorticoid receptor signaling and time course of cellular responses to stress hormones. **a.** After corticosterone binding to them, the multimeric protein complexes of mineralocorticoids and glucocorticoids (MR and GR, respectively) dissociate. For their genomic participation, both receptors interact with a GC-response element recruiting other molecules. The rapid responses, which involve steroid-induced conformational changes and re-aggregation with other proteins (such as heat-shock proteins), and a putative membrane steroid receptor, are still poorly understood. **b.** After GC binding, MR and GR

can regulate the transcription of several genes, which may control the properties of G protein-coupled receptors, ion channels, ionotropic receptors and ion pumps; this will alter the conductance of the plasma membrane. These steroid actions are conditional, slow in onset and long lasting. **c.** Stress-driven activation of the hypothalamic–pituitary–adrenocortical (HPA) axis leads to a temporary increase in circulating corticosteroid levels, as shown in the graph by the changes in hormone levels. In the early phases of the stress response, the GC rise and together with other fast-acting agents lead to an adequate response to the stressor. Then, slowly, gene-mediated corticosteroid effects start the transcriptional regulation of specific genes. This will affect the normal cellular function in cells that have these receptors. Typically, the dose-dependence curve of stress response is U-shaped (*Adapted from de Kloet 2005*).

As GC receptors can function as transcriptional regulators, they will alter the expression of responsive genes, leading ultimately, to an effect on adaptive behavior. This fact indicates that GC can modulate the expression of GR target genes that underlie aspects of cell metabolism, structure and synaptic transmission, such as enzymes and receptors for biogenic amines and neuropeptides (Sabban and Kvetnansky, 2001), growth factors (Schaaf et al., 1997) and cell-adhesion factors (Sandi, 2004). These facts underpin the relevance of corticosteroids as structural modulators in limbic areas. Additionally, corticosteroids are pleiotropic agents that regulate the transcription of several genes, although they are not able to change all cellular properties. The role of several proteins involved, for example, in voltage-gated ion-channel function or G-protein-coupled receptor signaling, are strongly affected by GC and stress. The stress effects on ionotropic receptors are commonly rapid and independent of the slow gene-mediated pathways. Furthermore, fast GC actions might be a link between the fast effects mediated by catecholamines and CRH, and the delayed actions mediated by corticosterone through gene transcription. It was also postulated that MRs and GRs could also function in a binary fashion at the cellular level (De Kloet et al., 1998). MR activation, on cellular communication, maintains the excitability and stability of networks, whereas GR (in addition to MR) activation leads to delayed suppression or normalization of network activity. The last might facilitate the retention of information by allowing more calcium influx into the cell on depolarization. However, this enhancement in calcium influx could also prime neurons for detrimental effects.

Basal levels of GC are associated with induction of mechanisms of synaptic plasticity in the hippocampus such as Long Term Potentiation (LTP) (Diamond et al., 1992), which is a well-documented neuronal substrate for memory formation (Martin et al., 2000). On the contrary, high levels of GC or new environment exposure have been shown to impair induced LTP and to facilitate long-term depression (LTD) (Kim and Diamond, 2002). In addition, very little is known

about the underlying mechanism through which stress induces changes, although *N*-methyl-d-aspartate (NMDA) receptors seem to be involved (Kim et al., 1996). Undoubtedly, modifications of the potential of synaptic plasticity can occur through changes in glutamate transmission and calcium influx mediated by GR or stress. At this stage, the timing of events reveals of great importance, i.e. if stress hormones reach limbic system, they might alter network function making the patterned stress independent-input less effective at strengthening the synapses. However, if stress hormones are released together with a learning situation, resulting in hormone release and network activation to concur, they might prime synapses, facilitating synaptic potentiation. Moreover, whereas elevations in GC seem to be highly relevant for consolidation of any demanding task (Oitzl and de Kloet, 1992), GR activation before retention seems to interfere with this process. The involvement of specific brain regions and neurotransmitter systems are fulcral for the outcome of hormonal effects. The combined effects of stress hormones in different, but interconnected, areas (which might be differentially involved depending on the type and severity of the stressor) determine the overall outcome.

Summarizing, the GR receptors help to maintain GC levels within physiological limits (Erdmann et al., 2008); this is important as GC plasma levels are under circadian and ultradian control (Liston et al., 2013; Qian et al., 2012). Together, MR and GR determine sensitivity of the brain to stress (Harris et al., 2013; Medina et al., 2013; Pruessner et al., 2010; Sousa et al., 2008) and thereby modulate several behavioral responses, such as memory formation and eventually adaptation and coping with stress; for instance, alterations in the normal GR expression have been implicated in stress vulnerability and the development of neuropsychological disorders such as anxiety and depression (de Kloet et al., 2005; Ridder et al., 2005).

1.2.3 Neuropathology of chronic stress

The complete and precise consequences of stress exposure are not easy to be understood as the respective stress response depends on individuals coping strategy and stress sensitivity. However, severe or prolonged stress is well known to increase the risk to develop psychopathologies, such as depression, dementia, schizophrenia or anxiety disorders in susceptible individuals.

Considering broader neuropathological aspects of stress, animal studies have reported brain damage upon prolonged periods of stress exposure. These structural changes were found mainly

in hippocampus, which displays increased GR levels in patients suffering of depression (Wang et al., 2012; Wang et al., 2013). Although much less is known about the MR, the abundant expression of GR in human brain makes it more susceptible and therefore important target for stress exposure (Joels et al., 2012). Whereas acute stress is generally beneficial and adaptive, chronic stress may cause an MR/GR imbalance or down-regulation (de Kloet et al., 2005), which can deregulate HPA feedback and result in overexposure of the brain and peripheral tissues to these powerful steroids.

For many years, stress-related brain disorders were explained by neurochemical imbalances. Recently, several studies have also demonstrated impairments in structural plasticity and volumetric changes of specific limbic areas, e.g. stress-driven dendritic atrophy and synaptic loss that may contribute to their pathophysiology (MacQueen and Frodl, 2011). However, it is still not clear whether the above substrates should be considered as truly pathological or whether they may represent neuroplastic (dynamic) adaptations to a stressor with the side effect of concomitant neuronal disconnection and related behavioral deficits.

1.2.3.1 Neuroplastic actions of glucocorticoids in the brain

1.2.3.1.a. Neuronal Remodeling

Functional plasticity in the brain is generally preceded by structural plasticity, typically, dendritic and synaptic remodeling. Stress affects spine density and brain morphology in a region-specific manner. One of the best-described forms of stress-evoked structural plasticity is dendritic retraction, first observed in CA3 and CA1 hippocampal and medial prefrontal cortex (PFC) (Cerqueira et al., 2007; Sousa et al., 2000). Sustained exposure to various types of chronic stress leads to damage of apical (but not basal) dendritic complexity (e.g. reduced dendritic length and arborization) of CA3 pyramidal neurons (Woolley et al., 1990). However, in other brain regions (e.g., orbitofrontal cortex, amygdala and BNST), repeated stress results in increased dendritic length (Liston et al., 2006; Schwabe and Wolf, 2009). In addition, chronic stress also triggers a loss of mossy fiber synapses, an increased surface area of the post-synaptic density, and rearrangements of synaptic mitochondria and vesicles at the presynaptic terminals (Sandi et al., 2003). Note that at least some of these dendritic rearrangements need several weeks to develop.

Dendritic spines are deeply involved in the storage of information and their density can be modulated by stress (Tasker and Herman, 2011). The ability to compile information about changes in spine shape and function with high spatiotemporal resolution provides greater insights not only into the dynamic reorganization of postsynaptic function during stress but also into the disconnection syndrome that may underlie stress-related diseases of the brain. Chronic stress and elevated GC levels are shown to decrease the number of spines in CA3 pyramidal dendrites that was rapidly reversible after a recovery period or subsequent training (Alfarez et al., 2009; Sandi et al., 2003); pyramidal cells of mPFC respond in a similar fashion. Both present reduced dendritic complexity and spine loss to chronic stress (Goldwater et al., 2009; Radley and Morrison, 2005), which are reversible following a recovery period (Goldwater et al., 2009; Radley et al., 2005). Note that not all brain areas are affected by exposure to chronic stress as some other regions appear to be resistant (Shansky and Morrison, 2009). Also, spine elimination, but not their formation, was shown to require MR activation (Liston et al., 2013). Together, these studies suggest that prolonged exposure to chronic stress and GC markedly alters the quantity and morphology of both pre- and post-synaptic structural elements and, consequently, the strength of excitatory synapses in the hippocampus.

1.2.3.1.b. Neural cell fate

A new variable into the equation of the structural and functional integrity of the brain was introduced with the discovery that new neurons can be generated (neurogenesis) throughout life in the hippocampal dentate gyrus and some other brain regions. It is now known that these newly-born cells integrate into the hippocampal circuitry of adult brain (Dranovsky et al., 2011; Sahay et al., 2011) and are functional insofar that they contribute to the formation of new memories (Kitamura et al., 2009; Sahay et al., 2011). In addition, imbalances in adult hippocampal neurogenesis received considerable attention during recent years once it has been associated with the pathophysiology of several neuropsychiatric disorders, such as depression (Kempermann et al., 2008). Furthermore, exposure to chronic stress inhibits the production of new neurons in adult brain (Lucassen et al., 2010; Schoenfeld and Gould, 2012). Both psychosocial (Gould et al., 1997) and physical stressors (Vollmayr et al., 2003) can inhibit at least one or more phases of the neurogenesis process (Gould et al., 1997; Heine et al., 2004). Stress and GC further interfere with other stages of neuronal renewal, proliferation, maturation

and survival (Heine et al., 2004; Schoenfeld and Gould, 2012). The underlying cellular mechanism that mediates the inhibitory effect of stress on neurogenesis is still unknown. MR, GR and NMDA receptors have been identified on progenitor cells (Gould et al., 1997). In the same line, some studies have identified a long lasting inhibition of neurogenesis after an initial stressor, despite later normalized GC levels (Schoenfeld and Gould, 2012), suggesting that while GC may be involved in the initial suppression of cell proliferation, particularly in early life, when neurogenesis is abundant, they are not always necessary for the maintenance of this effect (Gould et al., 1997). Some possible mechanisms that may underlie stress-evoked neurogenesis includes reduced expression of several growth factors, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) that all can influence neurogenesis (Galea, 2008). Besides stress effects on adult brain neurogenesis, some studies have provided some evidence about GC-induced hippocampal pyramidal cell death (Sapolsky et al., 1986), where they propose that GC could induce programmed cell death (apoptosis) of hippocampal granule neurons. These findings led to the idea that excess GC render neurons vulnerable to Ca^{2+} influxes (Landfield and Pitler, 1984) and excitotoxicity (Sapolsky et al., 1986), as well as oxidative stress (Behl et al., 1997). However, the incidence of apoptosis or cell loss in rodent stress models is rare (Heine et al., 2004; Lucassen et al., 2006) and only in the most extreme cases chronic stress leads to cellular death.

1.2.3.2 Chronic stress and brain diseases

Although stress is a necessary mechanism for survival, severe and/or long-term stress disrupts normal brain structure and function impairing mood and cognitive function in both animals and humans. Chronic stress has been implicated in the etiology of emotional disorders, such as depression (McEwen, 2003), representing one of the most common risk factors for the development of the disease with GC having a key role in pathophysiology of depression (Krishnan and Nestler, 2008; Sheline et al., 2003). High GC levels can reduce neurogenesis in the hippocampal dentate gyrus (Krishnan and Nestler, 2008) and induce the retraction of hippocampal apical dendrites (Magarinos and McEwen, 1995; Sapolsky, 2000). Furthermore, GC can also increase neuronal vulnerability to different neuronal insults, such as radical oxygen species (Behl et al., 1997), excitotoxins (Elliott and Sapolsky, 1992) as well as the neurotoxic peptide amyloid beta (Aβ) involved in Alzheimer's disease (AD) (see also below). Interestingly, a

large proportion of depressed patients present an overactivation of the HPA axis and high GC levels. Notably, these subjects show a remarkable heterogeneity in the neuroendocrine function and the proportion of depressed individuals demonstrating overt HPA axis abnormalities may range from 35 to 65 % (Lucassen et al., 2014).

Recently, clinical studies suggest prolonged stressful life experiences as part of the etiology of sporadic forms of AD, as it is shown that a considerable portion of AD patients hypersecrete GC (O'Brien et al., 1996; Rasmuson et al., 2001). Indeed, once chronic elevation of GC levels is known to impair memory and cognitive performance, it is speculated that GC play a role in progressive cognitive decline in AD. A major target of GC is the hippocampus, which is a main target area for AD pathology and chronic stress. The hippocampal dysfunction in AD has significant detrimental consequences on declarative, spatial and contextual memory processes. Interestingly, the GC levels generally correlate with the rates of cognitive impairment and the extent of neuronal remodeling in AD subjects (Huang et al., 2009; Lupien et al., 1998). Moreover, it is interesting to highlight the fact that at least two AD transgenic mouse strains display elevated corticosterone secretion (Touma et al 2004; Green et al 2006), although it is not clear (i) whether the hypercorticalism is causal to the deposits of Aβ, one of the "molecular culprits" of the disease, and cognitive impairments seen in this model, or (ii) whether the increased adrenal activity was as a result of the damaged brain areas (e.g. as hippocampus has a inhibitory role on HPA axis function) or a consequence of the genetic manipulation itself.

Based on the above, chronic stress, acting through GC, is described to initiate a cascade of cell signaling events that culminate in mood and cognitive disorders. As it will be discussed later, different studies suggest a strong association between depressive illness and increased risk for developing AD (Ownby et al., 2006), adding credence to the suggestion that chronic stress and GC could have a fundamental role in the development of both brain disorders (Sotiropoulos et al., 2008); yet, the appealing conceptual framework that links depression to AD pathology is still waiting for mechanistic evidence.

1.3 Chronic stress in depressive pathology

1.3.1 Depression: Epidemiology and Etiology

Depression is a very common neuropsychiatric illness with almost 1 in 5 individuals experiencing

a depressive episode during their lifespan (Kessler et al., 2003; Murray and Lopez, 1997). Currently, depression affects more than 121 million people worldwide a number predicted to highly increase with depression becoming the first cause of disability by 2020. Together, the economic and human suffering burdens of depression are also extremely high (World Health Organization 2012, DEPRESSION; A Global Public Health Concern http://www.who.int/mental_health /management/depression/who_paper_depression_wf mh _2012.pdf). The disease core symptomatology consists of depressed mood, pessimistic thinking, lack of enjoyment and lowered energy while poor concentration and memory, cognitive deficits, reduced confidence and self-esteem and feelings of guilt and self-blame are also commonly found in a depressive state (Cowen, 2013). Depression is a heterogeneous disorder with a highly variable course and inconsistent response to treatment. Several factors were identified as being associated with an increased risk for depression with family history of the disorder being among the stronger factors (Williamson et al., 2004). It is now believed that a combination of genetic, psychological, and environmental factors, such as repetitive experiences of stress episode, may also be involved in the onset and progression of a depressive disorder.

1.3.2 Depressive neuropathology and chronic stress

The multitude and heterogeneity of symptoms in depression suggests the involvement of different brain regions in its etiology. The limbic system has received extensive attention since it is thought to govern affective behavior and it is involved in processes such as learning and memory. This system comprises a complex set of structures including the hippocampus and PFC, which are highly interconnected (Sierksma et al., 2010). The hippocampus is known as the primary memory structure, necessary for declarative memory, or remembering facts, while the PFC is thought to exert top-down control over all emotions, thoughts and actions (Arnsten, 2009). Imaging studies have shown abnormalities in structure and function within these areas.

The molecular basis underlying pathophysiology of depression is largely unknown due to its complex nature; however, growing evidence suggests impaired neuroplasticity as a central issue of this disorder. Indeed, reduced neuroproliferation, dendritic atrophy and decreased volume in the hippocampus have been implicated in the onset, progression and remission of depressive symptoms. Adult hippocampal neurogenesis imbalances have also been shown to be involved in

pathophysiology of depression, as well as in the action of several antidepressants (Mateus-Pinheiro et al., 2013).

1.3.2.a Chronic stress and HPA axis deregulation as part of depressive pathology

Hyperactivity of the HPA axis is an important mechanism that has been suggested in the etiology of depression. Dysfunctional feedback may lead to an excessive response of the HPA axis, which can drive to detrimental effects on both body and brain. All physical, affective and cognitive changes in depression could thus be regarded as a consequence of this dysfunctional HPA axis; this theory is known as the 'glucocorticoid cascade hypothesis of depression' (Sapolsky et al., 1986). Several clinical studies have indicated that depressive patients show higher baseline levels of cortisol in body fluids such as plasma, serum, cerebrospinal fluid and urine (Juruena et al., 2006; Pfohl et al., 1985); indeed, hypercortisolemia is often seen in these patients (Modell et al., 1997; Weber et al., 2000). In addition, a study also found that in a dexamethasone-suppression test, designed to assess HPA-responsivity, depressive patients revealed a lower capacity to down-regulate the stress response, which is indicative of a dysfunctional feedback loop (Juruena et al., 2006). Thus, a malfunction of the HPA axis in regulating the stress response has been theorized to underlie the pathogenic mechanisms behind depression and prime the brain towards further depressive episodes (Checkley, 1996; Modell et al., 1997).

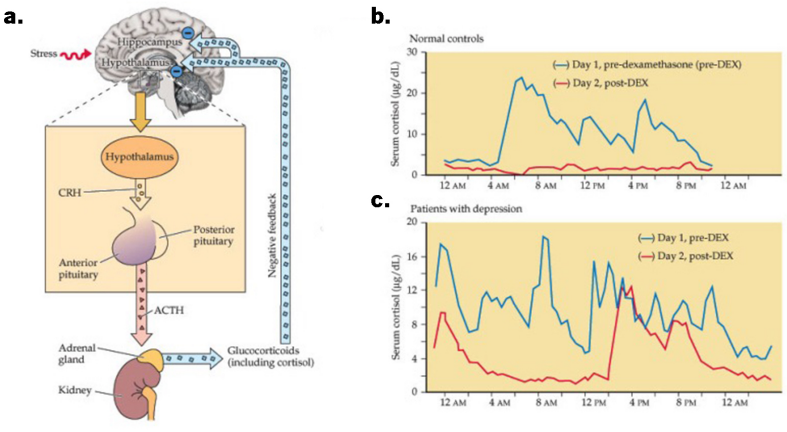

Figure 3. The HPA axis in Depression a. Schematic representation of the HPA axis involvement in depression. **b.** The normal circadian rhythm in the secretion of cortisol (day 1) is abolished by treatment with the synthetic GC dexamethasone (day 2) **c.** The same dose of dexamethasone is far less effective in patients with depression, as their brains are less responsive to the negative feedback effects (see part *a*) of steroids (Adapted from *http://www.mindsmachine.com/asf12.02.html*)

In line with clinical research, animal studies have shown that an antidepressant treatment has normalized GC levels in an animal model of depression (Yau et al., 2002). Furthermore, persistently elevated GC or dexamethasone exposure is associated with reactive glial cell proliferation, reduced dendritic branching in the CA3 area, as well as reductions in volume (Sapolsky, 1996). Accordingly, animal models of depression are mainly focused on prolonged exposure to different stressful stimuli that results in depressive behavior in these animals, accompanied by elevated GC levels and neuronal atrophy. Antidepressant treatment reverts all the above neuronal and behavioral deficits decreasing GC levels back to normal (Mateus-Pinheiro et al., 2013).

Interestingly, all cognitive deficits that occurred as a consequence of increased GC are highly specific for hippocampal or prefrontal dysfunction. In fact, hippocampus is one of the key structures in the brain for learning and memory processes, and alterations in its structure and function have been unequivocally established in patients with depression (Sierksma et al., 2010). Hippocampal volume reductions in depression are by now one of the best-replicated findings in biological psychiatry, but whether it is cause or consequence of the disorder it is still not clear. Indeed, reduced hippocampal volume has been described in depressive patients and the degree of hippocampal volume reduction correlates with total duration of depressive episode (Sheline et al., 2003) and severity of accompanying cognitive dysfunction (Frodl et al., 2006). Elevated plasma cortisol levels have been also related to the cognitive impairments seen in depressive patients (Van Londen et al., 1998), and increased cortisol levels in healthy elderly correlated negatively with explicit and declarative memory and selective attention performance (Lupien et al., 2005).

The PFC is another brain area found to be altered in patients who suffer from depression. The PFC participates in cognitive, socio-emotional and executive functions that are susceptive to stress (Arnsten, 2009; Cerqueira et al., 2008), and also modulates autonomic and neuroendocrine responses to stress (Herman et al., 2003). Similarly to hippocampus, repeated stressful experiences have a profound impact on neuronal plasticity in this brain region. The most thoroughly investigated neuromorphological change is the decrease of the total length of apical dendrites of pyramidal neurons in layers II/III of the mPFC, while sparing the structure of basal dendrites (Cerqueira et al., 2008). The main outcomes from these studies are a significant reduction in total dendritic length of 20–35 % with a significant decrease in branching and spine density of the distal apical dendrites. These chronic stress-induced effects are most probably

partially mediated by the activation of GR and NMDA receptors. Exogenous administration of high GC levels result in morphological changes similar to those seen following chronic stress exposure (Cerqueira et al., 2008), whereas blocking NMDA receptors could prevent these stress-induced effects (Martin and Wellman, 2011). Interestingly, it seems that these morphological alterations are plastic and not degenerative in nature, as in most of cases they reverse spontaneously after a period of recovery (Goldwater et al., 2009), at least in young animals (Bloss et al., 2010).

1.3.2.b Neuronal transmission and connectivity in depression and antidepressant action

As depressive pathology is thought to be causally related to neuronal atrophy, neuronal transmission and connectivity has also received a lot of attention with monoaminergic system being implicated in the etiology of depression. The 'monoamine hypothesis of depression' suggested that the cause of depression is based on decreased levels of monoamines in the brain, i.e. serotonin (5-HT) and noradrenaline. The neurotransmitter 5-HT is synthesized in the serotonergic neurons of the raphe nuclei from the amino acid L-tryptophan. It is transported throughout the brain with highest fiber densities in the hippocampus, hypothalamus and amygdala (Jacobs and Azmitia, 1992; Steinbusch, 1981). There are numerous receptors thought which serotonin produces its effects. However, 5-HT1A, 5-HT2A and 5-HT2C receptors have been the most targeted in depression and antidepressant research, and are thought to be in the regulation of depression, anxiety and cognition (Lanfumey et al., 2008). Due to its interaction with other neurotransmitter systems, serotonin was also described to have a role in several memory and learning processes. In fact, 5HT was shown to be a modulator of many cellular and synaptic functions necessary for proper memory and learning, such as dendritic and synaptic outgrowth and neurogenesis (Gaspar et al., 2003). Besides serotonergic receptors, a 5-HT transporter (5-HTT) reveals from great importance. It is primary locating at the nerve terminal of the pre-synaptic neuron, where it has as main function the clearance of the serotonin from the synaptic cleft. It is the main target of the selective 5-HT reuptake inhibitors (SSRIs).

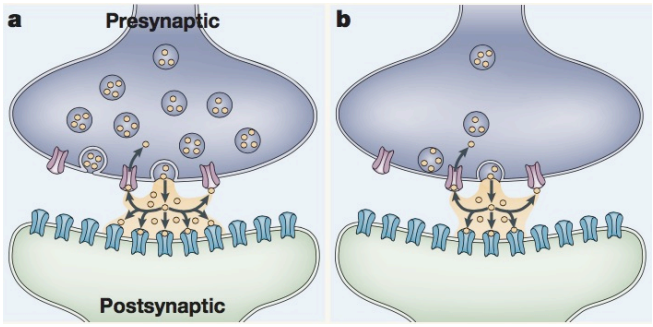

Figure 4. Monoamine hypothesis of mood disorders. a. In the normal brain, monoamine neurotransmitters are released and bind to receptors on the postsynaptic neuron. Transmission is terminated by re-uptake of the transmitter. **b.** In depression, the decreased concentration of monoamine at synaptic sites produces a mood disorder (*Adapted from Castrén, 2005*).

1.3.3 Antidepressants treatment

Currently, the first-line treatment for depression is pharmacotherapy with one of the different classes of antidepressants (ADs) available. However, the mechanisms behind drugs action are still not completely understood, although most of them share the propriety of increasing monoaminergic function. Experimental studies in animal models of depression show that antidepressant treatment revert both neuronal atrophy as well as depressive behavior and accompanying cognitive deficits (Mateus-Pinheiro et al., 2013).

Antidepressant drugs are categorized according to their mechanism of action on brain amines. The selective serotonin reuptake inhibitors (SSRI) are a class of antidepressants that selectively inhibit the reuptake of serotonin and subsequently increase the amount of serotonin available to bind to the postsynaptic receptor. SSRIs compounds have different pharmacokinetic profiles and chemical structures while all are primarily metabolized by oxidation prior to excretion (Spinks and Spinks, 2002). Examples of this class are fluoxetine, citalopram, sertaline, fluvoxamine and paroxetine (Berton and Nestler, 2006). SSRIs are the most commonly prescribed class of antidepressants once the major advantages of these drugs are their good safety and tolerability profiles.

The nonselective monoamine reuptake inhibitor class of antidepressants includes the tricyclic antidepressants (TCA), which is a group of antidepressants that inhibit the reuptake of both serotonin and noradrenalin. Some examples of this class of ADs are imipramine, clomipramine, amitriptyline and despiramine (Berton and Nestler, 2006). Some studies had suggested that dual

inhibitors might have higher efficacy and earlier response than selective reuptake inhibitors for a single monoamine (Thase et al., 2001). In addition to their therapeutic effects TCAs have presented an unwanted side effects, which had declined its prescription for new antidepressants with a better tolerability profile (Pacher and Kecskemeti, 2004).

Another class of antidepressants are the monoamine oxidase inhibitors (MAOI) (e.g. tranylcypromine) which act through the inhibition of monoamine oxidase (MAO), the enzyme responsible for the metabolism of monoamines (Berton and Nestler, 2006). In addition, atypical antidepressant agents are also in clinical use against depression such as bupropion, tianeptine and mirtazapine. Bupropion inhibits the reuptake of dopamine, tianeptine stimulates the uptake of monoamines, and mirtazapine antagonizes a_2 adrenergic receptors (Berton and Nestler, 2006).

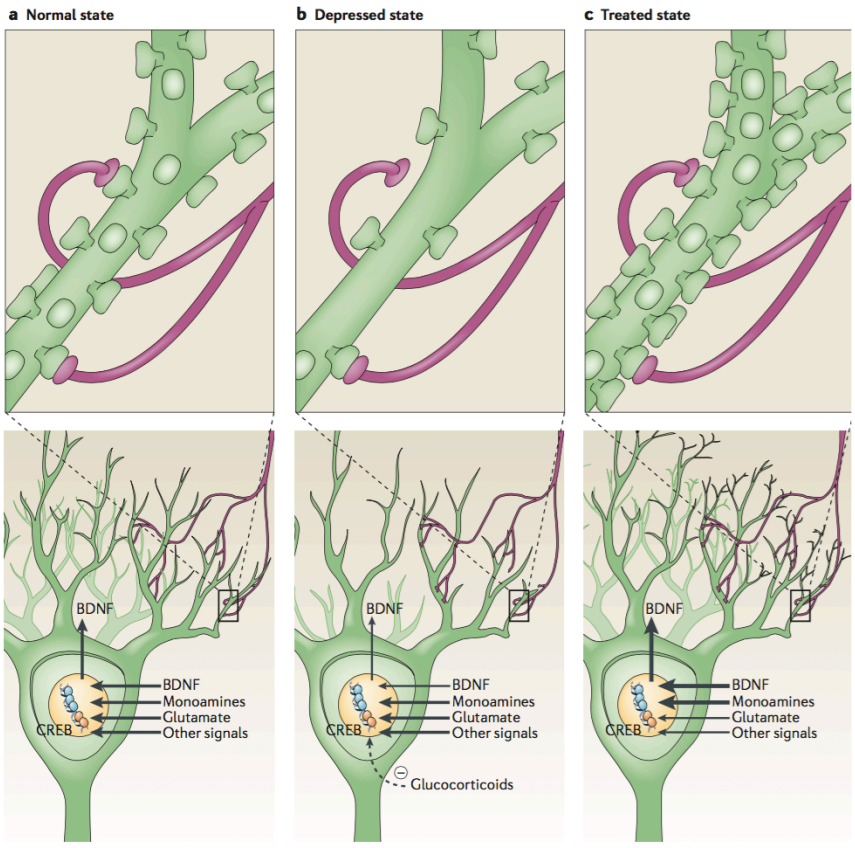

Figure 5. Neuroplasticity in depression and antidepressant action. a. The scheme show a hippocampal pyramidal neuron and its regulation by different signals/molecules e.g. brain- derived neurotrophic factor (BDNF), monoamines **b.** Chronic stress and elevated GC is suggested to regulate the above molecules causing reduction in their dendritic arborization **c.** Antidepressants may also exhibit their beneficial effect through the same molecules reversing neuronal atrophy and synaptic loss and thus, ameliorating depressive symptoms (*Adapted from Berton and Nestler, 2006*).

1.4 Chronic Stress and its role in Alzheimer's disease

1.4.1 Alzheimer's disease: Epidemiology and Etiology

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by cognitive deficits, including impaired judgment, decision-making and orientation and often-accompanied by mood impairments (e.g. depression). At later stages, it could also appear behavioral disturbances, as well as language impairments (Dubois et al., 2010). Currently, more than 26 million people are afflicted with AD worldwide, and these numbers are expected to radically increase by 2050 (*http://www.who.int/medicines/areas/priority_medicines/BP6_11Alzheimer.pdf*). Although the etiology of the disease is not yet fully understood, significant progress has been made over the last few decades in understanding both molecular mechanisms and risk factor of AD. Besides aging which is the highest risk factor, a variety of other factors such as genetic variations, exposure to gonadal and stress hormones, and environmental factors can contribute to the development and progression of the disease. As it will be discussed later, chronic stress and elevated GC levels are thought to play a precipitating role in the development and/or progression of AD. Indeed, stress and corticosteroids are thought to play a contributory role in AD since several studies describe elevated cortisol levels in AD patients, and hippocampal degeneration and memory impairment are found in patients with disturbed adrenocortical activity.

1.4.2 Molecular mechanisms of AD neuropathology

AD brain is characterized pathologically by cortical atrophy, neuronal cell death, synapse loss, neuroinflamation and the accumulation of two pathological lesions: neurofibrillary tangles (NFTs) and senile plaque (figure 6). NFTs deposit within neurons and are composed of hyperphosphoryated Tau protein, whereas senile plaques occur in the extracellular space and are made up of amyloid-beta (Aβ) peptide (Koffie et al., 2011). Build-up of Aβ-containing senile plaques and NFTs occurs over years, eventually causing lesions within neuronal circuits in the neocortex, hippocampus, and basal forebrain, being all essential for memory.

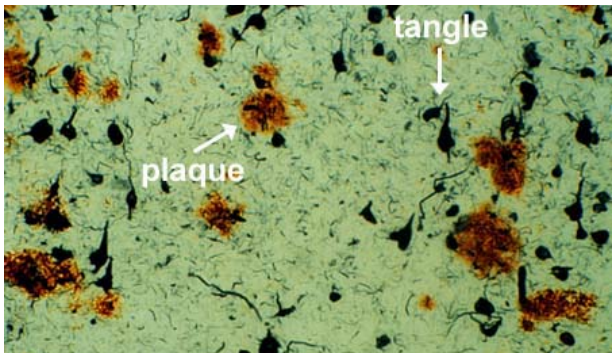

Figure 6. Histological staining of cortex from an AD patient showing both extracellular senile plaques composed of the peptide Amyloid β (brown) and intracellular NFTs (black) made of the microtubule-associated protein Tau (*http://ladulab.anat.uic.edu/images/ADstain.jpg*).

In contrast to senile plaques, which are heterogeneously distributed throughout the brain, NFTs develop at predictable sites and affect specific cell types in given cell layers and brain regions. In other words, Tau pathology spreads in a stereotypic, sequential, and hierarchical fashion (Braak and Braak, 1991), which show good correlation with impairment of memory and mental status (Henderson and Buckwalter, 1994). Of interest is the fact that plaques and NFTs were also observed in some non-demented aged individuals. This fact supports the view that the pre-clinical phase of AD is protracted, and that plaque and tangle formations may be normal events during aging; most likely, pathology only becomes overtly manifest once these lesions reach a 'critical mass' (Guillozet et al., 2003).

1.4.2.a. Amyloid Precursor Protein and its misprocessing in AD and their relationship with stress

The central core of senile plaques contains the Aβ peptide arranged as β-sheet filaments (Allsop et al., 1983). Aβ is also frequently deposited in the small blood vessels of the meninges and cerebral cortex, mostly in the outer walls of arterioles and capillaries and are found throughout brain. Aβ peptide is generated from cleavage of amyloid precursor protein (APP), which is an integral membrane protein that belongs to a family of transmembrane proteins with large extracellular domains. The APP gene is located on chromosome 21 and is expressed in many cell and tissue types including endothelia, glia and neuronal cells. Through alternative splicing of exons 7,8 and 15, it gives rise to 8 isoforms, of which 3 are most common: APP695, predominantly expressed in the CNS whereas APP 751 and 770 are more ubiquitously expressed (O'Brien and Wong, 2011).

APP is produced in large quantities in neurons and is metabolized very rapidly (Lee et al., 2008). After sorting in the endoplasmic reticulum and Golgi, APP is delivered to the axon, where it is transported by fast axonal transport to synaptic terminals (Koo et al., 1990). Key steps in APP processing occur at the cell surface and in the Trans-Golgi Network (TGN), where the highest concentration of APP is found, in neurons at steady state (Zhang et al., 2011). From the TGN, APP is further transported to the cell surface or directly to an endosomal compartment. Two alternate pathways exist for APP proteolysis; the non-amyloidogenic pathway (cleavage by α-secretase and γ-secretase), and the amyloidogenic pathway (cleavage by β-site APP-cleaving enzyme 1 (BACE1) and γ-secretase). On the cell surface, APP can be sequentially cleaved by α- and γ-secretase, a process that precludes Aβ generation, as the cleavage site is within the Aβ domain. Alternatively, APP can be reinternalized in clathrin-coated pits into another endosomal compartment containing the proteases BACE1 and γ-secretase. This results in the production of Aβ, which is then dumped into the extracellular space following vesicle recycling or degraded in lysosomes (O'Brien and Wong, 2011). The reason some surface APP is internalized into endosomes and some proteolyzed directly by α-secretase is still unclear, although segregation of APP and BACE1 into lipid rafts may be a crucial element (Ehehalt et al., 2003). Lastly, to complete the APP cycling loop, retrograde communication occurs between endosomal compartments and the TGN, mediated by a complex of molecules, the retromers (O'Brien and Wong, 2011).

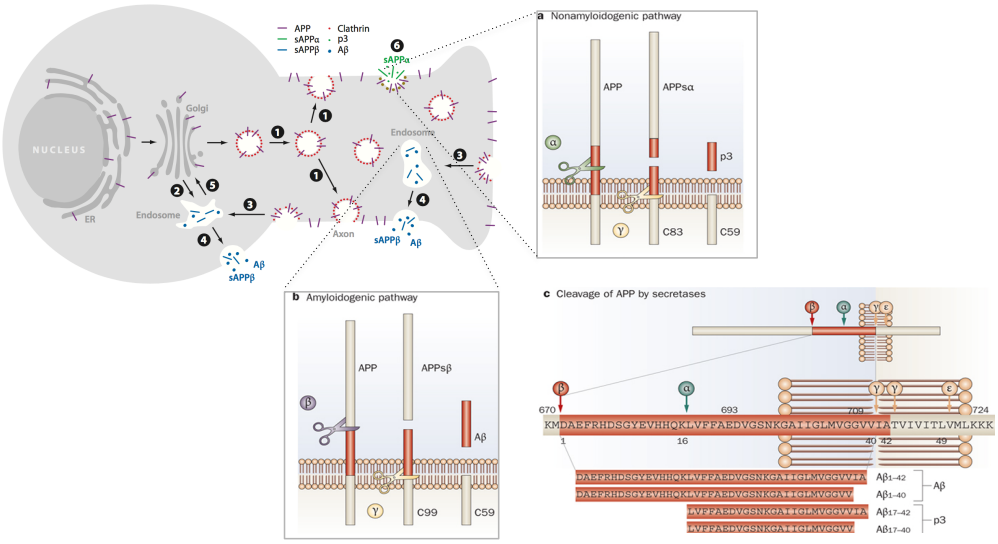

Figure 7. APP processing in neurons. Newly synthesized APP is transported from the Golgi down the axon (1) or into a cell body endosomal compartment (2). After insertion into the cell surface, some APP is cleaved by α-secretase (6) generating the sAPPα fragment, which diffuses away, and some is reinternalized into endosomes (3), where Aβ is generated. Following proteolysis, the endosome recycles to the cell surface (4), releasing Aβ and sAPPβ. Transport from the endosomes to the Golgi prior to APP cleavage can also occur, mediated by retromers (5). Sequential cleavage of the APP occurs by two pathways. **a.** Nonamyloidogenic processing of APP involving α-secretase followed by γ-secretase is shown. **b.** Amyloidogenic processing of APP involving Bace1 followed by γ-secretase is shown. Both processes generate soluble ectodomains (sAPPα and sAPPβ) and identical intracellular C-terminal fragments. **c.** The APP family of proteins has large, biologically active, N-terminal ectodomains as well as a shorter C-terminus that contains a crucial protein-sorting domain. The Aβ peptide starts within the ectodomain and continues into the transmembrane region (*Adapted from O'Brien and Wond, 2011*).

The enzymes that cleave APP have been extensively characterized. APP is sequentially cleaved by groups of enzymes or enzyme complexes termed α-, β- and γ- secretases. Three enzymes with α-secretase activity have been identified, all belonging to the ADAM family (a disintegrin and metalloproteinase-family enzyme): ADAM9, ADAM10 and ADAM17. Cleavage of APP by α-secretase releases a large soluble ectodomain of APP called sAPPα. The generation of sAPPα is a constitutive event but can also be regulated by various reagents. Several groups identified BACE1, which is a type I integral membrane protein belonging to the pepsin family of aspartyl proteases, as the β-secretase. BACE1 is directly involved in the cleavage of APP at the +1 (prior to amino acid 1) and +11 sites of Aβ resulting in the generation of an intermediate fragment called C99. Subsequent cleavage of C99 (between residues 38 and 43) by the γ-secretase liberates an intact Aβ peptide. Most of the full-length Aβ peptide produced is 40 residues in length ($A\beta_{40}$), whereas a small proportion (approximately 10%) is the 42 residue variant ($A\beta_{42}$). The $A\beta_{42}$ variant is more hydrophobic and more prone to fibril formation than $A\beta_{40}$; $A\beta_{42}$ is the predominant isoform found in senile plaques (LaFerla et al., 2007). The γ-secretase has been identified as a multiprotein complex composed of presenilin 1 or 2, (PS1 and PS2); nicastrin, a

type I transmembrane glycoprotein; and anterior pharynx defective (Aph-1) and presenilin enhancer 2 (Pen-2) (LaFerla et al., 2007; O'Brien and Wong, 2011).

While early studies on AD considered Aβ plaques to be causative for neurotoxicity (Lorenzo and Yankner, 1994), this idea has been modified in favor of Aβ oligomers as the prime source of Aβ-mediated neurotoxicity. Indeed, the degree of amyloid deposition in senile plaques correlates only poorly with cognitive decline in AD whereas memory loss and cognitive deifficts correlate better with the loss of synaptic terminals that are possibly caused by soluble Aβ (Lue et al., 1999; Mucke et al., 2000; Roselli et al., 2005). Further, new studies have shown that Aβ small oligomers and protofibrils also exert neurotoxic effects as they can rapidly block LTP in the hippocampus, increase oxidative stress partly by activating Fyn-related pathways, and stimulate GSK3β-mediated hyperphosphorylation of Tau, the other key AD pathomechanism.

Of particular importance and relevance to neuronal signaling is the ability of extracellular Aβ to impair calcium homeostasis, and thus, affecting nerve cell excitability by facilitation of membrane depolarization and Ca^{2+} influx. Aβ can alter Ca^{2+} concentrations by binding to either α7 nicotinic acetylcholine receptors or to L-type voltage-dependent Ca^{2+} channels and, thereby impact synaptic plasticity and transmission (Ekinci et al., 1999). Aβ could also modulate LTP and LTD, and alter dendritic architecture by remodeling the cytoskeleton through its activation of extracellular-signal-regulated kinase/mitogen activated protein kinase (ERK/MAP kinase) (Small et al., 2001). In addition, Aβ is also suggested to modulate NMDA receptor function in a cell type manner and depending on Aβ concentrations (Turner et al., 2003).

Previous studies show that elevated GC levels and exposure to chronic stress increase Aβ production in AD transgenic mouse models exacerbating their memory deficits (Green et al., 2006b; Jeong et al., 2006). Similarly, Green and colleagues (Green et al., 2006b) showed that prolonged treatment with the synthetic GC, dexamethasone, triggers APP misprocessing resulting in increased Aβ levels using both *in vitro* and *in vivo* AD models. 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). In line with previous studies, *in vitro* data have confirmed that GC trigger APP misprocessing without influencing the non-amyloidogenic pathway i.e. the other cellular cascade of APP cleavage/processing (Sotiropoulos et al., 2008a). 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 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 (Catania et al., 2009). This study also showed that chronic stress/GC increased BACE1 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 and co-workers (Catania et al., 2009) was that GC potentiate the APP misprocessing pathway in previously stressed animals of an AD model (Aβ-infused rats).

1.4.2.b. Tau biology and pathology: a possible target for chronic stress and GC

Hyperphosphorylated Tau protein is shown to be the main component of the NFTs. These protein aggregates accumulate in neuronal perikarya and as a neuropil threads or dystrophic neuritis in dendrites and axons. In contrast to amyloid deposits, NFTs correlate with the severity of dementia in AD in a similar way to synaptic and neuronal loss (Arriagada et al., 1992; McKee et al., 1991) (Arriagada et al., 1992). Although AD is characterized by co-existence of both plaques and NFTs, it has been reported that a small population of AD patients show abundant NFTs but very few amyloid plaques (Bancher and Jellinger, 1994), which may suggest a causal relationship between the accumulation of NFTs and the clinical manifestations of AD. However, the function of Tau protein and how its hyperphosphorylation can be pathological is still unknown.

Tau belongs to the family microtubule associated protein (MAPs) that together with heterodimers of α- and β- tubulin modulates the stability and assembly of microtubules (MT) (Gotz et al., 2013). Tau protein is expressed in the central and peripheral nervous system and, to a lesser extent, in kidney, lung, and testis (Gu et al., 1996). It is most abundant in neuronal axons (Lee et al., 2001) but can also be found in neuronal soma (Tashiro et al., 1997), dendrites and dendritic spines (Morris et al., 2011) as well as in oligodendrocytes (Klein et al., 2002). The MAPT gene located in chromosome 17 encodes Tau protein, which by alternative mRNA splicing of exons 2, 3 and 10, yields six main isoforms. Tau isoforms differ by the absence or presence of one or two acidic inserts at the N-terminal, and whether they contain three or four repeats of a conserved tubulin binding motif at the C-terminal (Lee et al., 1989). The repeat region, present within the microtubule-binding domain, binds to microtubules and promotes their assembly. Tau isoforms with four repeats (4R-tau) bind microtubules with a greater affinity than isoforms with three repeats (3R-tau), and can even displace the previously bound 3R-tau (Lu and Kosik, 2001).

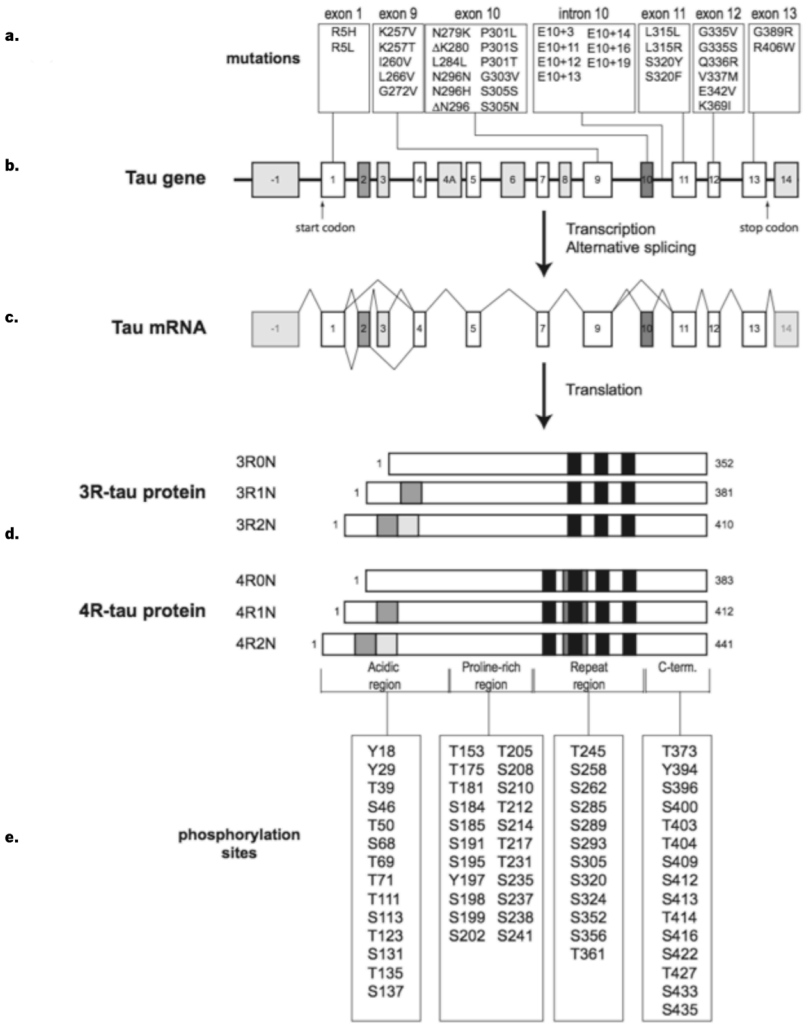

Figure 8. Schematic representation of the human tau gene, mRNA and protein isoforms. a-b. The human tau gene is located on chromosome 17q21 and contains 16 exons. White boxes represent constitutive exons and the gray boxes represent alternatively spliced exons. Identified mutations in exons 1–13, and intron 10, of the tau gene are shown using the numbering of the 441-amino acid isoform of tau. **c.** Exon 1 and 14 are transcribed but not translated. The different lines linking exons 2,3 and 10 represent the alternative splicing undergone by these specific exons. **d.** The alternative splicing lead to the generation of a total of six different mRNAs, which are translated into six different tau isoforms. These isoforms differ by the absence or presence of one or two N-terminal inserts encoded by exon 2 and 3, as well as the presence of either three or four repeat regions coded by exons 9, 10, 11 and 12 in the C-terminus. **e.** Sites in the acidic, proline-rich, repeat and C-terminal regions of tau reported to be phosphorylated *in vivo* or *in vitro* (*Adapted from Gendron and Petrucelli, 2009*).

The predominant Tau role at cellular level is based on its function to bind and stabilize MT assembly contributing to the establishment and maintenance of the overall neuronal cytoskeleton and cytoarchitecture (Allen et al., 1985; Vale et al., 1985; Nangaku et al., 1994; Trinczek et al., 1999). In addition, Tau protein is also involved in axonal cargo (e.g. mitochondria, components of synaptic vesicles) transported to and from pre- and postsynaptic sites, which is critical for synaptic maintenance and function (Gendron and Petrucelli, 2009; Vossel et al., 2015). Besides

its interaction with cytoskeletal proteins, Tau is recently shown to interact with synaptic proteins, such as scaffold proteins and receptor (Fyn, PSD-95, GluN2B) regulating synaptic function and plasticity (Gotz et al., 2013; Ittner et al., 2010; Kimura et al., 2013; Mondragon-Rodriguez et al., 2012).

Physiologically, tau has an intrinsically disordered structure and is subject to a complex array of posttranslational modifications, such as acetylation (Cohen et al., 2011), glycation (Ledesma et al., 1994), isomerization (Miyasaka et al., 2005), sumoylation (Dorval and Fraser, 2006), O-GlcNAcylation (Arnold et al., 1996) ubiquitination and phosphorylation (Cripps et al., 2006). The diversity of these modifications highlights tau as being extremely prone to regulation. Tau phosphorylation has received special attention due to its well-established role on regulating Tau binding capacity to MT. In its longest expressed (CNS) form (441 amino acids), Tau bears 80 putative serine and threonine residues at which phosphorylation can occur; some 60% of these are associated with neuropathology (Buee et al., 2000). While Tau dephosphorylation promotes rapid and extensive MT polymerisation, phosphorylation at certain sites results in conformational changes of Tau and subsequently, decreased MT binding assembly and stability. Phosphorylation at specific residues (e.g. Ser 262 or 396, located in the repeat domain and in the C-terminal region, respectively) can completely abolish MT- binding in vitro (Biernat et al., 1993), although other studies have shown that phosphorylation of Ser 262 alone is insufficient to eliminate Tau binding to MT (Seubert et al., 1995). Furthermore, phosphorylation of sites outside the MT-binding domain (e.g. within the proline-rich region) results in decreased *de novo* nucleation of MT in cell-free assembly reactions (Brandt et al., 1994). In AD brains, Tau is aberrantly hyperphosphorylated in different epitopes, which results in reduced MT-binding and conformational changes (Fischer et al., 2009) leading to Tau detachment from MT. The detached Tau accumulates in neuronal cell bodies and neurites, forming insoluble filaments and, ultimately, the NFTs (Lee et al., 2001; von Bergen et al., 2001). At least, 25 phosphorylation sites have been identified in paired helical filaments (PHF-tau) in AD brains (Hanger et al., 1998); most of these sites are clustered in the region flanking the MT-binding domain. While many of the phosphorylation sites are common to PHF-tau and native tau, the extent of phosphorylation as well as the number of phosphorylation sites in the affected brain far exceeds that in normal brain. Importantly, it should be highlighted that elevated levels of phosphorylated Tau are also found in healthy cells and its occurrence is not restricted to AD.

The phosphorylation state of Tau seems to influence the intraneuronal distribution of proteins (Pooler et al., 2014). Tau phosphorylated in its proline-rich region is mainly localized in the somatodendritic compartment, whereas dephosphorylation in this region or phosphorylation in the C-terminal domain of Tau is associated with predominant localization in the distal region of the axon (Mandell and Banker, 1996). More recently, hyperphosphorylated Tau was found to accumulate in dendritic spines of both AD brains and Tg mouse models representing the so-called missorting of Tau at synapses (Hoover et al., 2010; Ittner et al., 2010; Tai et al., 2012). Interestingly, hyperphosphorylation of Tau seems to be necessary for Tau missorting at synapses as only pseudophosphorylated Tau (which mimics hyperphosphorylated Tau), but not phosphorylation-deficient Tau, was mislocalized and accumulated in dendritic spines (Hoover et al., 2010).

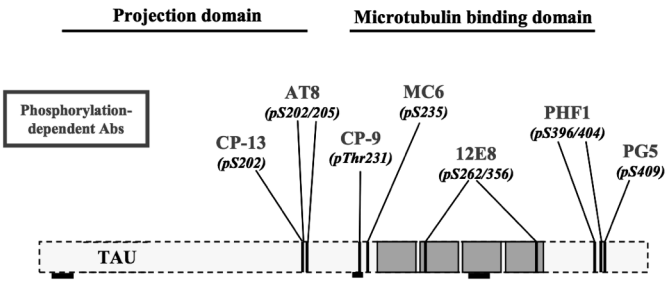

Figure 9. Schematic drawing of Tau protein showing some threonine (T) or serine (S) residues that are commonly found to be hyperphosphorylated in AD brain followed by some antibodies that recognize them (drawn by Sotiropoulos I.).

While the precise mechanisms through which Tau missorting facilitates neuronal and synaptic deficits are still under intense investigation, accumulating evidence suggest that both Aβ and glutamate trigger Tau missorting followed by synaptic atrophy and dendritic collapse (Zempel et al., 2010; Tsushima et al., 2016), probably involving NMDA receptor activation (Ittner et al., 2010; Talantova et al. 2013).

Using different AD animal models, others and we have shown that exposure to chronic stress and prolonged treatment with the dexamethasone aggravates Tau pathology. This leads to increase of Tau hyperphosphorylation and accumulation in neuronal somata affecting different Tau epitopes (Green et al., 2006a; Sotiropoulos et al., 2011) implicated in cytoskeletal pathology and synaptic loss in AD patients (e.g., pSer262) (Callahan et al., 2002; Lauckner et al., 2003); note that these epitopes are correlated with hippocampal atrophy in AD patients (e.g., pThr231) (Hampel et al., 2005). 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 (Augustinack et al., 2002). Furthermore, experimental studies also associate Tau hyperphosphorylation with synaptic malfunction and loss related to memory impairment (Kimura et al., 2007) suggesting that Tau and its hyperphosphorylation could be a key mediator of synaptic pathology.

1.5 Chronic stress as the connecting factor between depression and AD

As discussed above, neuronal atrophy and synaptic failure seems to be a common and critical mechanism in both depression and AD pathologies. Indeed, despite the fact that both disorders were considered completely distinct clinical entities for many years, growing clinical evidence supports a pathological link between them, as depressive disorder is commonly associated with increased risk for developing AD (Geerlings et al., 2000; Ownby et al., 2006). Some studies have focused in the temporal relationship between depression and AD trying to figure out whether depression is a simple prodromal symptom of AD, which precedes the onset of cognitive deficits, or if a history of depression might represent an independent risk factor for the development of AD. A systematic meta-analysis shows that the interval between diagnoses of the two diseases is positively related to an increased risk of developing AD. This fact suggests that rather than a prodrome, depression can be considered as a risk factor for AD (Ownby et al., 2006). Moreover, depression occurring early in life is highly associated with a late development of AD (Robert et al., 2003). Interestingly, other authors also found that the risk to develop AD increases with every new affective episode associated to mood disorders (Kessing, 1998; Kessing and Andersen, 2004), particularly, the rate of dementia tended to increase by 13% with every episode leading to medical assistance. In addition, depressive symptoms favor the conversion of (Mild Cognitive impairments) MCI into a more severe state (Houde et al., 2008), being patients with MCI and depression at more than twice in risk of developing AD than those without depression (Modrego and Ferrandez, 2004). On the other hand, depression may occur in 30–40% of the AD patients (Assal and Cummings, 2002) and thus affecting the clinical evolution of the pathology. AD patients with major depression usually present a greater and faster cognitive impairment (Lyketsos et al., 1997).

Interestingly, senile plaques and NFTs are more pronounced in the hippocampus of AD patients with comorbid depression as compared with AD patients without depression (Rapp et al., 2008).

Moreover, AD-related mechanisms such as APP misprocessing are also found in depressed patients (Post et al., 2006) but the appealing conceptual framework that links depression to AD pathology is still waiting for mechanistic evidence. Despite the commonalities in neurobiological substrates of depression and AD and the fact that chronic stress is causally related to both disorders (Czeh and Lucassen, 2007; Kendler et al., 1999; van Praag, 2004; Simard et al., 2009; Wilson et al., 2006), considerably less attention has been given to the suggested role of stress as a connecting risk factor (Sotiropoulos et al., 2008b). Accordingly, both AD and depressive patients exhibit neuronal atrophy and synaptic abnormalities and/or loss in hippocampus and PFC (Bessa et al., 2009; Sheline et al., 2003) while they often exhibit elevated GC levels (Hartmann et al., 1997; Weber et al., 2000). Furthermore, previous experimental studies in AD animal models showed that exposure to chronic stress and/or GC administration aggravated AD neuropathology trigger APP misprocessing towards the generation of Aβ (Catania et al., 2009) as well as Tau hyperphosphorylation (Sotiropoulos et al., 2011) in both hippocampus and PFC, two brain areas that suffer from neuronal atrophy and loss in both AD and depression.

Together with the recently described neuro- and synapto-toxic properties of Aβ and hyperphosphorylated Tau (Ittner et al., 2010; Zempel et al., 2010), the above findings suggest that APP misprocessing and Tau hyperphosphorylation could lie at the core of mechanisms through which chronic stress and elevated GC levels precipitates neuronal atrophy and synaptic damage underlying depressive pathology and cognitive impairments.

Aims

Clinical and experimental studies suggest a causal role of chronic stress for brain pathology and disorders e.g. depression and AD as stress is strongly associated with neuronal malfunction and atrophy resulting in impaired mood and/or cognition. Based on the suggested common neurobiological substrates between depression and AD and the fact that chronic stress is a risk factor for both disorders, this PhD thesis aims to investigate the potential role of AD-related cellular mechanisms, namely APP misprocessing and Tau hyperphosphorylation, in the establishment of stress-driven depressive pathology and cognitive impairments. Thus, this thesis aimed to address the following key objectives:

i) Provide mechanistic evidence about the potential mediation of Tau protein and its phosphorylation at the deleterious actions of chronic stress on mood and cognition as well as on related neurostructural deficits and synaptic damage **(Chapter 2.1 & 2.2)**;

ii) Obtain unequivocal evidence about the role of APP misprocessing on stress-driven depressive pathology as well as the influence of different classes of antidepressants on this AD-related cellular mechanism **(Chapter 2.3)**;

iii) Clarify the potential inter-relationship between loss of Tau function and age-related neurodegeneration **(Chapter 2.4)**

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Chapter 2

Chapter 2.1

Lopes S, Telptska L, Vaz-Silva J, Dioli C, Trindade R, Morais M, Webhofer C, Almeida OFX, Turck CW, Sousa N, Sotiropoulos I

Tau deletion prevents stress-induced dendritic atrophy in prefrontal cortex: role of synaptic mitochondrial pathways

Under revision at Cerebral Cortex

2015

Tau deletion prevents stress-induced dendritic atrophy in prefrontal cortex: role of synaptic mitochondrial pathways

Running title: Tau deletion attenuates stress effects

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Abstract

Tau protein in dendrites and synapses has been recently implicated in synaptic degeneration and neuronal malfunction. Chronic stress, a well-known inducer of neuronal/synaptic atrophy, triggers hyperphosphorylation of Tau protein and cognitive deficits. However, the cause-effect relationship between these events remains to be established. To test the involvement of Tau in stress-induced impairments of cognition, we investigated the impact of stress on cognitive behavior and neuronal structure as well as the synaptic proteome in the prefrontal cortex (PFC)

of Tau knock-out (Tau-KO) and wild-type (WT) mice by behavioral, neuroanatomical, neurochemical and quantitative proteomic approaches. Whereas exposure to chronic stress resulted in atrophy of apical dendrites and spine loss in PFC neurons and significant impairments in working memory in WT mice, such changes were absent in Tau-KO animals. Quantitative proteomic analysis of PFC synaptosomal fractions suggested a prominent role for mitochondrial pathways in the mediation of the effects of stress; specifically, stress-altered proteins were involved in mitochondrial transport and oxidative phosphorylation in a Tau-dependent manner. These findings provide evidence for a causal role of Tau in mediating stress-evoked neuronal atrophy and cognitive impairment and indicate that Tau exerts its effects through synaptic mitochondrial pathways.

Keywords: chronic stress, dendritic atrophy, mitochondria, prefrontal cortex, Tau knock-out

Introduction

Chronic stress has a significant impact on the prefrontal cortex (PFC) leading to impairments of high executive functions such as working memory, cognitive flexibility and decision-making (Cerqueira and Mailliet and et al., 2007; Sotiropoulos and Cerqueira and et al., 2008; Sousa and Almeida 2012; McEwen and Morrison 2013). The structural-functional correlates of these stress-induced behavioral impairments include neuronal atrophy and synaptic loss. A large body of evidence indicates that these changes presumably reflect the actions of glucocorticoids (GCs) and, in turn, altered glutamatergic activity and disrupted Ca^{2+} homeostasis (Cerqueira and Mailliet and et al., 2007; Sousa et al., 2012) that ultimately interfere with the stability of cytoskeletal proteins (Cereseto et al., 2006). We previously demonstrated that chronic stress triggers the hyperphosphorylation of the cytoskeletal protein Tau with concomitant cognitive deficits (Sotiropoulos et al., 2011); while previous work implicated synaptic loss in the latter (Cerqueira and Taipa and et al., 2007), the cellular and molecular underpinnings of these events are largely unknown. Hyperphosphorylated Tau is strongly associated with synaptic malfunction and loss as well as neuronal degeneration in Alzheimer's disease (AD) (Kimura et al., 2007; Ittner et al., 2010). Tau protein, which plays a key role in microtubule stabilization and intracellular cargo trafficking, has recently been shown to be important for the regulation of synaptic plasticity (Frandemiche et al., 2014 ; Ittner et al., 2010), albeit through mechanisms that are still poorly understood. Here, we tested the hypothesis that Tau mediates the actions of stress on

prefrontocortical structure and function by exposing mice lacking the Tau protein (Tau-KO) (Dawson et al., 2001) to a chronic unpredictable stress (CUS) paradigm. We subsequently used ^{15}N metabolic labeling-based quantitative proteomics (Filiou and Turck 2012) to explore the underlying molecular correlates of these effects in PFC synapses. Our data suggest that stress produces a decline in PFC-dependent memory by altering the synaptic mitochondrial localization and proteome and, subsequently, dendritic and synaptic atrophy in a Tau/dependent manner.

Materials and Methods

Animals

Tau-knock out (Tau-KO) and wild type (WT) mice (C57BL/6J background; 4-5 month-old males) (Dawson et al., 2001) 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. [ZT0] to 8 p.m. [ZT12]; room temperature 22 ^{o}C; relative humidity of 55%, *ad libitum* access to food and water).

Stress paradigm

A group of animals from each genotype was exposed to a chronic stress paradigm during light period (stressed; STR) while non-stressed animals remained undisturbed at their home cages (control; CON). Animals of both genotypes were submitted to a 6-weeks protocol of CUS. This stress protocol consists of four different stressors: restraint, vibrating platform, overcrowding and exposure to a hot air stream. Animals were exposed to one stressor per day for 3 h. The order of stressors as well as the time of the day that the stressor was applied was randomly chosen for each week as previously described (Cerqueira and Mailliet and et al., 2007; Sotiropoulos et al., 2011). Body weight was recorded throughout the study as an indication of stress efficacy. At the end of the stress protocol, blood was collected from all animals at 8:00 p.m. (peak time point of the circadian cycle), serum was isolated by centrifugation and serum corticosterone (CORT)

levels were measured using a radioimmunoassay kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. For CORT measurement after acute stressor (8:00 a.m.), blood was collected before and at two different time points after animals exposure to an acute stressor (4min restraint).

Behavioral analysis

Y-maze: For assessing PFC-dependent working memory, spontaneous alternations in the Y-maze arms were monitored using the Y-maze apparatus (33 cm x 7 cm x 15 cm). Animals (n = 10-12 per group) were placed in the center of the Y-maze apparatus and were allowed to explore freely for 8 min; 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.

Novel place recognition: To assess recognition memory we used the novel place recognition test. Briefly, animals (n = 10-12 per group) were habituated to an open arena (33 cm x 33 cm x 33 cm) for three days. Each animal was then 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. Recognition index was calculated based on the following formula [time spent in (novel place/novel place + familiar place) – familial place (novel place + familiar place)] x 100. Sessions were videotaped and evaluated using Kinoscope software (http://sourceforge.net/projects/ kinoscope/).

Open field: For monitoring locomotor activity we used an open field apparatus [square arena (43,2 cm x 43,2 cm)] surrounded by tall perspex walls (Med Associates Inc., St. Albans City, VT). Mice (n = 10 per group) were placed in the center and allowed to explore the area for 5 min. Infrared beams and manufacturer's software were used to automatically register animals' movements.

Neuronal structure analysis

For 3D morphometric analysis, animals (n=5 per group) were transcardially perfused with 0.9% saline under deep anesthesia [ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3

mg/kg)]. 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. Dendritic arborization was analyzed in the layer II/III of the infralimbic division of PFC. 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 (number of spines/dendritic length) was determined in distal branches of apical dendrites that were either parallel or at acute angles to the coronal surface of the section. 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 and Mailliet and et al., 2007; Bessa et al., 2009).

Transmission electron microscopy analysis

For monitoring number of mitochondria at synapses, animals (n=3 per group) were transcardially perfused with 4% PFA in microtubule stabilization buffer (65mM PIPES, 25mM HEPES, 10mM EGTA, 3mM MgCl2, pH=6.9) as previously described (David et al., 2005). Isolated brains were fixed in 4% PFA, 0.8% glutaraldehyde (GTA) in microtubule stabilization buffer (overnight; RT) and then, transferred to 4% PFA, 0.8 % GTA in 0.1 M of phosphate buffer (PB; pH 7.4) for 2 h and then, to 0.1 M PB (4 °C). Vibratome-cut coronal sections of the prefrontal cortex (PFC; 300 µm thick) were collected, and the medial PFC area was surgically removed, carefully oriented along the superficial-to-deep axis and embedded in Epon resin. Ultrathin sections (500 Å) were cut onto nickel grids. Images were obtained by JEOL JEM-1400 transmission electron microscope and Orious Sc1000 digital camera. 40-42 non-overlapping TEM (30000x) images of counterstained ultrathin-sections per group were used for analysis by experimenter blind to the samples source.

Statistical analysis of animal data

Unless otherwise specified, animal data were analyzed using two-way ANOVA and Tukey *post-hoc* comparisons (Graphpad Prism v 5.0, La Jolla, CA); differences were considered statistically significant when $p < 0.05$. Results are presented as mean ± SEM.

Proteomic sample preparation

C57BL/6 mice were *in vivo* metabolically labeled with a bacteria-based ^{15}N-labeled diet in the animal facility of the Max Planck Institute of Psychiatry as previously described (Frank et al., 2009) and a whole brain from a ^{15}N-labeled C57BL/6 adult mouse was used as internal labeled standard for quantitative proteomic comparisons. PFC from individual animals (n = 5 per group) were combined and homogenized in buffer A [0.32M sucrose, 4mM HEPES, complete protease cocktail inhibitor tablets (Roche Diagnostics, Mannheim, Germany)] and centrifuged twice for 10 min at 1000 g at 4 ^{0}C. For each animal, the two supernatants were combined. Five hundred μg from each PFC homogenate per group were combined (2500 μg in total) and were mixed at a 1:1 (w/w) ratio with the ^{15}N-labeled internal standard homogenate. Synaptosomes were then enriched from the combined WT-CON/^{15}N, WT-STR/^{15}N, Tau-KO-CON/^{15}N and Tau-KO-STR/^{15}N PFC homogenate mixtures according to (Filiou et al., 2010). Protein content in the synaptosomal fraction was estimated by Bradford Assay. One hundred μg of each WT-CON/^{15}N, WT-STR/^{15}N, Tau-KO-CON/^{15}N and Tau-KO-STR/^{15}N synaptosomal fractions were loaded on a SDS gel. Each lane was cut in 4 mm slices. In-gel digestion and peptide extraction were performed as previously described (Frank et al., 2009). Peptide extracts were lyophilized and dissolved in 0.1% HCOOH for mass spectrometry (MS) analysis.

Mass spectrometry (MS)

Peptide extracts from each gel slice were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) using a nanoflow HPLC-2D system (Eksigent, Dublin, CA) coupled online to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples were loaded onto an in house packed fused silica 3 μm RP-C18 column (Maisch, Monheim, Germany) followed by a 20min wash with 0.1% HCOOH and

elution with a 95% AcN/ 0.1% HCOOH gradient from 2% to 45% over 90 min at a flow rate of 200 nl/min. All other MS parameters were as previously described (Filiou et al., 2010).

Proteomics data analysis

MS raw files were analyzed as previously described (Filiou and Teplytska and et al., 2012). In brief, data were searched twice against a concatenated decoy Swiss Prot mouse database v 3.46 using BioWorks v 3.3.1 (ThermoFisher Scientific, San Jose, CA) and SEQUEST v 28 (ThermoFisher Scientific, San Jose, CA). Parameters for SEQUEST search were as described previously (Filiou 2011). DTA select v 1.9 was used to filter and assemble peptides into proteins. Ion chromatograms were extracted and peptide/protein ratios were calculated by ProRata v 1.0 (Pan et al., 2006). Non-synaptosomal protein contaminants with altered protein levels were not included. Proteins with fold change > 1.3 and $p < 0.05$ (corrected for multiple testing) were considered differentially expressed. All MS quantification data are provided in **Supplementary Material, Table S1.**

Results

Tau deletion does not interfere with peripheral biomarkers of the stress response

The hypothalamus-pituitary-adrenal (HPA) axis, including the secretion of corticosteroids, comprises the physiological response to stress; chronic stress is characterized by hyperactivity of the HPA axis and body weight loss. We subjected Tau-KO and WT mice to a 6-week chronic unpredictable stress (CUS) protocol (**Figure 1A**) and body weight and serum corticosterone levels were monitored at the end of the paradigm, just before behavioral testing. Two-way ANOVA showed a highly significant effect of *Stress* on body weight ($F_{1,75} = 21.83$, $p < 0.0001$); body weight was significantly decreased in stressed WT animals (WT-STR; $p = 0.013$) and stressed Tau-KO animals (Tau-KO-STR; $p = 0.004$) compared to control animals of the same genotype (Tau-KO-CON and WT-CON, respectively) (**Figure 1B**). Furthermore, we found an overall effect of *Stress* on basal circulating corticosterone levels at the onset of the dark period (ZT12 / 8:00 p.m.; $F_{1,43} = 18.68$, $p < 0.0001$) (**Figure 1C**); *post-hoc* analysis revealed a significant increase in circulating corticosterone levels in both WT-STR ($p = 0.041$) and Tau-KO-STR ($p = 0.009$) mice

when compared to the corresponding non-stressed animals. To monitor HPA axis function, we assessed corticosterone levels at different time points after exposure of mice to an acute stressor (restraint, 4 min at 8:00 a.m.; **Figure 1D**). We found an overall effect of *Stress* at 30 min after this stressor ($F_{1, 45}$ = 4.352, p = 0.043). Notably, there was no *Genotype* effect in any of the above comparisons, indicating that the effects of stress on these parameters were not influenced by the absence of Tau.

Absence of Tau confers resilience to stress-induced deficits in memory and neuronal atrophy in the prefrontal cortex (PFC)

Chronic stress is known to impair working memory, a function that requires PFC integrity and the intactness of its connections with other brain areas (Cerqueira and Mailliet and et al., 2007; Sotiropoulos and Cerqueira and et al., 2008). Here, we monitored spontaneous alternations in the arms of the Y maze as a measure of working memory (Sarnyai et al., 2000) in WT and Tau-KO mice that were exposed to CUS. Statistical analysis (two-way ANOVA) of correct alternations in the Y-maze showed a clear interaction between *Stress* and *Genotype* ($F_{1,38}$ = 4.677, p = 0.037) (**Figure 1E**), with *post-hoc* analysis revealing significant differences between stressed and control WT (p = 0.041), but not Tau-KO (p = 0.998), animals. Similarly, using novel place recognition test, we found that stress decreased the (%) recognition index of WT (p = 0.048) but not of Tau-KO (p = 0.548) animals (**Figure 1F**). These findings indicate that Tau protein is essential for stress to elicit cognitive deficits. Furthermore, we found no changes in locomotor activity in animals exposed to stress from both genotypes as assessed by total distance traveled in open field (**Figure 1G**). Neuronal atrophy and synaptic loss are robust correlates of impaired cognitive behavior, including that induced by chronic stress (Cerqueira and Mailliet and et al., 2007; Sousa et al., 2012). Using unbiased 3D morphometric analysis of Golgi-impregnated pyramidal neurons, we observed a significant interaction between *Stress* and *Genotype* when total dendritic length of PFC neurons was analyzed ($F_{1,77}$ = 4.918, p = 0.030); strikingly, stress resulted in a reduction in total dendritic length in WT (p = 0.019) but not Tau-KO (p = 0.998) animals (**Figure 2A**; **Figure 2F**); no difference was found between control WT and Tau-KO animals (p = 0.627). Further analysis showed that atrophy was confined to apical dendrites, again in a manner reflecting an interaction between *Stress* and *Genotype* ($F_{1,74}$ = 4.512, p = 0.037) and with stress only causing dendritic shrinkage in WT animals (p = 0.040) (**Figure 2B**;

cf. data for basal dendrites in **Figure 2C**); both apical and basal dendritic lengths were also similar between control animals of both genotypes (p = 0.892 and p = 0.9713, respectively). Similarly, neurons of CA3 hippocampal area of WT, but not Tau-KO, animals display a significant reduction of dendritic length after exposure to chronic stress (**Supplementary Material Table 2**). These data, and in particular with respect to the role of genotype, were confirmed by Sholl analysis of neuronal dendritic tree where we monitor the number of dendritic intersections as a function of their distance from the soma (Cerqueira and Taipa and et al., 2007). 2-way ANOVA analysis revealed an interaction between *Stress* x *Genotype* at 80 μm ($F_{1,75}$ = 3,279, p = 0.07), 140 μm ($F_{1,74}$ = 3,749, p = 0.05), at 220 μm ($F_{1,75}$ = 4.469, p = 0.03), 300 μm ($F_{1,75}$ = 4.627, p = 0.03) and 320 μm ($F_{1,75}$ = 5.432, p = 0.02). In addition, a Stress effect was found at 100 μm ($F_{1,74}$ = 6.756, p = 0.01) and at 120 μm ($F_{1,75}$ = 4.132, p = 0.0456). Specifically, further analysis showed that stressed WT neurons exhibit decreased branching at 80 μm, 100 μm, 120 μm and 360 μm compared to control WTs (p_{80} = 0.021, p_{100} = 0.026, p_{120} = 0.003, p_{360} = 0.05 respectively). These differences of dendritic tree are exemplified in **Figure 2E**. When monitoring spine density in PFC neurons, we found a significant interaction between *Stress* x *Genotype* ($F_{1,88}$ = 4.991, p = 0.028); spine density was significantly lower in WT-STR mice as compared to their controls (WT-CON; p = 0.008) but significant effects of stress were not observed in Tau-KO mice (p = 0.986) (**Figure 2D**); neurons of WT and Tau-KO animals under control conditions exhibit similar spine density (p = 0,649). The above Golgi-based findings of spine loss in stressed WT animals were confirmed by TEM-based monitoring of synaptic density. As shown at Fig2 G, H, chronic stress reduced synaptic density of mPFC in WT (p < 0.0001), but not Tau-KO (p < 0.99) (*Stress x Genotype* interaction $F_{(1,\ 161)}$ =11,77; p = 0.0008); synaptic density of control animals of both genotypes were similar (p = 0.9654).

Altogether, both behavioral and neurostructural findings suggest that, while it has no clear phenotype on adult animals as previously suggested (Harada et al., 1994; Ke et al., 2012), absence of Tau attenuated the detrimental effects of chronic stress.

Tau deletion effects on PFC synaptosomal protein expression

Synaptic malfunction and atrophy represent core features of stress-driven neuroplasticity underlying stress-related pathologies. To investigate the molecular underpinnings of the

differential neuroplastic responses of WT and Tau-KO mice to stress, we compared the synaptic proteomes of control and stressed Tau-KO and WT animals. The following comparisons were performed for PFC synaptosomes using a proteomic workflow based on *in vivo* ^{15}N metabolic labeling and quantitative mass spectrometry (Frank et al., 2009; Filiou and Turck 2012; Filiou 2013): Tau-KO-CON vs. WT-CON, WT-STR vs. WT-CON, Tau-KO-STR vs. Tau-KO-CON and Tau-KO-STR vs. WT-STR.

We compared the synaptic proteomes of control (unstressed) Tau-KO and WT mice (Tau-KO-CON vs. WT-CON) and found decreased expression of three proteins in Tau-KO mice, namely tubulin beta-4A, tenascin-R and 2',3'-cyclic nucleotide 3'-phosphodiesterase (**Supplementary Material, Table S2**). Interestingly, these proteins are functionally related to cytoskeletal components; tubulin beta-4A is a core cytoskeletal protein, tenascin-R induces actin-rich microprocesses and branches along neurite shafts (Zacharias et al., 2002), and 2', 3'-cyclic nucleotide 3'-phosphodiesterase is a microtubule-associated protein (Bifulco et al., 2002). Our findings indicate that absence of Tau affects the expression of other cytoskeletal proteins adding to our limited knowledge about interrelationship of Tau and other cytoskeletal proteins in cytoskeletal integrity ((Takei et al., 2000; Ke et al., 2012).

Chronic stress alters expression of cytosolic proteins in WT PFC synaptosomes

To investigate the effect of stress in WT animals, we compared PFC synaptosomes from WT-STR vs. WT-CON mice and found eight proteins which were differentially expressed; seven of these are cytosolic and one (Aco2) is of mitochondrial localization (**Supplementary Material, Table S3**). We observed significantly decreased expression levels of Na^+/K^+- and Ca^{2+}-transporting ATPase proteins (Atp1a1, Atp2b2, both involved in neurotransmission). We also found increased expression levels of creatine-kinase B (Ckb, involved in intracellular energy transfer), clathrin heavy chain 1 and Rab GDP dissociation inhibitor alpha (Cltc and Gdi1, respectively, implicated in synaptic vesicle recycling and intracellular receptor trafficking), ubiquitin-like modifier-activating enzyme 1 (Uba1, involved in degradation regulation), and heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP-A2/B1, an RNA-binding protein implicated in transport of dendrite-localized mRNAs in activated synapses).

Chronic stress alters mitochondrial protein expression in Tau-KO PFC synaptosomes

Chronic stress resulted in the differential expression of 23 proteins in PFC synaptosomes of Tau-KO-STR vs. Tau-KO-CON mice (**Table 1**). All of these proteins were expressed at higher levels in Tau-KO-STR compared to Tau-KO-CON mice and are all mitochondrial. It should be noted that expression levels of these proteins were not affected when WT animals were exposed to stress. The differentially expressed proteins were assigned to the following categories, based on their known functions:

Electron transport chain (ETC): Increased expression of ETC subunits of complexes I (Mtnd4, Ndufs1), III (Cyc1, Uqcrc2, Uqcrfs1), IV (Cox6a) and V (Atp5a1, Atp5b) was observed in Tau-KO-STR mice.

Mitochondrial transport: Increased expression of six members of the mitochondrial carrier family Slc25 was found in Tau-KO-STR mice, including a phosphate carrier (Slc25a3), ATP/ADP translocases (Slc25a4, Slc25a5), glutamate transporters (Slc25a12, Slc25a22) and a mitochondrial carrier with unknown function (Slc25a46). Increased expression of two members of the iron transporting sideroflexin family (Sfxn1, Sfxn3) was also observed.

Moreover, the scaffold mitochondrial proteins prohibitins (Phb, Phb2), the porins Vdac1 and Vdac3, an endonuclease (Endogl1) as well as proteins involved in energy metabolism (Ckmt1, Gpd2) were also found at increased levels in Tau-KO-STR mice.

In addition, to investigate how the absence of Tau affects stress response, we then compared the PFC synaptosome proteomes of Tau-KO-STR vs. WT-STR mice. We found 29 differentially expressed proteins (**Table 2**), 25 of which are located in mitochondria and are mainly classified in two functional categories:

Electron transport chain (ETC): Increased expression of ETC subunits of complexes I (Mtnd4, Ndufa5, Ndufa9, Ndufs1, Ndufs2, Ndufs6, Ndufv2), III (Cyc1, Uqcrc2, Uqcrfs1) and V (Atp1a2, Atp5b, Atp5a1) was observed in Tau-KO-STR mice.

Mitochondrial transport: Five differentially expressed proteins in Tau-KO-STR mice belong to the Slc25 mitochondrial carrier family, including Slc25a3, Slc25a4, Slc25a5, Slc25a22 (also found in higher levels in Tau-KO-STR compared to Tau-KO-CON PFC synaptosomes), as well as

the 2-oxoglutarate/malate carrier protein Slc25a11. The Sfxn1 and Sfxn5 sideroflexin members were also higher expressed.

Results of the comparative proteomic analysis are summarized in **Table 3** where all differentially expressed proteins across group comparisons are categorized according to subcellular localization. As shown at Table 3. mitochondrial proteins are prominently affected in synaptosomal fractions of Tau-KO mice both when compared to unstressed Tau-KO and stressed WT mice.

As previous evidence suggests that Tau deletion could be protective against disruption of mitochondria motility in neuronal dendrites (Zempel et al., 2013) and axons (Vossel et al., 2010; Vossel et al., 2015), we next monitor the mitochondria density in mPFC synapses using TEM microscopy (Figure 3). While we found no differences between WT and Tau-KO under control (non-stressed) conditions (p=0.934), stressed Tau-KO, but not WT, animals exhibited increased mitochondria density in postsynaptic compartment (p=0.019) indicating synaptic enrichment of mitochondria in Tau-KO after stress exposure [2 way-ANOVA ($F_{1,158}$= 6.345, p = 0.012)]

Discussion

Tau is a cytoskeletal protein implicated in various neuronal processes such as microtubule stabilization, axonal maintenance and transport while recent evidence suggest novel function for Tau in synaptic signaling and structure (Gotz et al., 2013). Despite the essential role of Tau on regulating cytoskeletal assembly and dynamics, evidence based on conventional Tau-KO models from different research teams (including ours) suggests that young/adult Tau-KO animals has no obvious behavioral or neuronal alterations (Dawson et al., 2001; Ke et al., 2012; Morris et al., 2013; Ma et al., 2014) (Lopes et al., Aging Cell in press) and neurons of animals lacking Tau do not exhibit axonal abnormalities (Yuan et al., 2008; Vossel et al., 2010). Accordingly, our current behavioral analysis show no cognitive or locomotive deficits in adult (4-5 months old) Tau-KO mice compared to WTs accompanied by absence of structural differences of PFC neurons as assessed by 3D neuronal reconstruction and TEM analyses. However, this luck of obvious phenotype in Tau-KO animals may be attributed to the occurrence of compensatory mechanisms due to altered expression of other cytoskeletal proteins (e.g. MAPs) (Harada et al., 1994; Dawson et al., 2001; Ma et al., 2014). Accordingly, our proteomic data provide novel evidence about alterations in cytoskeletal proteins of adult Tau-KO animals (e.g. tubulin 4A) adding further

support to the close interrelationship between Tau and other proteins on cytoskeletal integrity as previously suggested (Takei et al., 2000).

Previous evidence suggests that Tau ablation has a neuroprotective role against neurotoxic insults such as amyloid beta (Aβ) (Rapoport et al., 2002; Roberson et al., 2007). The present study shows that, although WT and Tau-KO mice do not differ in their overt responses to chronic stress (endocrine, body weight), Tau-KO mice are protected against the well-known central effects of stress, namely, impairment of working memory and dendritic remodeling and synaptic loss in the PFC (Cerqueira and Mailliet and et al., 2007; Sousa et al., 2012; McEwen et al., 2013). Furthermore, the altered levels of PFC synaptic mitochondrial proteins between Tau-KO-STR and WT-STR animals provide novel insights into the molecular and cellular events that underlie stress-triggered neuronal atrophy and cognitive dysfunction. The neuroplastic effects of stress on brain structure and function are causally linked to elevated levels of glucocorticoids (GCs) (Cerqueira and Mailliet and et al., 2007; Sousa et al., 2012) as the current and previous studies demonstrate that both chronic stress and elevated GC levels selectively induce morphological rearrangements in proximal and distal portions of apical dendrite of pyramidal neurons in PFC without affecting the structure of basal dendrites (Cerqueira and Taipa and et al., 2007). With the exception of a few studies (Cereseto et al., 2006), the effects of stress on cytoskeleton proteins have received little attention, despite the fact that the cytoskeleton is important for maintaining neuronal architecture and function (Morris et al., 2011). Extending our previous demonstration that chronic stress and/or GCs lead to the abnormal hyperphosphorylation of Tau protein (Sotiropoulos and Catania and et al., 2008; Sotiropoulos et al., 2011), the present findings show that Tau protein plays a key role in stress-induced dendritic remodeling, synaptic loss and cognitive impairment. Recently, localization of the Tau protein at the synapses has been reported where Tau is suggested to be involved in the NMDA signaling (Ittner et al., 2010; Gotz et al., 2013). These new data on the relationship between Tau protein and NMDA receptors support the results of earlier work suggesting the essential role of NMDA, but not AMPA, receptors in stress-triggered morphofunctional alterations in neurons (Magarinos and McEwen 1995, 1995; Pawlak et al., 2005; Martin and Wellman 2011). Indeed, apical dendrites, which display stress-evoked atrophy, are enriched in GluN2B-contaning NMDA receptors (Rudolf et al., 1996) whereas AMPA receptors, known to offer neuroprotection against glutamate (Wu et al., 2004), are clustered in basal dendrites and soma (Vickers et al., 1993).

While chronic stress is known to lead to dendritic remodeling and synaptic atrophy in the PFC of WT mice (Cerqueira and Mailliet and et al., 2007; McEwen et al., 2013), there is a conspicuous lack on information on the contributing pathways and mechanisms. The present quantitative proteomic analysis of PFC synaptosomes now reveals that stress results in the differential expression of a number of cytosolic proteins located within synapses. We observed decreased expression of two membrane proteins, ATPases Atp1a1 and Atp2b2, which are implicated in synaptic homeostasis, neurotransmission and buffering of neurons against Ca^{2+}-dependent excitotoxic damage. Decreased ATPase levels are involved in synaptic pathology related to the neurotoxic peptide amyloid beta and Ca^{2+} in Alzheimer's disease (AD) patients and mouse models of the disease (Chauhan et al., 1997; Vitvitsky et al., 2012), while ATPases have been suggested to offer neuroprotection against AD pathology (Lu et al., 2014). Here, it is pertinent to recall that previous studies revealed that chronic stress and stress hormones (glucocorticoids) increase production of amyloid beta (Aβ), a peptide linked to AD related to synaptic malfunction and atrophy (Catania et al., 2009). Reduced levels or absence of processed Atp1a1 have been associated with hypersecretion of corticosterone (Moseley et al., 2007; Mozhui et al., 2010). Among the proteins with increased expression in WT-STR compared to WT-CON mice, Rab GDP dissociation inhibitor alphaGdi1 has been reported as a candidate marker of stress reactivity in a chronic mild stress rat model of depression (Bisgaard et al., 2012) whereas increased creatine kinase B-type (Ckb) levels were found in acutely stressed rats (Yang et al., 2014). Generation of overexpressing or knock-out mouse models for the proteins with altered expression levels identified here and their subsequent characterization would shed light on the functional interdependencies of these proteins on synaptic atrophy and malfunction.

Our results demonstrate that Tau is essential for the manifestation of the effects of stress on dendritic and synaptic atrophy in the PFC. In order to obtain an insight into the underlying molecular processes, we compared the PFC synaptosomes of stressed and control Tau-KO animals using a state-of-the-art comparative proteomic approach that provides high relative quantification accuracy (Filiou and Turck 2012; Filiou 2013). Synaptosomes are artificially isolated synapses that also include cytosol, membranes and mitochondria (Schrimpf et al., 2005). The specificity of the synaptosome enrichment protocol has been previously addressed (Filiou et al., 2010). We observed that while stress alters the expression levels of mainly cytosolic proteins in the synaptosomal fraction of WT mice, chronic stress only affected mitochondrial proteins in the synaptosomes of Tau-KO mice (**Table 3**). This finding strongly suggests that

stress-induced neuronal damage and behavioral impairments depend on an interaction between Tau and mitochondrial proteins. Oxidative phosphorylation (ETC complexes I, III, IV and V) and mitochondrial transport (Slc25 and sideroflexin family) were the main processes affected by chronic stress in Tau-KO mice. Interestingly, these two processes were also affected when stressed Tau-KO and WT mice were compared. Mitochondrial dysfunction and alterations in ETC complexes have been linked to pathological oxidative stress and apoptotic pathways (Einat et al., 2005; Szego et al., 2010) and is implicated in psychiatric disorders in which stress is a main etiopathogenic factor (Shao et al., 2008; Sousa et al., 2012). Indeed, altered expression of proteins involved in oxidative phosphorylation and mitochondrial transport have been reported in mice selectively bred for high vs. low anxiety (Filiou 2011), transgenic mice with schizophrenia-like symptoms (Otte 2011; Filiou and Teplytska and et al., 2012) and in patients suffering from major psychiatric disorders (Gardner et al., 2003; Rezin et al., 2009).

Our previous work showed that chronic stress increases the generation of Aβ in WT animals and results in Tau hyperphosphorylation (Catania et al., 2009; Sotiropoulos et al., 2011). The latter has been recently reported to damage mitochondria mobility (Shahpasand et al., 2012). Indeed, Tau ablation was shown to prevent Aβ-induced defects of mitochondrial motility in dendrites (Zempel et al., 2013) and axons (Vossel et al., 2010; Vossel et al., 2015) as mitochondria movement in neurons lacking Tau remained normal after Aβ treatment. In agreement with the above view, our TEM-based findings suggest the exposure to stress in Tau-KO resulted in enriched synaptic localization of mitochondria, an effect that was attenuated in WT animals. Based on this, it is highly plausible that stress-induced Tau hyperphosphorylation attenuates synaptic mitochondrial localization and subsequent proteome changes after stress (MacAskill et al., 2009). Notably, Tau malfunction-associated neuronal damage has been suggested to involve mitochondria (Atlante et al., 2008; McInnes 2013) and a causal link between mitochondrial, abnormal Tau phosphorylation and impaired synaptic plasticity in AD pathology has been previously proposed (Lee et al., 2012; Schulz et al., 2012). The present findings add support to previous suggestions that Tau reduction or prevention of Tau malfunction could be neuroprotective (Roberson et al., 2007; Vossel et al., 2010) introducing both Tau and synaptic mitochondria in the underlying mechanisms of stress-induced synaptic damage and neuronal malfunction. Indeed, it is suggested that mitochondria play a key role in conserving synapses (Cho et al., 2009) while disruption of mitochondrial proteins has been shown to reduce dendritic arborization (without major changes in axon morphology) (Chihara et al., 2007), indicating a

critical role of mitochondria in neuronal architecture. Furthermore, besides their role in generating energy necessary for synaptic maintenance and activity, mitochondria are also involved in Ca^{2+} homeostasis and signaling, play a role in cytoprotection and neuronal plasticity (Toescu and Verkhratsky 2004; Cheng et al., 2010) and are dynamically regulated by GC (Du et al., 2009). Altogether, the above findings suggest an association between the absence of synaptic loss and enriched mitochondria at synapses of stressed Tau-KO animals.

In summary, the current study demonstrates a lack of stress-induced neuronal atrophy and cognitive deficits in the absence of Tau protein, providing a solid proof that Tau plays an essential role in the stress-directed orchestration of cellular cascades involved in dendritic and synaptic atrophy/loss and subsequent cognitive deficits. The implication of mitochondria in the mediation of stress effects via Tau protein encourage exploration of the potential importance of mitochondrial pathways in the search for means to prevent, delay or treat neurodegenerative conditions; it is worth noting that mitochondrial pathways have already been shown to be amenable to pharmacological manipulation in various disease settings (Fulda et al., 2010; Edeas and Weissig 2013).

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Figures

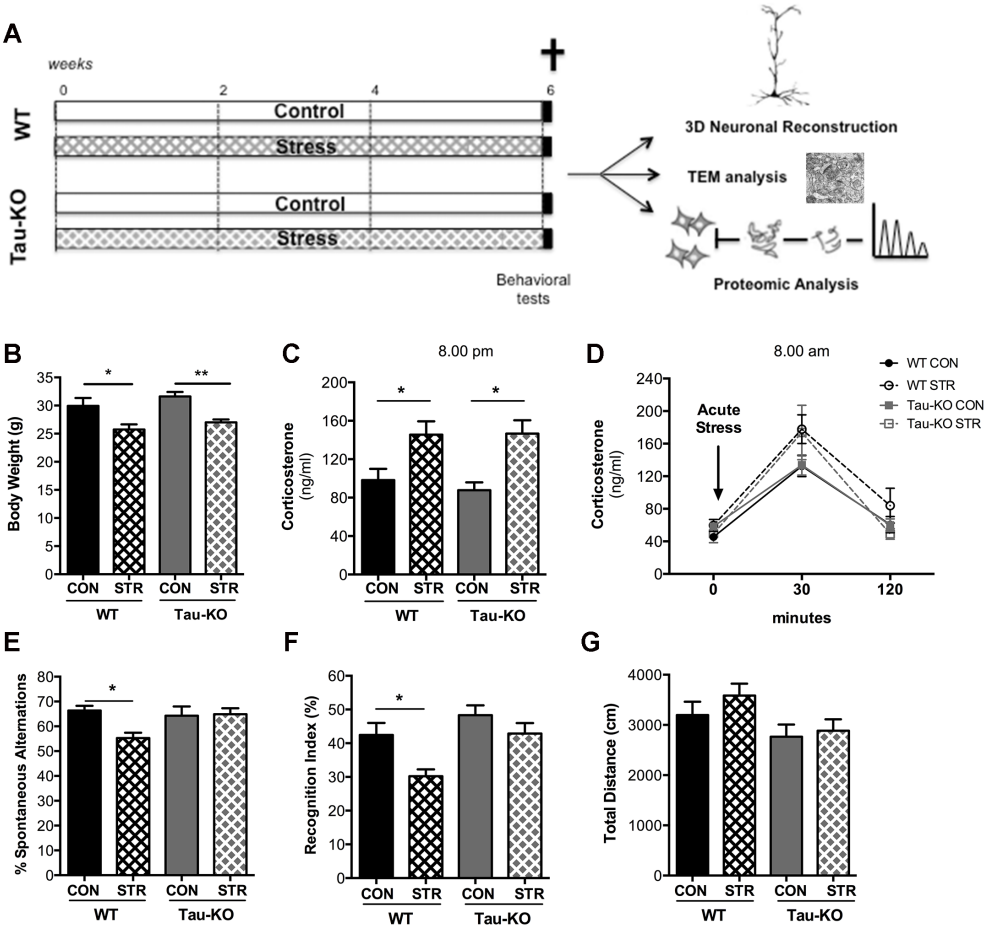

Figure 1: Tau deletion prevents stress-induced decline in working memory without interfering with endocrine response to stress

A. The experimental design is represented schematically. Control and stressed WT and Tau-KO mice were used. Analysis included behavioral, neurostructural, TEM and quantitative proteomic analyses. **B.** Exposure of WT and Tau-KO animals to 6 weeks of chronic stress (Stress; STR) exhibited lower weight gain, as compared to non-stressed (Control; CON) counterparts. **C.** Chronic stress resulted in elevated circulating corticosterone levels in both WT and Tau-KO animals (measured at usual peak of corticosterone secretion, ZT12/08:00 p.m). **D.** Previously stressed animals of both genotypes (WT and Tau-KO) respond similarly to an acute stressor (3-4 min restraint); both groups showed peak responses 30 min after the stressor, and displayed basal levels of corticosterone secretion after 120 min. **E.** WT-STR animals exhibited a reduced percentage of spontaneous alternations in the arms of a Y-maze, as compared to WT-CON animals. This deficit in working memory was not observed in Tau-KO animals. **F.** Similarly, stress reduced % recognition index in WT but not in Tau-KO animals indicating deficits of short-term memory only in WT mice. **G.** Total distance traveled in open field apparatus did not differ in animal groups of both genotypes under stress and control conditions. All numerical data are shown as mean ± SEM. (* $p < 0.05$; **$p < 0.001$).

Abbreviations: WT CON: wild-type control; WT-STR: wild-type stressed Tau-KO-CON: Tau knock-out control; Tau-KO-STR: Tau knock-out stressed

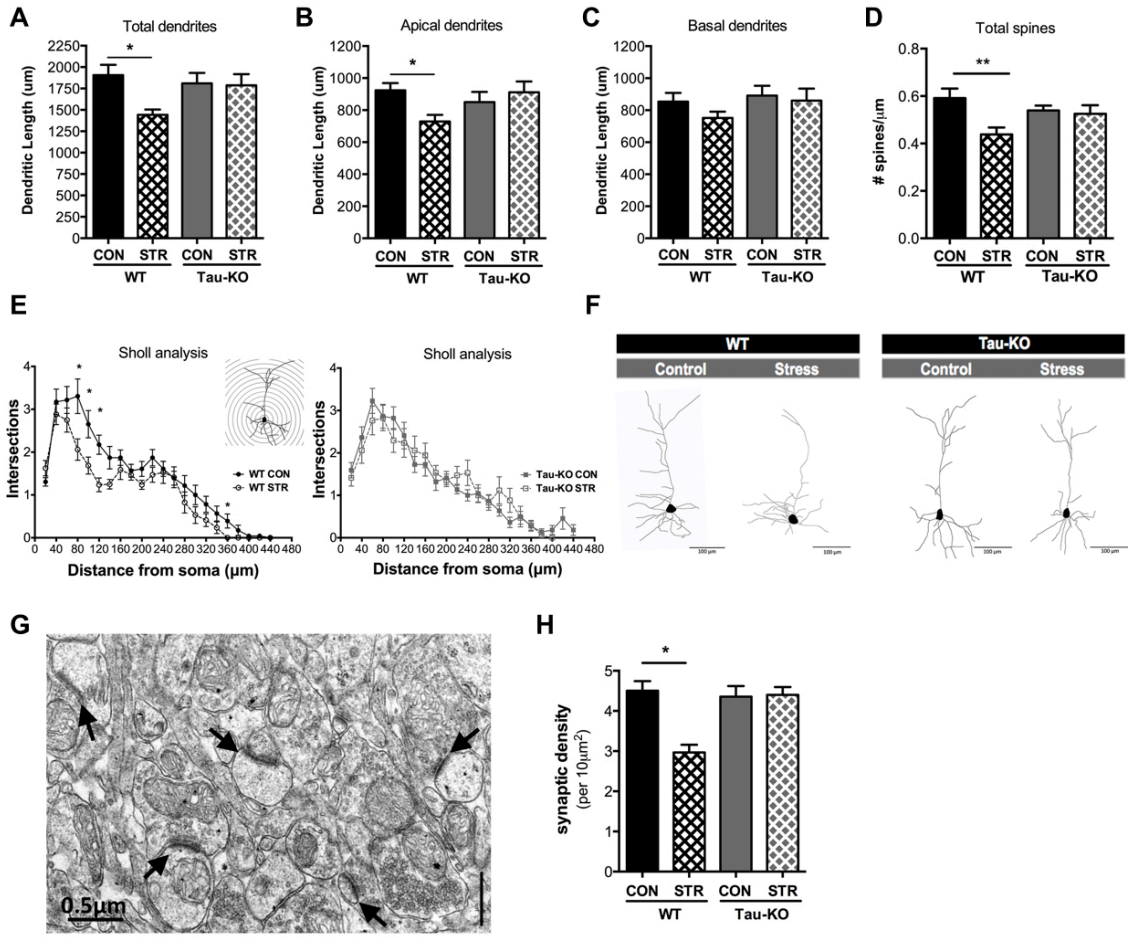

Figure 2: Stress-induced neuronal atrophy and synaptic loss depends on Tau protein
A-B. Exposure to chronic stress resulted in reduced total dendritic length in PFC neurons of WT but not Tau-KO animals (**A**); atrophy was pronounced in apical (**B**) but not basal (**C**) dendrites. **D.** Spine density was reduced in WT-STR but not in Tau-KO-STR mice, as compared to their respective unstressed controls. **E.** Sholl analysis demonstrated a reduction of dendritic arborization in neurons of WT-STR compared to WT-CON mice; no marked differences were found between neurons of Tau-KO-STR and Tau-KO-CON mice. **F.** 3D reconstruction of neurons from control (CON) and stressed (STR) WT and Tau-KO animals. **G.** TEM microphoto of layers II and III of mPFC with postsynaptic density (PSD) marked with arrow. **H.** Chronic stress decreased synaptic density in WT, but not Tau-KO animals. All numerical data are shown as mean ± SEM. (* $p < 0.05$; **$p < 0.001$).
Abbreviations: WT CON: wild-type control; WT-STR: wild-type stressed Tau-KO-CON: Tau knock-out control; Tau-KO-STR: Tau knock-out stressed

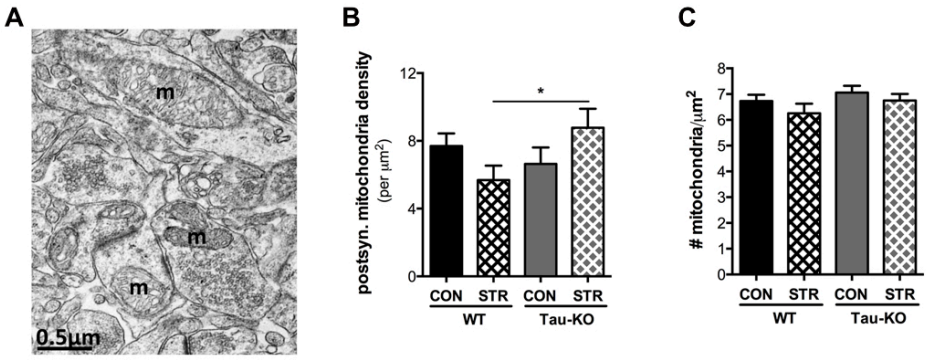

Figure 3: Chronis stress results in enriched synaptic density of mitochondria in Tau-KO, but not WT animals.

A. TEM microphoto showing the presence of mitochondria (m) at synapses. **B-C.** Exposure to chronic stress in Tau-KO, but not WT, animals resulted in increased postsynaptic density of mitochondria (B) whereas total mitochondria density was not altered among groups (C). All numerical data are shown as mean ± SEM. (* $p < 0.05$).

Tables

Table 1: Stress-driven changes in PFC synaptosomes of WT mice (WT-STR vs. WT-CON).

WT-STR/ WT-CON abundance ratio	Adjusted p-value	Protein name	Protein full name	Uniprot IDs
1.52	0.028390278	Aco2	Aconitate hydratase, mitochondrial	ACON_MOUSE
0.76	7.95385E-05	Atp1a1	Sodium/potassium-transporting ATPase subunit alpha-1	AT1A1_MOUSE
0.76	0.006002401	Atp2b2	Plasma membrane calcium-transporting ATPase 2	Q3UHH0_MOUSE, AT2B2_MOUSE, Q3UHJ3_MOUSE
1.62	2.55587E-10	Ckb	Creatine kinase B-type	KCRB_MOUSE
1.32	0.000642042	Cltc	Clathrin heavy chain 1	Q80U89_MOUSE, Q5SXR6_MOUSE, CLH_MOUSE
1.62	0.009163899	Gdi1	Rab GDP dissociation inhibitor alpha	GDIA_MOUSE
2.83	8.70414E-05	HnRNPA2b1	Heterogeneous nuclear ribonucleoprotein A2/B1	B7ZP22_MOUSE, ROA2_MOUSE
1.62	0.009163899	Uba1	Ubiquitin-like modifier-activating enzyme 1	UBA1_MOUSE, B9EHN0_MOUSE

Table 2: Stress-evoked protein expression changes in PFC synaptosomes of Tau-KO mice (Tau-KO-STR vs. Tau-KO-CON)

Tau-KO-STR/ Tau-KO-CON abundance ratio	Adjusted p-value	Protein name	Protein full name	Uniprot IDs
1.41	2.60577E-06	Atp5a1	ATP synthase subunit alpha, mitochondrial	ATPA_MOUSE
1.41	2.60577E-06	Atp5b	ATP synthase subunit beta, mitochondrial	ATPB_MOUSE
1.62	2.60577E-06	Ckmt1	Creatine kinase U-type, mitochondrial	Q545N7_MOUSE, KCRU_MOUSE
1.62	0.018849463	Cox6c	Cytochrome c oxidase subunit 6C	COX6C_MOUSE
1.41	0.005100139	Cyc1	Cytochrome c1, heme protein, mitochondrial	CY1_MOUSE
1.74	0.014929679	Endogl1	Nuclease EXOG, mitochondrial	EXOG_MOUSE
1.41	0.00140646	ENSMUSG00000064363, ENSMUSG00000065947, Mtnd4,ND4,Nd4	NADH-ubiquinone oxidoreductase chain 4	NU4M_MOUSE, Q7JCZ9_MOUSE, Q5GA75_MUSMM, B9V1K8_MOUSE, Q7JCY6_MOUSE, A3R4A0_MUSMM, Q9ME04_MOUSE
1.41	0.005119749	Gpd2	Glycerol-3-phosphate dehydrogenase, mitochondrial	GPDM_MOUSE
1.32	0.004233795	Ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Q3UQ73_MOUSE
1.52	0.009622831	Phb	Prohibitin	PHB_MOUSE
1.52	0.005160328	Phb2	Prohibitin-2	PHB2_MOUSE, Q3V235_MOUSE
1.52	0.020752947	Sfxn1	Sideroflexin-1	SFXN1_MOUSE
1.32	0.036446008	Sfxn3	Sideroflexin-3	SFXN3_MOUSE
1.41	0.00140646	Slc25a12	Calcium-binding mitochondrial carrier protein Aralar1	CMC1_MOUSE
1.62	7.08077E-05	Slc25a22	Mitochondrial glutamate carrier 1	GHC1_MOUSE
1.41	4.7627E-05	Slc25a3	Phosphate carrier protein, mitochondrial	MPCP_MOUSE, Q3THU8_MOUSE
1.41	1.2941E-05	Slc25a4	ADP/ATP translocase 1	ADT1_MOUSE, Q8BVI9_MOUSE
1.87	0.040072784	Slc25a46	Solute carrier family 25 member 46	S2546_MOUSE
1.52	0.006828915	Slc25a5	ADP/ATP translocase 2	ADT2_MOUSE, Q545A2_MOUSE
1.41	0.001477532	Uqcrc2	Cytochrome b-c1 complex subunit 2, mitochondrial	QCR2_MOUSE
1.41	0.001124486	Uqcrfs1	Cytochrome b-c1 complex subunit Rieske, mitochondrial	UCRI_MOUSE
1.41	5.95591E-05	Vdac1	Voltage-dependent anion channel 1	Q3THL7_MOUSE
1.41	0.033850501	Vdac3	Voltage-dependent anion channel 3	Q5EBQ0_MOUSE

Table 3: Stress-evoked protein expression changes between Tau-KO and WT mice (Tau-KO-STR vs. WT-STR).

Tau-KO-STR/ WT-STR abundance ratio	Adjusted p-value	Protein name	Protein full name	Uniprot IDs
0.71	0.047353553	Aco2	Aconitate hydratase, mitochondrial	ACON_MOUSE
1.52	0.007560066	Anxa5	Annexin A5	ANXA5_MOUSE
1.32	1.92521E-05	Atp1a2	Na/K-transporting ATPase subunit alpha-2	Q6ZQ49_MOUSE, Q3UHK5_MOUSE, AT1A2_MOUSE
1.41	1.92521E-05	Atp5a1	ATP synthase subunit alpha, mitochondrial	ATPA_MOUSE
1.41	2.36468E-07	Atp5b	ATP synthase subunit beta, mitochondrial	ATPB_MOUSE
0.71	6.36725E-05	Ckb	Creatine kinase B-type	KCRB_MOUSE
1.32	0.02713824	Ckmt1	Creatine kinase U-type, mitochondrial	Q545N7_MOUSE, KCRU_MOUSE
1.32	0.045709527	Cyc1	Cytochrome c1, heme protein, mitochondrial	CY1_MOUSE
1.41	0.01299402	ENSMUSG00000064363, ENSMUSG00000065947, Mtnd4,ND4,Nd4	NADH-ubiquinone oxidoreductase chain 4	NU4M_MOUSE, Q7JCZ9_MOUSE, Q5GA75_MUSMM, B9V1K8_MOUSE, Q7JCY6_MOUSE, A3R4A0_MUSMM, Q9ME04_MOUSE
1.32	0.02713824	Gpd2	Glycerol-3-phosphate dehydrogenase, mitochondrial	GPDM_MOUSE
0.31	0.000797218	Hnrnpa2b1	Heterogeneous nuclear ribonucleoproteins A2/B1	B7ZP22_MOUSE, ROA2_MOUSE
0.66	0.02713824	Hspd1	60 kDa heat shock protein, mitochondrial	CH60_MOUSE
0.57	0.00033385	Mtap2	Microtubule-associated protein 2	B2KGT6_MOUSE
1.52	0.010772537	Ndufa5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	NDUA5_MOUSE
1.41	0.002434666	Ndufa9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	Q6GTD3_MOUSE
1.41	0.000833242	Ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Q3UQ73_MOUSE
1.41	0.0335448	Ndufs2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial	NDUS2_MOUSE
1.74	0.028572087	Ndufs6	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	NDUS6_MOUSE
1.74	0.030732182	Ndufv2	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	NDUV2_MOUSE
1.52	0.01299402	Sfxn1	Sideroflexin-1	SFXN1_MOUSE
1.52	0.030732182	Sfxn5	Sideroflexin-5	SFXN5_MOUSE
1.41	0.030732182	Slc25a11	Mitochondrial 2-oxoglutarate/malate carrier protein	Q5SX53_MOUSE, M2OM_MOUSE
1.52	0.007560066	Slc25a22	Mitochondrial glutamate carrier 1	GHC1_MOUSE
1.32	0.002663516	Slc25a3	Phosphate carrier protein, mitochondrial	MPCP_MOUSE, Q3THU8_MOUSE
1.41	0.000151019	Slc25a4	ADP/ATP translocase 1	ADT1_MOUSE, Q8BVI9_MOUSE
1.41	0.025563981	Slc25a5	ADP/ATP translocase 2	ADT2_MOUSE, Q545A2_MOUSE
1.32	0.04343625	Slc3a2	4F2 cell-surface antigen heavy chain	4F2_MOUSE
1.32	0.03623825	Uqcrc2	Cytochrome b-c1 complex subunit 2, mitochondrial	QCR2_MOUSE
1.32	0.030732182	Uqcrfs1	Cytochrome b-c1 complex subunit Rieske, mitochondrial	UCRI_MOUSE

Table 4: Summary of differentially expressed proteins across all group comparisons. Proteins are presented according to their subcellular localization.

Protein name	Tau-KO vs. WT	WT-Str vs. WT	Tau-KO-Str vs. Tau-KO	Tau-KO-Str vs. WT-Str
mitochondria-related proteins				
Atp5a1			↑	↑
Atp5b			↑	↑
Ckmt1			↑	↑
Cox6c			↑	
Cyc1			↑	↑
Endogl1			↑	
Gpd2			↑	↑
Hspd1				↓
Nd4			↑	↑
Ndufa5				↑
Ndufa9				↑
Ndufs1			↑	↑
Ndufs2				↑
Ndufs6				↑
Ndufv2				↑
Phb			↑	
Phb2			↑	
Sfxn1			↑	↑
Sfxn3			↑	
Sfxn5				↑
Slc25a11				↑
Slc25a12			↑	
Slc25a22			↑	↑
Slc25a3			↑	↑
Slc25a4			↑	↑
Slc25a46				↑
Slc25a5			↑	↑
Uqcrc2			↑	↑
Uqcrfs1			↑	↑
Vdac1			↑	
Vdac3			↑	
Aco2		↑		↓
cytosol/membrane-related proteins				
Anxa5				↑
Atp1a1		↓		
Atp1a2				
Atp2b2		↓		
Ckb		↑		↓
Cltc		↑		
Gdi1		↑		
Hnrnpa2b1		↑		↓
Slc3a2				↑
Uba1		↑		
cytoskeleton-related proteins				
Cnp	↓			
Mtap2				↓
Tnr	↓			
Tubb4	↓			

Supplementary Material

Materials and Methods

Mass Spectrometry

Samples were loaded onto an in house packed fused silica 3 μm RP-C18 column (Maisch, Monheim, Germany) followed by a 20min wash with 0.1% HCOOH and elution with a 95% AcN/ 0.1% HCOOH gradient from 2% to 45% over 90 min at a flow rate of 200 nl/min. All other MS parameters were as previously described (Filiou *et al*, 2010).

Proteomic data analysis

MS raw files were analyzed as previously described (Filiou *et al*, 2012a). In brief, data were searched twice against a concatenated decoy Swiss Prot mouse database v 3.46 using BioWorks v 3.3.1 (ThermoFisher Scientific, San Jose, CA) and SEQUEST v 28 (ThermoFisher Scientific, San Jose, CA). Parameters for SEQUEST search were described previously (Filiou *et al*, 2011). DTA select v 1.9 was used to filter and assemble peptides into proteins. Ion chromatograms were extracted and peptide/protein ratios were calculated by ProRata v 1.0 (Pan *et al*, 2006). Non-synaptosomal protein contaminants with altered protein levels were not included. Proteins with fold change > 1.3 and $p < 0.05$ (corrected for multiple testing) were considered differentially expressed. The MS raw data and the corresponding protein quantification files are available upon request.

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Table S1: Protein expression changes in PFC synaptosomes between Tau-KO-CON and WT-CON mice.

Tau-KO-CON / WT-CON abundance ratio	Adjusted p-value	Protein name	Protein full name	Uniprot IDs
0.54	0.00109632	Tubb4	Tubulin beta-4A chain	TBB4_MOUSE
0.57	0.02120841	Tnr	Tenascin-R	A2RT70_MOUSE, TENR_MOUSE
0.76	0.046446354	Cnp	2',3'-cyclic-nucleotide 3'-phosphodiesterase	CN37_MOUSE, Q3TYV5_MOUSE

Chapter 2.2

Lopes S, Vaz-Silva J, Pinto V, Dalla C, Kokras N, Bedenk BT, Mack N, Czisch M, Almeida OFX, Sousa N, Sotiropoulos I

Tau protein is essential for stress-induced brain pathology

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Tau protein is essential for stress-induced brain pathology

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Exposure to chronic stress is frequently accompanied by cognitive and affective disorders in association with neurostructural adaptations. Chronic stress was previously shown to trigger Alzheimer-like neuropathology, which is characterized by Tau hyperphosphorylation and missorting into dendritic spines followed by memory deficits. We here demonstrate that stress-driven hippocampal deficits in wild-type mice are accompanied by synaptic missorting of Tau and enhanced Fyn/GluN2B-driven synaptic signaling. In contrast, mice lacking *Tau* (Tau-KO) do not exhibit stress-induced pathological behaviors and atrophy of hippocampal dendrites or deficits of hippocampal connectivity. These findings implicate Tau as an essential mediator of the adverse effects of stress on brain structure and function.

The cytoskeletal protein Tau is implicated in the establishment of Alzheimer's disease (AD)[1] as well as excitotoxicity[1] and, more recently, epilepsy[2-4]. Exposure to stressful conditions induces depressive behavior and memory deficits in both rodents and humans[5-11]. Studies in rodents have shown that chronic stress triggers Tau hyperphosphorylation, a key pathogenic mechanism in AD, and results in cognitive and mood deficits[12-16], albeit the absence of direct evidence for a role of Tau in stress-evoked brain pathology. Given that Tau plays an important role in regulating neuronal architecture and function through its interaction with various cellular targets (e.g. tubulin, Fyn)[17], we hypothesized that Tau mediates the deleterious actions of stress on brain structure and function.

To test the above hypothesis, we compared the impact of chronic unpredictable stress (CUS)[14, 18] in mice carrying a null mutation of the *Tau* gene (Tau-KO)[19] with their wild-type (WT) littermates. Three well-characterized behavioral endpoints (cognitive, mood and anxiety) that are disrupted by CUS served as the primary assay endpoints; these were complemented with measures of hippocampal structural and functional integrity. The hippocampus is a central component of the neurocircuitries that control these behaviors and displays overt lesions in both stress- and Tau-related pathologies; in the latter, the hippocampus is one of the earliest brain regions to show signs of neurodegeneration[1, 6, 9, 13-16, 20].

Results

Deleterious effects of stress on memory and mood are abrogated in the absence of Tau protein

Cognition, mood and anxiety are inter-dependent behavioral domains that exhibit complex interactions[7]. Different forms of memory were assessed after exposure of WT and Tau-KO mice to the CUS paradigm; the test battery included the Y-maze, Morris water maze (MWM) and the novel object recognition test (NOR). Anxiety was evaluated using the elevated plus maze (EPM), whereas depression-like behavior was assessed using the forced swim test (FST) and the sucrose consumption test (SCT).

Two-way ANOVA analysis of the Y-maze data revealed *CUS X Genotype* interactions for both, distance travelled [$F_{(1,65)} = 4.024$; $p = 0.04$] and time spent [$F_{(1,65)} = 4.614$; $p = 0.03$] in the novel

arm of the apparatus. Exposure to CUS resulted in deficits in spatial memory in WT (p_{dist} = 0.02; p_{time} = 0.02), but not Tau-KO (p_{dist} = 0.98; p_{time} = 0.95), mice; no differences were found between WT and Tau-KO control (non-stressed) animals (p_{dist} = 0.84; p_{time} = 0.77) (Fig. 1a, Supplementary Fig. 1). Total distance travelled in the three arms of the maze did not differ between any of the groups (Fig. 1b). Results from the MWM *test c*onfirmed that CUS induces impairments in spatial learning/memory in WT, but not Tau-KO, mice (significant *CUS x Genotype* interaction in distance swum to reach the escape platform [$F_{(1,35)}$ = 7.467; p = 0.01]); CUS increased the distance swam in WT mice only ($p \leq 0.05$) (Fig. 1c). The NOR test showed that recognition memory was also disrupted by CUS in WT, but not Tau-KO, mice. Specifically, we found a *CUS x Genotype* interaction [$F_{(1,37)}$ = 4.387; p = 0.04] on the discrimination index. Importantly, this index was reduced by CUS in WT (p_{WT} = 0.01), but not Tau-KO (p_{KO} = 0.83), mice; control WT and Tau-KO mice did not differ on this parameter (p = 0.60) (Fig. 1d).

Testing in the EPM (Fig. 1e) showed that CUS elicits an anxiogenic phenotype in WT, but not Tau-KO, animals (2-way ANOVA, *CUS x Genotype* interaction: $F_{(1,84)}$ = 4.004; p = 0.04; overall effect of CUS [$F_{(1,84)}$ = 6.296; p = 0.01], with WT animals spending significantly less time in the open arms of the maze (p_{WT} = 0.01 *vs.* p_{KO} = 0.98). Further, use of the FST to assess learned helplessness, an index of depressive-like behavior in rodents, revealed a *CUS x genotype* interaction [$F_{(1,73)}$ = 4.071; p = 0.04] and an overall effect of CUS [$F_{(1,73)}$ = 6.229; p = 0.01] on time of immobility. Furthermore, CUS-treated WT mice displayed longer periods of immobility (p = 0.01) than Tau-KO (p = 0.98) mice, as compared to their respective non-CUS-treated (control) counterparts (Fig. 1f); these findings were confirmed by measuring latency to immobility (Supplementary Fig. 2). Results from the SCT, which provides an index of anhedonia (a cardinal symptom of depression in humans), showed a significant *CUS x Genotype* interaction [$F_{(1,30)}$ = 5.906; p = 0.02], with post hoc analysis revealing reduced sucrose consumption in WT (p = 0.03), but not Tau-KO (p = 0.94), mice (Fig. 1g – right panel).

The above sets of data demonstrate that Tau-KO mice are resistant to the memory-, mood- and anxious behavior-impairing effects of CUS.

Tau deletion does not interfere with the endocrine response to stress

The ability of chronic stress to interfere with cognitive and affective functions is largely attributable to the actions of glucocorticoids (e.g. corticosterone; CORT) which are released in response to stress[8]. In contrast to the genotype-specific behavioral responses to CUS, both WT and Tau-KO mice displayed similar elevations in blood CORT and body mass loss following the CUS paradigm (Supplementary Table I) and responded to an acute stressor (4 min restraint) with similar increases in CORT secretion (Supplementary Table II). These observations suggest that the above Tau-dependent detrimental effects of stress on behavior are not due to differential regulation of the endocrine response to stress in WT and Tau-KO animals.

Tau ablation attenuates stress-induced disruption of neuronal connectivity

Reductions in monoaminergic tone, specifically of noradrenaline (NA) and serotonin (5-HT), play a central role in mediating the detrimental effects of stress on cognition and mood[20,21]. Consistent with the failure of CUS to generate anxiety- and depressive-like behavior in Tau-KO mice, NA and 5-HT levels in the hippocampus were found to be altered in WT, but not Tau-KO, mice (Fig. 2). Specifically, we detected significant *Genotype* x *CUS* interactions [$F_{(1,16)}$NA = 7.639; p = 0.01; $F_{(1,16)}$5-HT = 6.954; p = 0.02], and post hoc analysis revealed significant CUS-induced reductions in hippocampal monoamine levels in WT (NA: p = 0.02; 5-HT: p = 0.04) vs. no changes in Tau-KO (NA: p = 0.96; 5-HT: p = 0.97) animals (Fig. 2a, b). Genotype did not influence the effect of CUS on NA and 5-HT levels (NA: p = 0.95; 5-HT: p = 0.97). Calculations of 5-HT turnover ratio from parallel measurement of the 5-HT metabolite, 5-hydroxyindole acetic acid (5-HIAA) confirmed that CUS altered 5-HT turnover in WT (p = 0.04), but not Tau-KO (p = 0.99), animals, indicated by an interaction effect between *CUS* and *Genotype* [$F_{(1,16)}$ = 4.628; p = 0.04] (Supplementary Fig. 3). Monitoring hippocampal levels and turnover of dopamine (DA) did not reveal any significant effect of CUS in either WT or Tau-KO mice (Fig. 2a and Supplementary Fig. 3).

We next monitored the impact of stress on hippocampal synaptic plasticity by measuring the inducibility of long-term potentiation (LTP) in slice preparations (Fig. 2c, d)[22]. Analysis of the LTP data revealed a significant *CUS* x *Genotype* interaction [$F_{(1,27)}$ = 6.283; p = 0.018] and overall

effects of *CUS* [$F_{(1,27)}$ = 11.24; p = 0.002] and *Genotype* [$F_{(1,27)}$ = 41.35; p < 0.0001]. In addition, post-hoc analysis revealed that LTP was reduced in slices from CUS-treated WT (p = 0.002), but not CUS-treated Tau-KO (p = 0.92), mice when compared to their controls. Interestingly, LTP levels differed between WT and Tau-KO animals (p < 0.0001). These changes were accompanied by altered synaptic release probability, as assessed by monitoring paired-pulse (PP) facilitation at different inter-stimulus intervals (Fig. 2e, f)[20]: PP ratios (25 and 50 ms interpulse intervals) were subject to *CUS* x *Genotype* interactions [$F_{(1, 27)}$25 ms = 7.100; p = 0.012; $F_{(1, 27)}$50 ms= 9.148; p = 0.005] and *Genotype* had an overall effect on this parameter [$F_{(1, 27)}$25 ms= 4.777 p = 0.037; $F_{(1, 27)}$50 ms = 5.034; p = 0.033]. Post-hoc analysis revealed that CUS decreases the probability of synaptic release in slices of WT (p_{50ms} = 0.02), but not Tau-KO (p_{50ms} = 0.66), mice (Fig. 2e, f). Interestingly, basal synaptic transmission, assessed using input-output curves, did not differ between any of the groups tested (Supplementary Fig. 4).

Next, we compared the effects of CUS on hippocampal neuronal activity using manganese (Mn)-enhanced magnetic resonance imaging (MEMRI)[23] to detect hippocampal neuronal activity; the method allows evaluation of local neural activity since Mn enters into neurons through calcium channels[23]. Hippocampal MEMRI intensity was normalized to a non-brain region (masseter muscle)[24]. Data analysis showed a significant *CUS* x *Genotype* interaction [2-way ANOVA $F_{(1,44)}$ = 10.64; p = 0.0021], an overall *CUS* [$F_{(1,44)}$ = 25.00; p < 0.0001] and *Genotype* effect [$F_{(1,44)}$ = 56.63; p < 0.0001] (Fig. 3h); it should be noted that the *CUS* x *Genotype* interaction resulted from the stronger CUS-induced reduction in neuronal activity in WT (p<0.001) *vs.* Tau-KO (p = 0.02) mice. Lastly, a significant difference of hippocampal neuronal activity was found between non-stressed WT and Tau-KO mice (p < 0.001).

Neuronal atrophy in stressed hippocampus is Tau-dependent

Chronic stress exerts profound effects on neuronal architecture in the hippocampal formation, in particular on dendritic arborization in the dentate gyrus, CA1 and CA3 neurons[6,8-10]; these structural changes correlate with CUS-induced emotional and cognitive impairments[6,9,25]. Examination of dendritic lengths in the various subfields of the rostral hippocampus by 3D reconstruction analysis of Golgi-stained tissues showed significant *CUS x Genotype* effects on the dendritic lengths of dentate granule [$F_{(1,117)}$ = 3.944; p = 0.04], CA1 (specifically, apical dendrites)

[$F_{(1,94)}$ = 4.440; p = 0.03] and CA3 pyramidal [$F_{(1,75)}$ = 8.481; p = 0.004] neurons. Overall effects of *CUS* were found on dendritic lengths in the dentate [$F_{(1,117)}$ = 11.31; p = 0.001] and CA1 [$F_{(1,94)}$ = 7.7040; p = 0.006] subfields and *Genotype* influenced dentate [$F_{(1,117}$ = 7.109; p = 0.008] and CA3 [$F_{(1,75)}$ = 6.6415; p = 0.01] neuron dendritic lengths. Post-hoc analysis revealed that CUS significantly reduced dendritic lengths of hippocampal neurons in WT (p_{DG} = 0.001; p_{CA3} = 0.02; p_{CA1} = 0.005), but not Tau-KO (p_{DG} = 0.75; p_{CA3} = 0.62; p_{CA1} = 0.96), mice (Fig. 3a-f). No differences in apical dendritic length were found between control WT and Tau-KO animals (p_{DG} = 0.96, p_{CA3} = 0.99, p_{CA1} = 0.27). Consistent with previous findings, CUS did not influence the structure of the basal dendrites of CA1 and CA3 pyramidal cells[25]. These alterations in dendritic atrophy were complemented by the results of Sholl analysis which showed that CUS reduces dendritic arborization in the hippocampus of WT, but not Tau-KO, animals (Fig. 3g and Supplementary Fig. 5).

Chronic stress evokes Tau hyperphosphorylation and missorting in synapses

Tau hyperphosphorylation and missorting in dendrites and synapses are considered key mechanisms in the neuronal damage and atrophy that characterize AD[26-29]. In light of the above evidence that Tau is required for the manifestation of CUS-induced neuronal atrophy and dysfunction (Fig. 2 and 3), we next monitored the impact of CUS on Tau and its phosphorylation status in cytosolic and synaptosomal fractions from the hippocampi of WT mice (Fig. 4a-c). CUS induced a significant increase in cytosolic levels of total Tau (t test, p = 0.03), accompanied by increased levels of Thr231-, Ser262- and Ser396/404-Tau phospho-epitopes (p_{231} = 0.03; p_{262} = 0.0001; $p_{396/404}$ = 0.018; Fig. 4d-e); these findings indicate cytoplasmic accumulation of Tau. While Tau is mainly found in the neuronal axon and soma, previous work suggests that, Tau is missorted and accumulated at synapses in pathological conditions[26, 30]. We show here that CUS elevates total Tau levels (p = 0.02) as well the levels of pSer262-Tau and pSer396/404-Tau isoforms (p = 0.04, p = 0.03, respectively) in synaptosomal fractions from WT hippocampi indicating synaptic accumulation of these phospho-Tau forms (Fig. 4d-e). Since the effects of chronic stress on neuronal structure and function are largely attributed to glucocorticoids (GC)[8, 9], we next examined whether the actions of CUS could be reproduced by chronic administration of a glucocorticoid (dexamethasone). Immunoblotting analysis of fractionated hippocampal tissue (see Fig. 4a) showed that GC treatment increases both cytosolic and synaptosomal levels of total

Tau, as well as of phosphorylated Tau (Supplementary Fig. 6). These findings were substantiated by transmission electron microscopy (TEM) analysis of immunogold Tau-stained hippocampal sections (Fig. 4g-h; Supplementary Fig. 7). This analysis demonstrated that GC exposure elevates the density of total Tau staining in dendrites and synapses ($p = 0.0002$ and $p = 0.0001$, respectively). Together, the above biochemical and TEM results suggest that CUS and GC lead to cytoplasmic accumulation of Tau as well as its missorting to hippocampal synapses, possibly leading to disturbed synaptic function, as recently suggested[26, 30].

Tau was recently found to participate in synaptic signaling by interacting with Fyn[31], a Src family kinase, that selectively modulates the function of the GluN2B-containing NMDA receptor by phosphorylating the Y1472 residue of the GluN2B subunit[32]. Synaptic missorting of Tau has been suggested to underpin synaptic toxicity by enhancing the postsynaptic targeting of Fyn[26], subsequently linking NMDA receptors (NMDARs) to downstream synaptic excitotoxic signaling[26, 32]. We show here that exposure to CUS increases levels of Fyn in post-synaptic density (PSD) fractions obtained from the hippocampi of WT ($p = 0.02$), but not Tau-KO ($p = 0.95$), mice ($F_{(1,51)} = 5.94$; $p = 0.01$; Figure 4i-k). Moreover, in line with previous reports[26], we found that Tau-KO animals express lower levels of PSD-associated Fyn ($F_{(1,51)} = 17.2$; $p = 0.0001$). Further, we show that only WT mice respond to CUS with higher PSD levels of Y1472-phosphorylated GluN2B ($p = 0.01$; $F_{(1,50)} = 4.10$; $p = 0.04$) and elevated levels of total GluN2B receptors in the PSD ($p = 0.03$; Fig. 4j, k). Briefly, these observations are consistent with the view that Tau plays an essential mediatory role in Y1472 phosphorylation of GluN2B, an event that helps stabilize GluN2B receptors within the PSD enhancing its synaptic localization (see ref [26]), whereas the absence of Tau attenuates the above stress-driven signaling.

In summary, the neurochemical, electrophysiological, molecular and neuroanatomical evidence reported in this section demonstrates that CUS differentially influences the structural and functional integrity of the hippocampus in WT and Tau-KO mice; the latter "escape" stress-induced disruption of the hippocampal circuitry (cf. ref [9]).

Discussion

The experiments reported here demonstrate that Tau protein is a critical mediator of the neuronal dysfunction and associated cognitive and affective impairments seen after the experience of chronic stress; they introduce a Tau-dependent cellular mechanism to explain the well-known causal relationship between stress and hippocampal malfunction[6, 8].

Clinical and preclinical studies have shown that prolonged exposure to stressful conditions impairs structural and functional plasticity of the hippocampal formation related to stress-driven cognitive and mood deficits[5,9,11,25]. A key finding of this animal study is that Tau is essential for chronic stress to induce dendritic atrophy and interrupt neuronal connectivity in the hippocampus. Consistent with these structural and functional observations, animals lacking Tau were spared from the deleterious behavioral effects of chronic stress. Although our knowledge about the cellular mechanisms through which stress induces structural and functional remodeling of the hippocampus is limited, we previously showed that chronic stress increases the levels of two kinases (GSK3β, cdk5) that play a key role in the generation of aberrantly hyperphosphorylated Tau[14,33]. We now show that chronic stress leads to an accumulation of Tau and different isoforms of hyperphosphorylated Tau in the cytosolic and synaptic compartments of hippocampal neurons. Notably, two of these isoforms, pThr231 and pSer262, reduce the microtubule-binding capacity of Tau which subsequently results in destabilization of the neuronal cytoskeleton, disrupted intracellular trafficking and hippocampal atrophy in AD[28,34-37]. Recent evidence demonstrated that the intracellular distribution of Tau depends critically on the phosphorylation status of the protein[38,39]. Accordingly, 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[27]. To date, amyloid β is a well-known trigger of Tau missorting and dendritic collapse[30,40]. Our findings represent the first demonstration that an exogenous stimulus – chronic stress – can also induce Tau missorting; they add to our mechanistic understanding of the impact of stressful conditions on the development of non-familial forms of AD as previously suggested[41].

Tau missorting at dendritic spines is suggested to represent an early event in AD, preceding the manifestation of detectable neurodegenerative processes[27,28]. While the precise mechanisms through which endogenous Tau facilitates neuronal deficits are still under intense investigation, a

recently suggested pathway involves enhanced Tau-mediated postsynaptic targeting of Fyn[26]; the latter is known to selectively modulate the function of GluN2B-containing NMDARs, by phosphorylation of the GluN2B at its Y1472 epitope[26], an event that stabilizes GluN2B at the postsynaptic density, thus linking NMDARs to downstream excitotoxic cascades[26,32]. Besides adding support for the view that stress and AD share common neurobiological substrates[42], the results presented here suggest a plausible Tau-dependent mechanism (Fig. 5) through which chronic stress initiates a signaling cascade that culminates in neuronal damage. Interestingly, the same mechanism was previously proposed to operate in other neuropathological conditions such as stroke and AD[26,43]. Note that NMDARs are also shown to be involved in stress-driven neurotoxicity[44] as blockade of NMDARs, but not AMPARs, attenuates neuroremodeling actions of stress[45,46].

The current study reveals the neuroprotective role of Tau reduction against the establishment of stress-driven pathology. This observation is in line with other approaches using Tau-lowering strategies to tackle neuropathologies with diverse etiology[1,3,4,26]. Interestingly, the absence of Tau does not interfere with the endocrine response to stress and thus does not pose a threat to the organism's survival. Together, our findings highlight Tau protein and its synaptic missorting as an essential mediator of the deleterious effects of stress on brain structure and function.

Online Methods

Animals and treatments

Wild-type (WT) and Tau knock-out (Tau-KO) male mice (C57BL/6J background), aged 4-6 months, were used in this study[19]; 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. Animals from each genotype were either a) exposed to a chronic unpredictable stress (CUS) paradigm during the daily period of light or b) left undisturbed in their home cages (control; CON). The CUS paradigm consisted of different stressors (restraint, vibrating platform, overcrowding, exposure to a hot air stream; one

stressor per day) for 6 weeks[14, 18]. To monitor the efficacy of CUS, body weights were measured weekly and blood serum was collected at the end of stress period and assayed for corticosterone levels (ICN Biomedical, Costa Mes, CA, USA). In one experiment, male Wister rats, aged 4-5 months, were treated with the synthetic glucocorticoid dexamethasone for 15 days (daily subcutaneous injections of 300µg/kg dissolved in sesame oil containing 0.01% ethanol); control animals received sesame oil; rats were used here because dexamethasone does not penetrate the mouse brain[47].

Behavioral analysis

Y-Maze: All animals were subjected to a 2-trial test to assess spatial memory using a Y-maze (33cm x 7cm x 15cm). The 3 arms were randomly designated as start, novel and old arm; visual cues were placed on the edges of each arm. In the first trial (10 min), animals were allowed to explore only two arms. For the second trial (5 min), mice were placed back in the start arm but had free access to all arms of the maze. Trials were captured using a video-tracking system (Viewpoint, Champagne-au-Mont-d'Or, France); data are expressed as percentage of distance travelled and time spent in the novel arm.

Morris Water Maze**:** The maze consisted of a white circular pool (diameter: 170 cm; depth: 50 cm), filled with tap water (23± 1°C; 25cm of depth) and was divided into quadrants by imaginary lines; the maze was placed in a dimly-lit room with extrinsic clues. During testing, a transparent escape platform (14cm in diameter; 30cm high), invisible to the animals, was placed in the same quadrant during five consecutive days. Each test session consisted of four trials (maximum 120 sec). Trials were captured on a video-tracking system (Viewpoint). The distance that animals swam to reach the hidden platform was recorded and used to evaluate learning and memory performances[14, 48].

Novel Object Recognition: NOR was tested using an open arena (33cm x 33cm x 33cm). After a habituation period (3 sequential days), 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). All sessions were videotaped and scored manually using Kinoscope software (http://sourceforge.net/projects/kinoscope/). A

discrimination index was calculated using the formula: [(Novel Object/Novel Object + Familiar Object)- Familiar Object (Novel Object + Familiar Object)] x 100.

Elevated Plus Maze: Animals were placed in an elevated-plus maze (EPM), consisting of two open arms (50.8 x 10.2 cm) and two closed arms (50.8 x 10.2 x 40.6 cm), elevated 72.4 cm above the floor in a dimly- illuminated room. The time spent in the open arms was monitored with an infrared camera (MedPCIV, Med Associates Inc.) over a total of 5 min[48].

Forced Swim test: Learned-helplessness was assessed using the forced swimming test (FST)[27]. Briefly, mice were individually placed into transparent cylinders filled with water (24°C; depth 30cm). A 5-min test session for each mouse was recorded and trials were manually scored using Kinoscope software (http://sourceforge.net/projects/kinoscope/). Depression-like behavior was evaluated by immobility time and latency to immobility[27, 48].

Sucrose Preference test: Sucrose preference was tested in all (individually-housed for 48 h) animals before the CUS paradigm was started. They received two drinking bottles, one containing water, the other 2% sucrose. At the end of the CUS paradigm, animals were again monitored for sucrose preference. Anhedonia (reduction in sucrose preference) was calculated according to the formula: sucrose preference = sucrose intake/total intake] × 100.

Neurostructural analysis

For 3D morphological analysis, animals (n=5 per group) were transcardially perfused with 0.9% saline. Brains were immersed in a Golgi-Cox solution for 14 days and transferred to 30% sucrose before being cut on a vibratome (coronal sections) and further proceeded as previously described[18, 25]. Dendritic arborization was analyzed in the dentate gyrus, CA1 and CA3 of the dorsal hippocampus[49]. Dendritic trees of individual neurons (25-30 neurons/area/experimental group) were reconstructed at 600x (oil) magnification using a motorized microscope (BX51, Olympus). A three-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield) which also provided dendritic lengths. Three-dimensional Sholl analysis was used to evaluate the arrangement of the dendritic tree. For that purpose, the number of dendritic intersections with concentric spheres positioned at radial

intervals of 20 mm was determined using NeuroExplorer software (MBF Bioscience, Germany) as previously described[18, 25].

HPLC analysis

Monoamines (NA, DA and 5-HT) levels were measured using high performance liquid chromatography with electrochemical detection (HPLC-ED). Dorsal hippocampi were homogenized, deproteinized (0.1N perchloric acid solution, 7.9 mM $Na_2S_2O_5$, 1.3 mM Na_2EDTA]. After centrifugation (20000g, 45min), supernatant was analyzed using a GBC LC 1150 HPLC pump (GBC Scientific Equipment, Australia) coupled with a BAS-LC4C (Bioanalytical Systems Inc., USA) electrochemical detector (+800mV), as previously described[48,49]. Reverse phase ion pairing chromatography was used to assay monoamines and their metabolites using an Aquasil C18HPLC column (250x4.6mm, 5μm; ThermoElectron, UK). Samples were quantified by comparison of the area under the curve against known external reference standards using a PC-compatible HPLC software package (Chromatography Station) as previously described[48,49]. The limit of detection was 1 pg/20 μL (injection volume). Turnover rates of 5-HT and DA (5-HIAA/5-HT as well as HVA/DA and DOPAC/DA, respectively) were calculated as indices of serotonergic and dopaminergic activity, respectively. Altered turnover rates are indicative and more reliable indices than neurotransmitter/metabolite levels of neurotransmission activity of the cells, integrating synthesis, release, reuptake, and metabolism of neurotransmitters[48].

Electrophysiological recordings

After brain removal in ice-cold sucrose-based artificial cerebrospinal fluid solution [in mM: 2.5 KCl, 7 $MgCl_2$, 1.25 NaH_2PO_4, 110 sucrose, 26 $NaHCO_3$, 10 glucose, bubbled with carbogen gas (95% O_2, 5% CO_2)], 300 μm axial slices of dorsal hippocampus were prepared and recorded [31°C] in standard artificial cerebrospinal fluid (in mM): 124 NaCl, 2.5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 Glucose, bubbled with carbogen gas (95% O_2, 5% CO2)]. Extracellular recordings [Multiclamp 700B amplifier (Axon Instruments) and borosilicate glass pipettes with saline (3-5MΩ)] and stimulation (bipolar tungsten electrode) were both performed in the middle of the stratum radiatum of CA1. Signals were low-pass filtered at 3 KHz and

sampled at 10 KHz. The input-output relation was monitored using a STG4002 stimulus isolating unit for Shaffer collaterals stimulation [0,03Hz (2-8V); stimulus strength at 30-50% of the maximum slope of the fEPSP]. The paired pulse ratio was assessed before LTP induction by giving two close stimulus of varying interpulse interval (25, 50, 100 and 300 ms); ratio was calculated by dividing the slope of fEPSP 2 by fEPSP 1 (baseline 0.03 Hz). LTP was elicited by delivering 3 1s-long 100 Hz burst stimulus intervaled by 15s. Final slopes were calculated offline using the LTP software[22]. All points of each individual curve were normalized to the average value of the last 10 minutes baseline. Averages of the slopes of the last 6 min recordings were used for comparison of the LTP curves.

Manganese enhanced MRI scanning

MEMRI scans were acquired on a 7 T Avance Biospec 70/30 scanner (Bruker BioSpin, Ettlingen, Germany). For T_1 contrast enhancement animals were injected i.p. with manganese chloride (50 mM $MnCl_2$ x $4H_2O$ (Sigma, Germany) solution in 0.9 % NaCl, adjusted to pH 7.0) using a fractionated injection protocol to minimize toxic side effects[24]. Eight doses of 20 mg/kg $MnCl_2$ were injected. Animals were positioned on a saddle-shaped receive-only coil in a prone position, with stereotaxtical fixation. Body temperature was controlled by a rectal thermometer (Thermalert TH-5, Physitemp Instruments, USA) and kept at 37°C. Additionally the pulse was monitored with a plethysmographic pulse oxymeter (Nonin 8600V, Nonin Medical Inc., USA). T_1w images were acquired using a 3D gradient echo pulse sequence (TE = 3.2 ms, TR = 50 ms, matrix size = 128 x 106 x 106, zero filled to 128 x 128 x 128, number of averages = 10, field of view (FOV) = 16 x 16 x 18 mm, resulting in a spatial resolution of 125 x 125 x 140.6 μm^3; experimental duration 90 min). For MRI scanning, mice were anaesthetized (isoflurane 1.3-1.6 vol% in an oxygen flow of 1.2-1.4 l/min) and 3D T1-weigthed images were acquired. Images were reconstructed in Paravision (Bruker BioSpin, Ettlingen, Germany) and further processed in SPM8 (www.fil.ion.ucl.ac.uk/spm), including spatial normalization to an in-house template. On the basis of the anatomical brain atlas of the C57BL/6 mouse by Franklin and Paxinos, a bilateral hippocampal (HPC) region was defined in normalized space on the master template. HPC masks were back-transformed to match the individual's native space, and mean signal intensities were extracted. To account for possible individual differences, HPC intensity was normalized to the intensity of the masseter muscles[24].

Subcellular fractionation and Western blot analysis

An established protocol was used to obtain subcellular fractions[49,50] (Fig. 4a). Briefly, hippocampal 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). To obtain the postsynaptic density (PSD) fraction, LP1 was incubated with 1% Triton for 5-6 minutes before ultracentrifugation (176, 000 *g*); the resulting pellet contained the PSD fraction (Fig. 4i).

The various fractionated samples were electrophoresed and semi-dry transferred onto nitrocellulose membranes (*Trans-Blot® Turbo™* 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), p202-Tau (1:1000, Abcam), pThr231-Tau (1:1000 Abcam), pSer262-Tau (1:1000, Santa Cruz), PHF1 (1:2000, recognizes p396/404-Tau; kind gift from Dr Peter Davies), GluN2B (1:1000, Abcam), pY1472-GluN2B (1:1000, Millipore), Fyn (1:500, Santa Cruz), PSD-95 (1:10000, NeuroMab), synaptophysin (1:1000 NeuroMab) 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 instrument and and ImageLab software from Bio-Rad. All values were normalized and expressed as a percentage of control values.

Electron microscopy

Hippocampi were fixed in 4% PFA and post-fixed in 4% PFA/0.8% glutaraldehyde in 0.1M of phosphate buffer (PB; pH 7.4) or 1h. The CA1 areas were dissected from vibratome-cut axial sections of the dorsal hippocampus and embedded in Epon resin. Ultrathin sections (500 Å) were placed on nickel grids: Following antigen retrieval (boiling in citrate buffer (30min) and 5% BSA treatment (30 min), sections were stained for Tau, using an immunogold technique. Briefly, specimens were incubated overnight with Tau5 antibody (1:30, Abcam), followed by appropriate

gold-labelled secondary antibody (1:15; Abcam), and inspected with a JEOL JEM-1400 transmission electron microscope. Images were obtained using a Orious Sc1000 digital camera. Fifty non-overlapping TEM (30000 X) images of counterstained ultrathin-sections per treatment group were analyzed by an experimenter blind to the *in vivo* treatments.

Statistical analysis

Data were analyzed using two-way ANOVA analysis before application of post hoc comparisons (SPSS Inc., USA and GraphPad Software Inc., USA); 2-way repeated measures ANOVA was used to analyze the MWM data. Differences were considered statistically significant when $p < 0.05$.

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Author Contributions

S.L., J.V-S, V.P., CD, NK, BB, NM, MC, IS performed experiments, analyzed data, prepared figures. IS, OFXA, NS contributed to the data analysis and wrote the manuscript. All coauthors approved a prefinal version of the manuscript.

Conflict of interest

None of the authors have any conflicts of interest to declare. The funders did not influence the design, execution, interpretation or writing up of the results reported in this paper.

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Figures

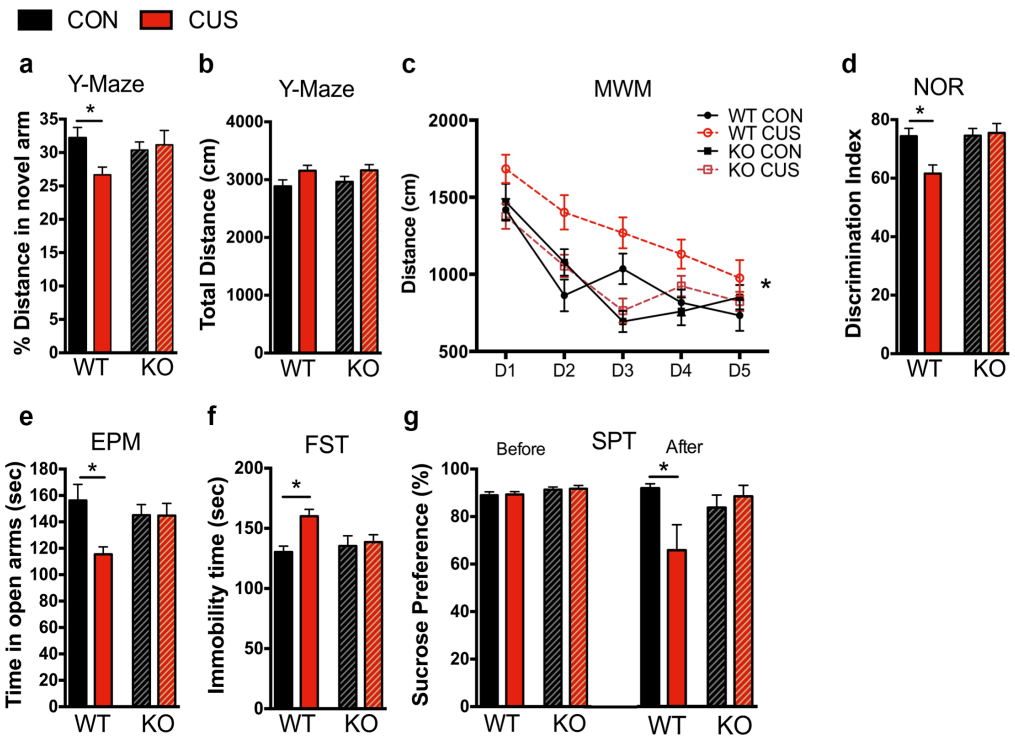
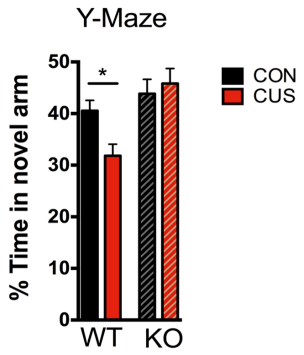


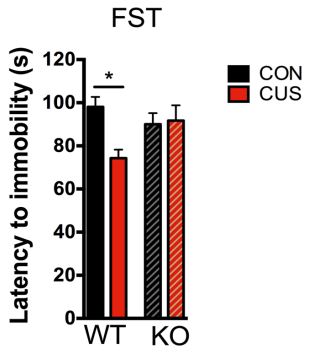
Figure 1 Tau ablation blocks stress-driven depressive-like behavior and cognitive impairments. a-b) Y-maze memory test showed that chronic stress (CUS) decreased % distance that WT animals travelled in the novel arm of Y-maze apparatus indicating cognitive deficits (see also Suppl. FigS1); this CUS effects was not found in Tau-KO animals (a). No CUS-evoked differences of total distance travelled in Y-maze apparatus in both WT and Tau-KO animals (b). c) In Morris Water Maze (MWM) test, WT animals exposed to CUS travelled more distance in order to reach the escaping platform when compared with WT controls (CON) suggesting spatial learning and memory deficits. d) CUS decreased discrimination index in WT animals when tested in Novel Object Recognition (NOR) indicating memory deficits while Tau-KO animals were not affected by CUS. e) Exposure to CUS resulted in reduced time in open arms of Elevated Plus Maze (EPM) apparatus in WT, but not Tau-KO, animals indicating increased WT anxiety levels. f-g) WT animals exposed to CUS exhibited an increase in immobility time at forced swimming test (FST) as well as reduced preference to sucrose in sucrose preference test (SPT) (g; right panel) indicating stress-driven depressive-like behavior; no differences in sucrose preference were found among all groups before the 6-weeks period of CUS (g; left panel). Note that Tau-KO animals were resilient to CUS effects on both depressive parameters tested. All data shown represent mean ± SEM (* $p<0.05$).

Supplementary figure 1



Supplementary Figure 1: Time in novel arm of Y-maze. 2-way ANOVA revealed an interaction between *CUS* and *Genotype* ($F_{1,65}$ = 4.614, p=0.03). Post hoc analysis showed that exposure to CUS reduced the percentage of time spent by WT animals in the novel arm of Y-maze (*p=0.02). Data are expressed as group mean ± SEM.

Supplementary figure 2



Supplementary Figure 2: Latency to immobility in forced swim test (FST). 2-way ANOVA demonstrated a *CUS x Genotype* interaction ($F_{1,73}$ = 4.984, p=0.02); post hoc analysis revealed that latency to immobility in WT animals is reduced after exposure to CUS (*p=0.03). Data are expressed as group mean ± SEM.

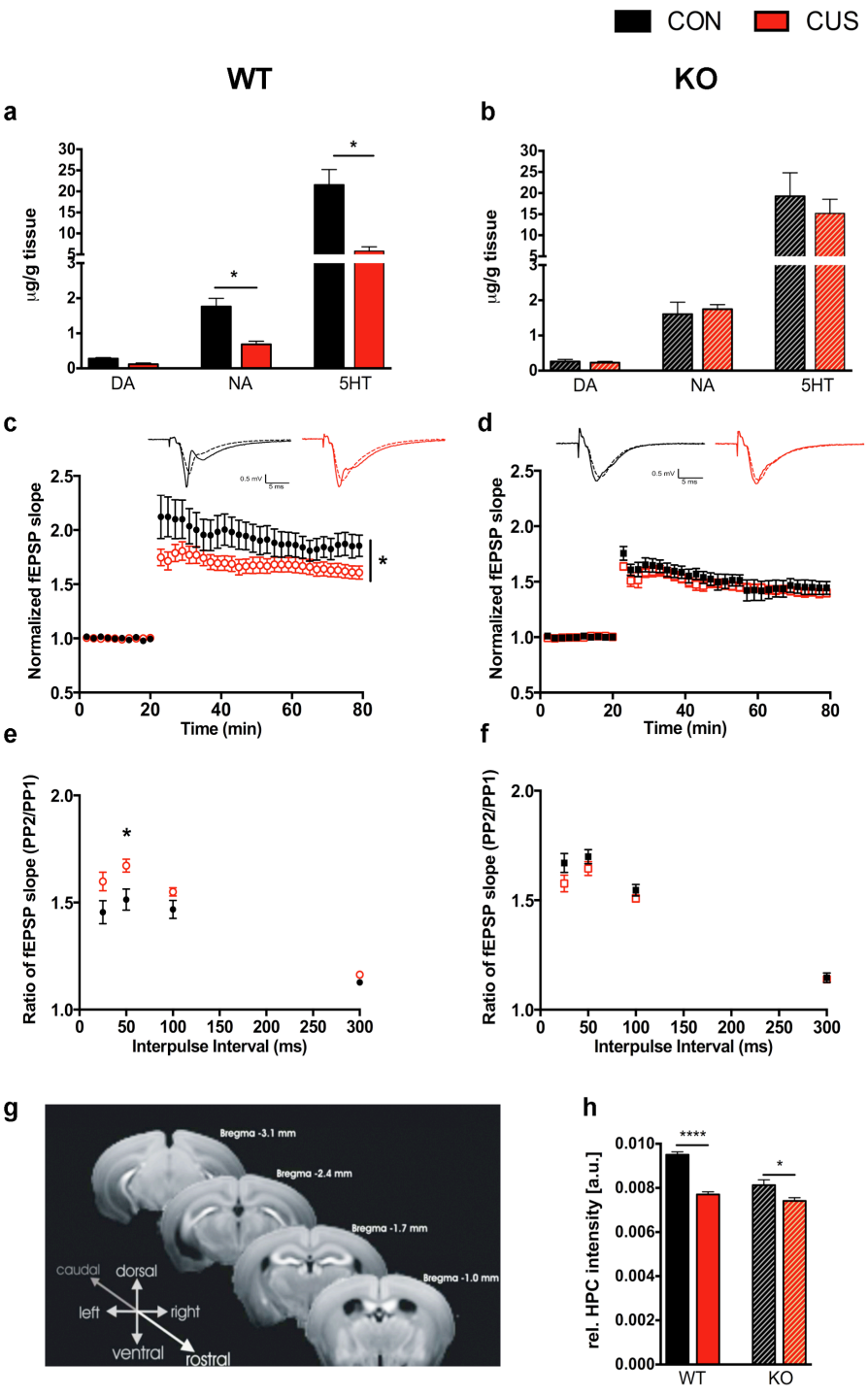
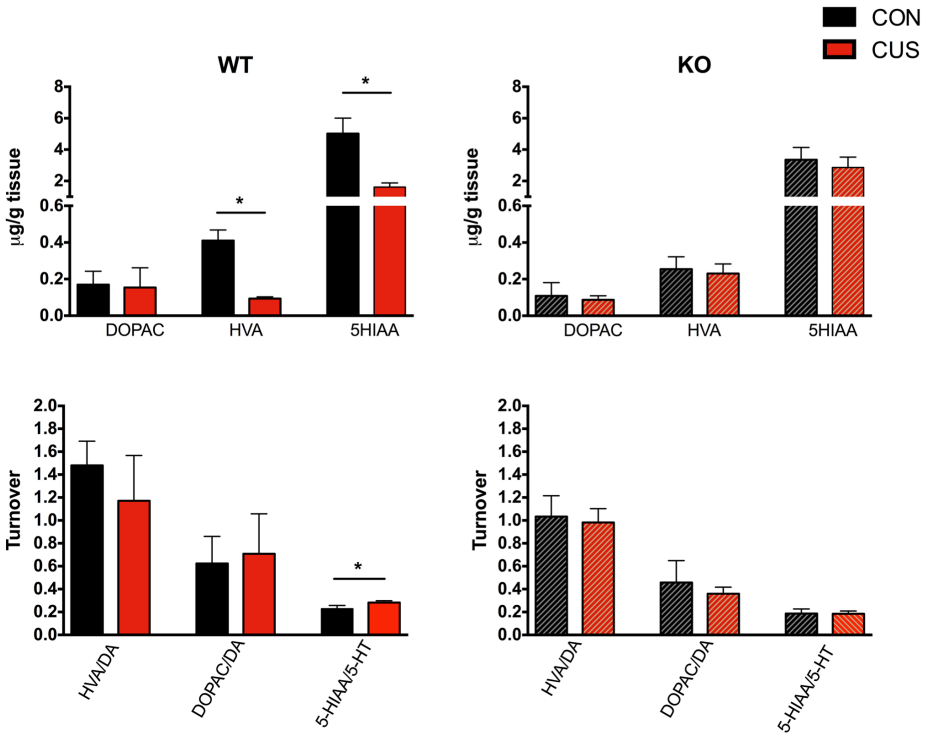


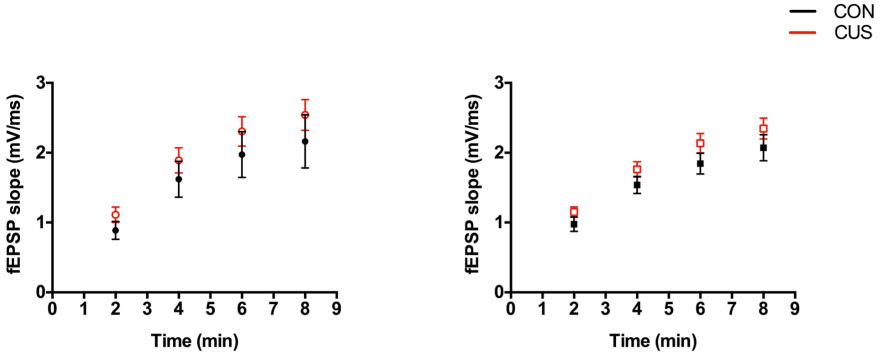
Figure 2. Differential impact of stress on hippocampal function and activity in WT and Tau-KO animals a-b) HPLC-analyzed levels of different monoamines in hippocampus of WT (a) and Tau-KO (b) animals under control and stress (CUS) conditions. CUS resulted in decreased levels on noradrenaline (NA) and serotonin (5HT) in WT animals whereas not significant effects were found in Tau-KO hippocampus. c-d) 100Hz induction of long-term potentiation (LTP) was significantly diminished in WT CUS animals when compared to WT CON but this CUS-triggered change was not found in LTP of Tau-KO animals (d). e-f) Paired pulse facilitation was increased by CUS in WT but not Tau-KO animals. g) Manganese-enhanced MRI analysis of neuronal activity in hippocampus revealed that CUS reduced hippocampal activity in both WT and Tau-KO animals but this effect was stronger than in WT. All data shown represent mean ± SEM (* $p<0.05$; **** $p<0.0001$).

Supplementary figure 3



Supplementary Figure 3: Monoamine metabolites and turnover. HPLC levels of dopamine (DA) metabolites, DOPAC and HVA, as well as 5-HT metabolite, 5HIAA, followed by DA and 5-HT turnover ratios. CUS did not change the levels of DA metabolite, DOPAC, in both genotypes; yet, the hippocampal levels of HVA, the other metabolite of DA, was affected by CUS in WT ($p=0.002$) but not in Tau-KO animals ($p=0.98$), also exhibiting a *CUS x Genotype* interaction ($F_{(1,16)}=8.078$ $p=0.02$). No change was detected in DA turnovers (both DOPAC/DA and HVA/DA). In addition, both the 5-HT metabolite, 5HIAA, and 5-HT turnover (5HIAA/5-HT) were altered by CUS in WT animals ($p_{5HIAA}=0.02$; $p_{turn}=0.04$) but not in Tau-KO animals ($p_{5HIAA}=0.38$; $p_{turn}=0.14$). Data are expressed as group mean ± SEM.

Supplementary figure 4



Supplementary Figure 4: Basal synaptic transmission. None of the groups displayed differences in their input–output curves, indicating that CUS does not influence basal synaptic transmission. Data are expressed as group mean ± SEM.

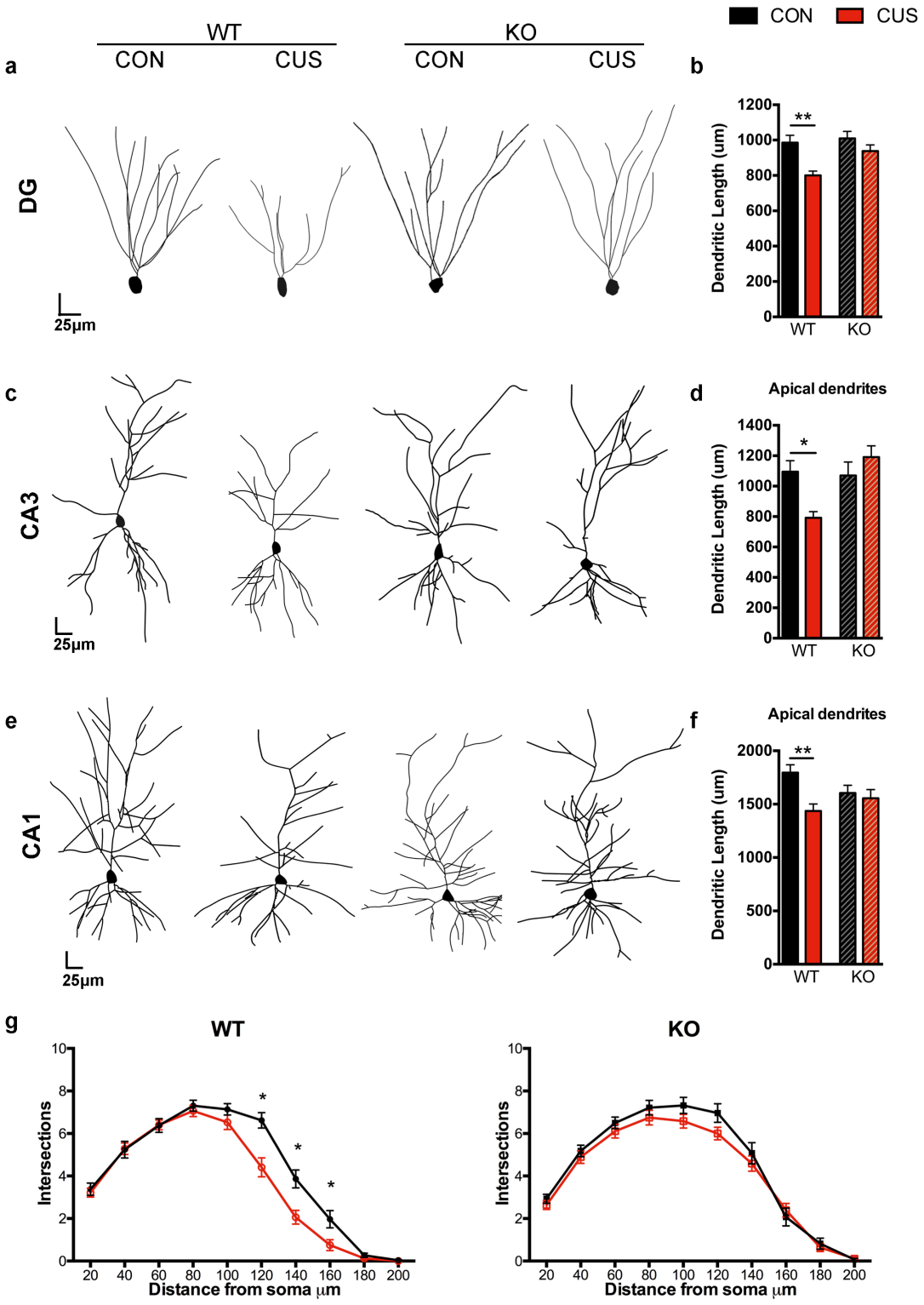
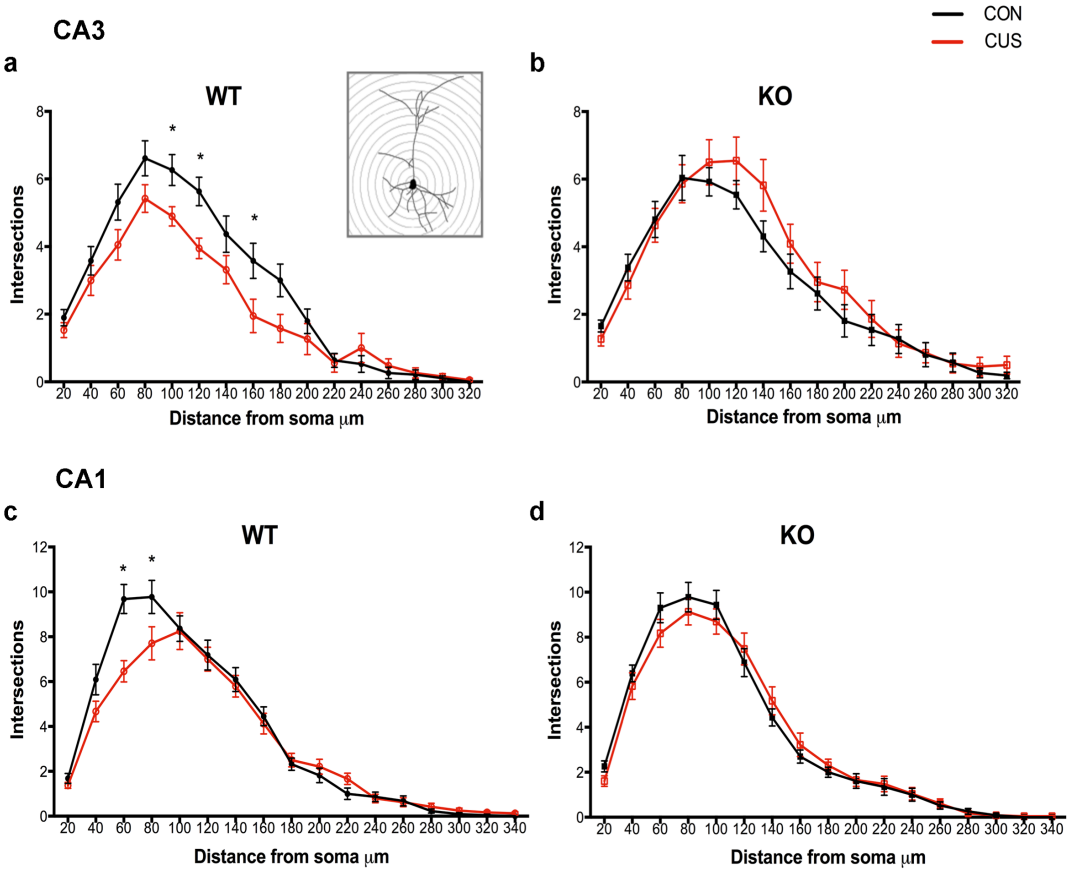


Figure 3: Neuroremodeling properties of stress depend on Tau protein. a-f) Exposure to chronic stress (CUS) resulted in neuronal atrophy in dentate gyrus, CA3 and CA1 hippocampal areas of WT animals as shown by 3D neuronal reconstruction (a, c, e) as well as by apical dendritic length measurement (b,,d, f). No significant CUS-evoked changes were found in DG, CA3, CA1 neurons of Tau-KO brains. g) Dendritic arborization of DG neurons was also affected by CUS as shown by reduced number of intersections in CUS WT animals when compared to CON WT. No significant differences were found between CON and CUS Tau-KO animals- see also Supplementary Fig. 5. All numeric data represent mean ± SEM (* $p<0.05$).

Supplementary figure 5



Supplementary Figure 5: Sholl analysis of CA3 and CA1 hippocampal neurons. The graphs present the number of intersections in the dendritic trees of CA1 and CA3 neurons (for details, see methods). As shown in panels **a** and **c**, CUS reduced the number of dendritic intersections at different distances from the neuronal soma, indicating decreased neuronal arborization in CA1 and CA3 hippocampal areas of WT animals. In contrast, CUS did not influence dendritic tree branching of CA1 and CA3 in Tau-KO mice. Data are expressed as group mean ± SEM. * $p < 0.05$).

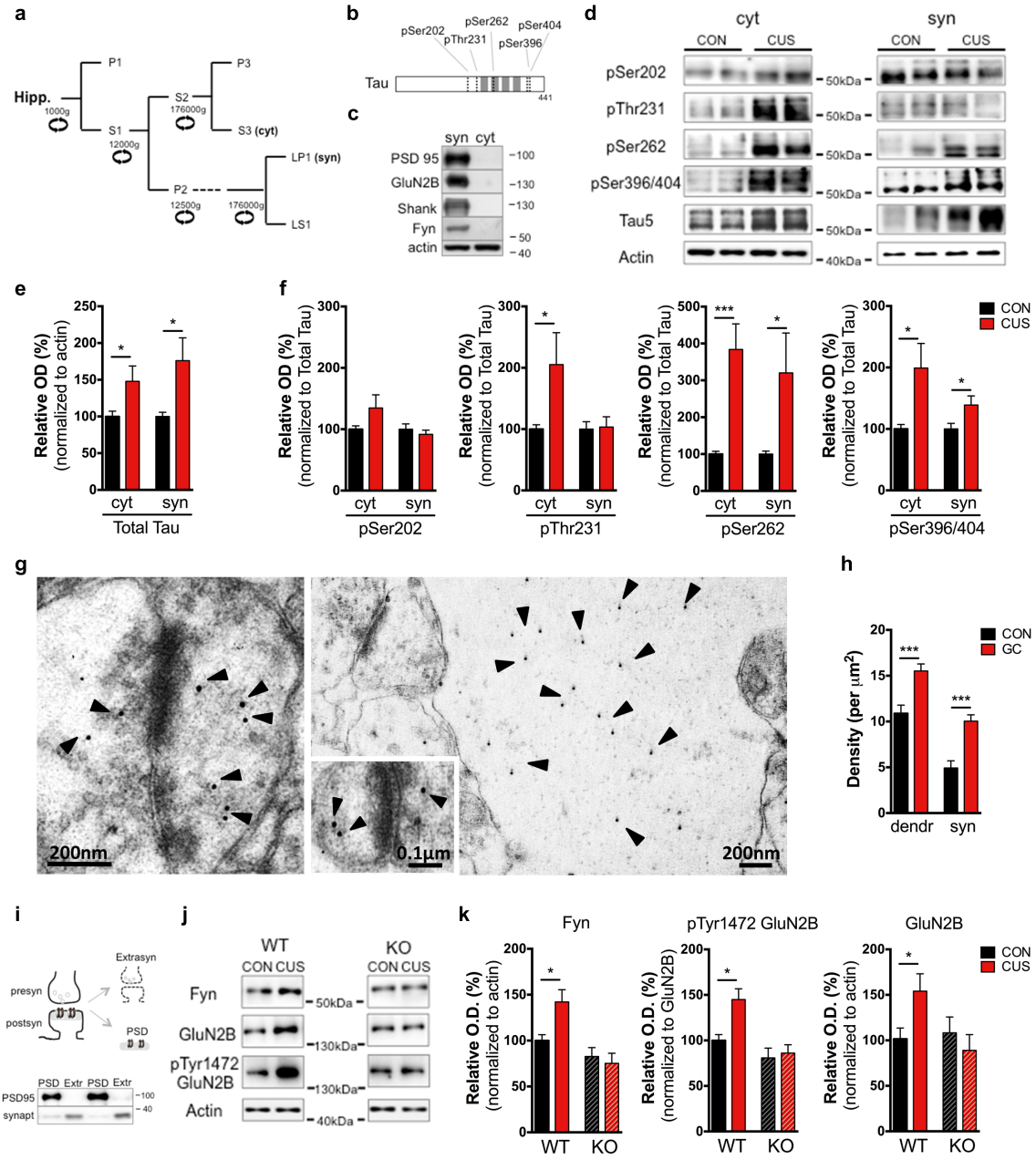
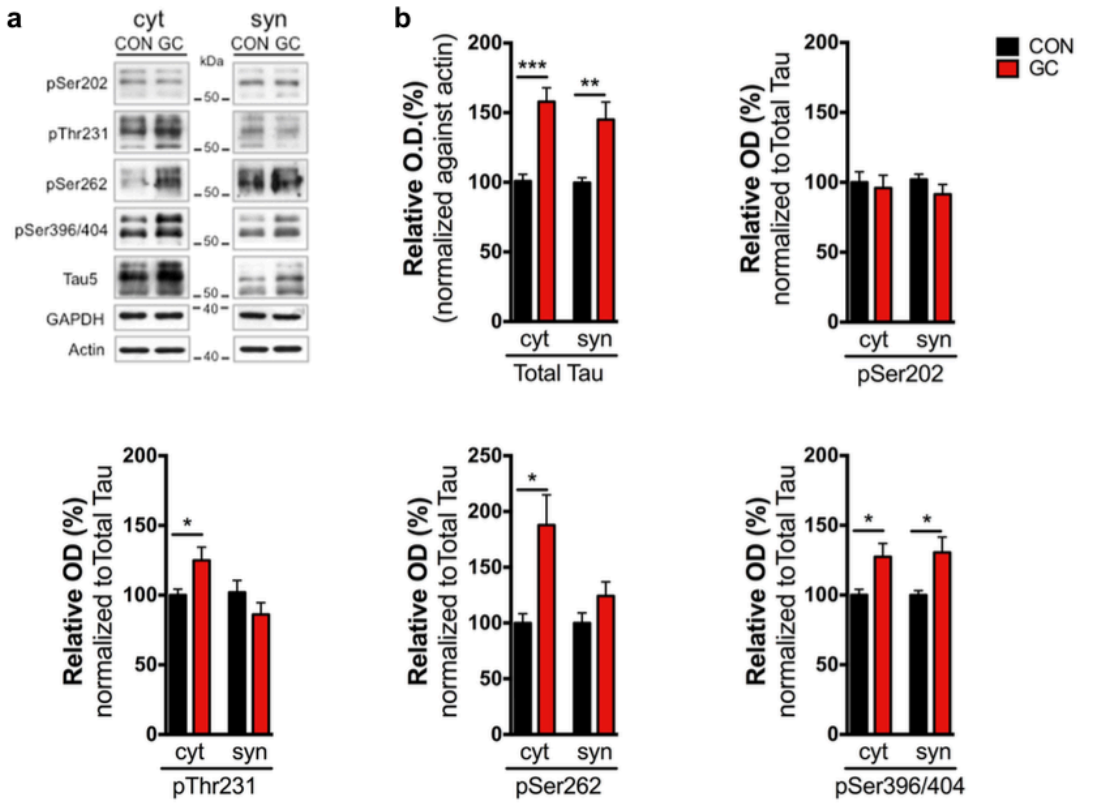
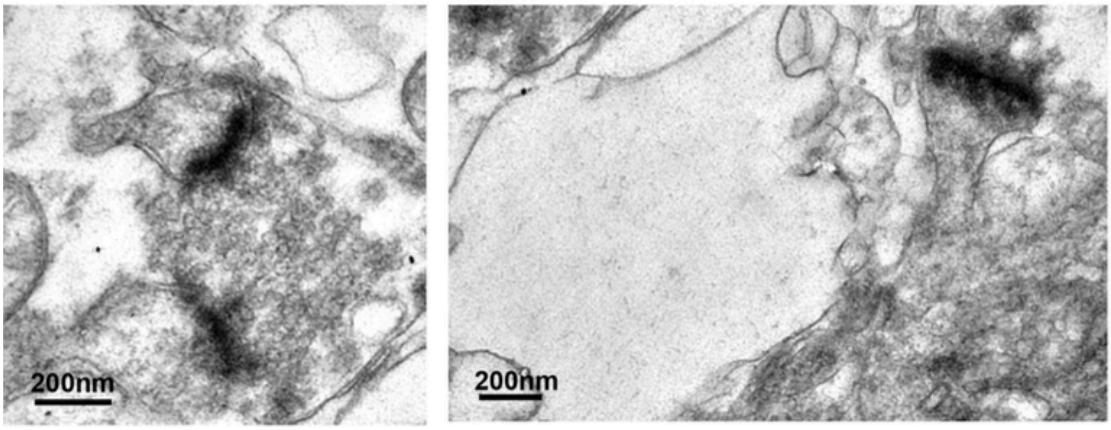


Figure 4: Chronic stress triggers Tau accumulation and missorting in synaptic compartment. a) Schematic representation of subcellular fractionation protocol of preparation of synaptosomes (LP1) and cytosolic fractions (S3) and b) representation of the different phosphorylation epitopes of Tau protein monitored in this study. c) WB-based detection of synaptic proteins (e.g. PSD-95) and receptors in synaptosomal, but not cytosolic, preparation. d-f) CUS increased cytosolic levels of total Tau and pThr231, pSer262 and pSer396/404 Tau isoforms in WT hippocampus. In addition, CUS also boosted overall Tau levels in synaptosomic fraction, while only pSer262 and pSer396/404 Tau isoforms were elevated by CUS in this fraction. g-h) TEM images of Tau immunogold staining in hippocampus (g) showing that glucocorticoid (GC) treatment increased Tau density of both dendritic and synaptic compartments (h). i) Schematic representation of postsynaptic density (PSD) and extrasynaptic (Extra) fractionation followed by WB-based detection of PSD95 and synaptophysin in PSD and Extra fractions, respectively. j-k) CUS triggered an increase of PSD levels of Fyn protein followed by increased total and pY1472 levels of GluN2B subunit at PSD fraction in WT, but not Tau-KO, hippocampus. All numeric data represent mean ± SEM (* $p<0.05$).



Supplementary Figure 6: Prolonged GC treatment triggers Tau accumulation in both cytosolic and synaptosomal compartments. GC treatment for 15 days elevated total Tau levels in both cytosolic and synaptosomal fractions while this was followed by increased phosphorylation of Tau at pThr231, pSer262 and p396/404 in cytosolic fraction. In contrast, GC increased p396/404-Tau synaptosomal levels while this GC effect didn't reach significance for p262-Tau (p=0.07). No effect was detected in p202-Tau isoform in both fractions. All numeric data represent mean ± SEM (* p<0.05).



Supplementary Figure 7: TEM microphotos of negative control for Tau-immunogold staining showing both dendrites and synapses in hippocampus.

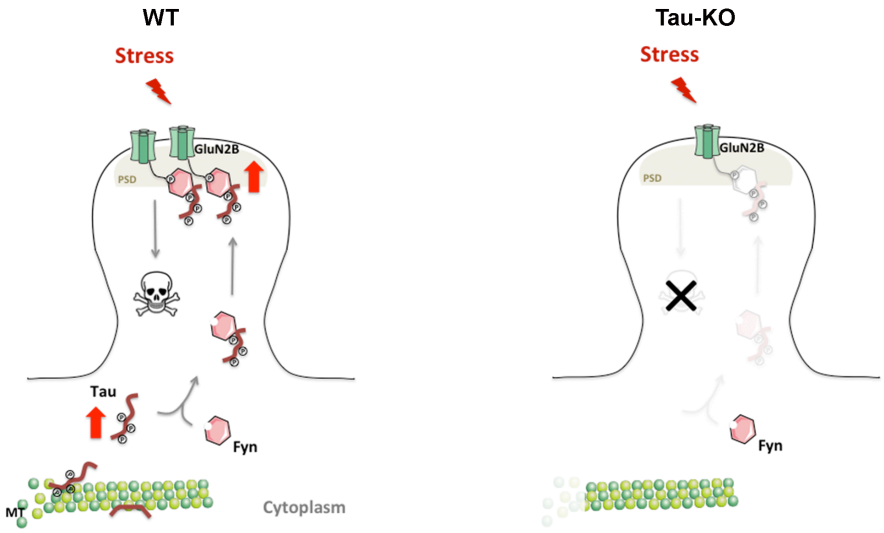


Figure 5: Working model of Tau-mediated cellular processes underlying stress-driven neuronal malfunction. The diagram describes a hypothetical model where chronic stress triggers the hyperphosphorylation of the cytoskeletal protein Tau that dissociates from microtubules (MT) resulting to its cytoplasmic accumulation as well as its missorting at dendritic spines. It leads to increase of Tau-dependent targeting of the Src kinase Fyn at postsynaptic density (PSD; gray box) where Fyn phosphorylates the NMDA receptor subunit GluN2B at Y1472 epitope. This phosphorylation is known to increase the stability of NMDA receptors within the PSD coupling them to the induction of GluN2B-mediated neurodamaging cascades as previously described. In contrast, absence of Tau blocked the above stress-driven pathway providing neuroprotection against the detrimental effects of chronic stress.

Supplementary Table 1: Body weights and blood corticosterone levels. While CON (control, non-CUS) WT and KO animals showed body weight gains during the 6 weeks of the experimental protocol, CUS-exposed WT and KO animals showed a net loss in body weight. 2-way ANOVA analysis showed an overall CUS effect on body weight ($F_{1,43}$ = 56.65, p<0.0001); body weight was significantly decreased in CUS animals when compared to control animals of the same genotype (***p≤0.001 for both WT and KO animals). In addition, CUS resulted in elevated circulating corticosterone levels in both WT and KO animals. 2-way ANOVA revealed an overall effect of CUS on peak (dark-phase) circulating corticosterone levels ($F_{1,43}$ = 18.68, p<0.0001). Both WT and KO mice responded to CUS animals with increased levels of corticosterone (* $p < 0.05$ for both genotypes). Data are expressed as group mean ± SEM.

	WT		KO	
	CON	CUS	CON	CUS
% Δ Body Weight	2.44 ± 0.65	−1.48*** ± 0.83	2.67 ± 0.44	−2.95*** ± 0.49
Corticosterone (ng/mL)	98.2 ± 11,8	145.5* ± 13,9	87.7 ± 8,2	146.7* ± 13,8

Supplementary Table 2: Acute stress activates HPA axis of WT and Tau-KO animals in a similar way. Animals were exposed to an acute stressor (restraint, 4 min at 8:00 a.m) and circulating corticosterone (CORT) levels were monitored as read-out of HPA function. All animals showed a peak of their CORT levels at 30 minutes after the acute stressor and return to basal at 120 minutes. Note that both CUS WT and Tau-KO animals exhibited higher CORT levels compared with their CON (non-stressed) littermates at 30min time point [2-way ANOVA, overall CUS effect at 30min ($F_{1,45}$ = 4.36, p = 0.04] but no difference between CUS groups of both genotypes.

		WT		KO	
	After acute stress	CON	CUS	CON	CUS
Corticosterone (ng/mL)	0'	45.4 ± 4.7	59.6 ± 7.3	58.3 ± 4.2	49.5 ± 11.1
	30'	132.5 ± 13	177,7 ± 17.6	133,8 ± 12.4	180,4 ± 35.6
	120'	60.5 ± 10	83.9 ± 21.4	59.9 ± 7.7	49.2 ± 6.5

Chapter 2.3

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BACE-1 and APP misprocessing as molecular sculptors of stress-driven depressive pathology

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BACE-1 and APP misprocessing as molecular sculptors of stress-driven depressive pathology

Running title: BACE-1 role in depressive pathology

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ABSTRACT

Clinical and experimental studies suggest a causal role of chronic stress for brain pathology and disease e.g. depression and Alzheimer´s disease (AD) as stress is strongly associated with neuronal atrophy and synaptic loss, resulting in impaired mood and/or cognition. Indeed, neuronal atrophy and synaptic malfunction are key underlying pathomechanisms in both disorders, while depression is now an established risk factor for AD. Whereas clinical evidence suggests shared neurobiological mechanisms between both disorders, it remains unclear whether APP misprocessing, a key AD mechanism, is involved in the establishment of depressive

pathology. Using a well-validated animal model of depression, chronic unpredictable stress (CS), we found that CS-driven depressive symptomatology and neuronal atrophy are accompanied by APP misprocessing and increased expression of BACE-1, the first enzyme responsible for this neuronal pathway. Notably, treatment with 2 different types of antidepressants, fluoxetine and imipramine, during the last 2 weeks of CS reversed APP misprocessing, followed by neuronal and behavioral recovery. Furthermore, genetic deletion of *BACE-1* blocked the CS-driven depressive pathology, providing neuronal protection against chronic stress. Our findings provide a solid proof of the essential role of BACE-1 and APP misprocessing in the establishment of stress-driven depressive pathology adding to our limited knowledge about the cellular mechanisms underlying the detrimental impact of chronic stress on brain structure and function.

Keywords

BACE-1, APP misprocessing, depression, chronic stress, antidepressants, neuronal atrophy

INTRODUCTION

Improvements in lifespan over the last decades have not been matched by improvements in the mental health span. World Health Organization indicators point depression and AD as possible leading causes of mental disability in the next decades, with high social and financial impact of modern societies and world economy. Growing clinical evidence supports a pathological link between depression and AD pointing to shared neurobiological underpinnings and pathogenic mechanisms[1] e.g. depressive symptomatology is often comorbid with cognitive deficits[2] while AD-related mechanisms are also found to be affected in depression[3]. Amyloid precursor protein (APP) processing through its sequential cleavage by BACE-1 and γ-secretase complex that results in the generation of amyloid βeta (Aβ), so called APP misprocessing, constitute one of the major pathogenic mechanisms in AD brain. Interestingly, recent evidence suggests that APP misprocessing might also be observed in depressive patients, highlighting the diagnostic potential of various APP cleavage products for discrimination between subjects undergoing normal aging from those suffering from depression and AD[1,4,5]. Furthermore, clinical studies suggest that depression predisposes individuals to develop AD[2], while history of depression is correlated with increased AD histopathology e.g. amyloid plaques that are produced by Aβ aggregates[1].

Both AD and depressive patients exhibit neuronal atrophy and synaptic abnormalities and/or loss in hippocampus and prefrontal cortex (PFC)[6,7] followed by elevated levels of stress hormones, glucocorticoids (GC)[8]. Indeed, chronic stress and GC are suggested to increase susceptibility to brain pathology, as it is associated with neuronal and synaptic atrophy/loss, impaired cognition, as well as affective disorders, such as depression. Indeed, suporting the prevailing idea of impaired neural plasticity, which embraces dendritic atrophy and synaptic loss, as a key process in the etiopathology of depressive disorder[6-8]. Clinical studies have also implicated chronic stress in the pathogenesis of AD[9], now viewed as a disease of synaptic dysfunction and loss, i.e. a type of "disconnection syndrome"[10], while previous experimental studies showed that exposure to chronic stress in AD animal models aggravated AD neuropathology and triggered APP misprocessing[11,12]. Although clinical evidence suggests shared neurobiological mechanisms between both depression and AD, and exposure to prolonged stressful conditions is suggested as a risk factor for both disorders, it remains unclear whether APP misprocessing, a key AD mechanism is involved in the establishment of stress-driven depressive pathology. Our results show that APP misprocessing and BACE-1, the first enzyme responsible for APP cleavage towards the amyloidogenic pathway, is necessary for the stress-triggered depressive pathology and accompanying cognitive deficits, while fluoxetine and imipramine antidepressants exhibited a beneficial effect reversing APP misprocessing, followed by recovery of neuronal atrophy in both hippocampus and PFC brain areas. These findings provide new insights about potential role of APP misprocessing and BACE-1 on the establishment of stress-driven depressive state extending our mechanistic knowledge of the neurobiological substrates of stress-related brain pathology.

RESULTS

Stress-driven depressive symptomatology is accompanied by APP misprocessing and neuronal atrophy while antidepressant beneficial impact reverses both molecular and structural changes

To monitor the potential role of APP misprocessing in depressive state, we used a well-established animal model of depressive-like pathology based on the exposure to different stressful conditions for 6 weeks, called chronic unpredictable mild stress (CS). In addition, two

different antidepressant drugs commonly used in clinical practice, fluoxetine and imipramine, were administered to animals during the last 2 weeks of CS (see Figure 1). First, the CS impact in depressive and cognitive dimension was analyzed using a battery of different tests. For monitoring anhedonic behavior, a key depressive symptom in humans, sucrose preference test (SPT) was performed in different time points during the 6 weeks of experiment. As expected, SPT revealed reduced sucrose preference in CS animals ($F_{(1,44)}$ = 92.95, $p < 0.001$), while treatment with antidepressants (fluoxetine or imipramine) reversed the CS-reduction of sucrose preference back to control levels, confirming the beneficial effect of antidepressant treatment ($F_{(2,44)}$ = 8.23, p = 0.001); Figure 1b). Furthermore, we monitored another core symptom of depressive pathology, the learned helplessness by the use of forced swim test (FST). As shown at Figure 1c, exposure to CS resulted in a significant increase in immobility time ($F_{(1,\ 44)}$ = 18.56, $p < 0.001$), which was reverted by the administration of fluoxetine (p = 0.006) and imipramine ($p < 0.001$). Evaluation of spatial learning in Morris Water maze (MWM) test did not reveal any CS-induced significant differences in the distance swum by animals until they find the escaping platform, which was positioned in the same position during the first 3 days of the experiment ($F_{(1,44)}$ = 0.119, p = 0.73) (Figure 1d). Accordingly, neither imipramine nor fluoxetine induced changes in this parameter of MWM ($F_{(2,44)}$= 0.23, p = 0.8). In contrast, when the platform was re-positioned in the opposite quadrant of WMW swimming pool at day 4 (reversal learning task), CS animals exhibited decreased percentage of distance swum in the new quadrant ($F_{(1,44)}$ = 8.2, p = 0.006), followed by increased percentage of distance swum in the old quadrant where the platform had been the previous 3 days ($F_{(1,44)}$ = 12.7, p = 0.001). These findings suggest that CS elicited impairment in behavioral flexibility reflected the reverse learning task (Figure 1e). However, both antidepressants reversed this phenotype with animals treated with fluoxetine or imipramine travelling significantly lower distances in the “old” quadrant and longer distances in the “new” quadrant when compared to CS animals ($F_{(2,44)}$ = 5.3, p = 0.009).

Hippocampus and PFC atrophy is critically involved in the establishment of depressive behavior, as both brain areas are known targets of chronic stress and GC. Thus, we next performed three-dimensional morphometric analysis of Golgi-impregnated neurons in both brain areas. Our analysis revealed that CS induced a significant decrease in total dendritic length of granule cell dendrites of Dentate gyrus (DG) ($F_{(1,20)}$ = 32.06, $p < 0.001$; Figure 2a) and CA3 pyramidal neurons of hippocampus ($F_{(1,20)}$ = 46.35, $p < 0.001$; Figure 2b). Moreover, administration of antidepressant

fluoxetine or imipramine during the last two weeks of CS period reversed the CS-evoked reduction in dendritic length in both hippocampal subareas ($p < 0.005$ in both cases). Significant reduction in total dendritic length was also observed in pyramidal neurons in the PFC after exposure of animals to chronic stress ($F_{(1, 20)} = 130.09$, $p < 0.001$; Figure 2c); these changes were attenuated by treatment with imipramine, but not fluoxetine. The dendritic atrophy observed in CA3 and PFC pyramidal neurons and its reversal by antidepressant treatment were both confined to apical dendrites; basal dendritic lengths were not significantly altered by any of the treatments (data not shown).

Given the suggested common neurobiological substrates in depression and AD and the clinical data suggesting APP misprocessing in depressed patients, we next monitor the cellular pathway of APP misprocessing in hippocampal homogenates by immunoblotting of different target molecule that actively participate in its processing towards the production of the Aβ. As shown in Figure 2 e-f, WB analysis revealed that APP levels were up regulated by CS ($p = 0.04$); this CS effect was attenuated by treatment with either fluoxetine or imipramine (Figure 2e). Similarly, CS increased the levels of C-terminal fragments of APP ($p = 0.001$; Figure 2f). Importantly, antidepressant treatment was able to restore this CS effect back to control levels. Next, we also monitored the levels of BACE-1 levels, the first enzyme that cleaves APP towards the amyloidogenic pathway. Our results demonstrated that CS induced an increase of BACE-1 protein levels ($p = 0.04$; Figure 2g) supporting the induction of the amyloidogenic pathway. Interestingly, this CS effect on BACE-1 levels was also completely reversed by the fluoxetine or imipramine treatment. In addition, we also monitor the protein levels of nicastrin, an essential protein in the regulation of γ-secretase complex. Along with the above impact of CS, nicastrin levels were also increased after exposure to stress ($p = 0.032$; Figure 2h), while fluoxetine and imipramine countered the stress effects, lowering nicastrin protein to the normal (control) levels.

In summary, using a well-validated stress model of depression, the above results suggest that stress-drive depressive symptomatology and related neuronal atrophy is accompanied APP misprocessing, where 2 different antidepressants exhibited their beneficial effect reversing both neurostructural damage and APP misprocessing, thus highlighting a potential role for APP misprocessing in stress-driven brain pathology.

BACE1 ablation blocks the establishment of stress-driven depressive symptomatology and neuronal atrophy.

In order to clarify the essential role of APP misprocessing in stress-driven depressive pathology, we next investigate the behavioral and neurostructural impact of chronic stress paradigm on wild-types (WT) and mice lacking *BACE-1* gene (BACE-KO), the first enzyme responsible for APP cleavage, and thus lacking APP misprocessing as previously described. As shown at Figure 3, FST data analysis revealed an interaction between *Stress* and *Genotype* (2-way ANOVA; $F_{(1,55)}$ = 10.80, p = 0.001). Post hoc analysis showed that WT animals submitted to the stress protocol showed an increase of immobility time when compared to their controls (p = 0.003) indicating depressive behavior (Figure 3b). In contrast, BACE-KO animals were not affected by chronic stress (p = 0.74). Moreover, we used tail suspension test (TST) (Figure 3c) monitoring the time of animals climbing as an index of depressive behavior. 2-way ANOVA analysis showed a *Stress x Genotype* interaction in the climbing time ($F_{(1,80)}$ = 17.94, p < 0.0001) with stressed WT animals, but not BACE-KO group, animals exhibiting a decrease in time spent climbing (p < 0.0005). Assessment of cognitive behavior was based on Novel Object Recognition (NOR) and Y-maze tests. As shown at Figure 3d, 2-way ANOVA analysis revealed an interaction between *Stress* and *Genotype* in recognition index of NOR ($F_{(1,37)}$ = 17.15, p = 0.0002) with post-hoc analysis providing a significant reduction in stressed WT animals vs control WT (p = 0.0004) indicating short-term memory deficits; no significant effect of stress was found in BACE-KO animals (p = 0.5). Similarly, in Y-maze test (Figure 3e), we found a significant reduction in percentage of spontaneous alternations animals performed in three arms of Y-maze apparatus in stressed in WT animals compared to control WT (p = 0.02), suggesting cognitive impairment; note that stressed and control BACE-KO animals were similar in this parameter of Y-maze. Altogether, these findings suggest that lack of BACE-1 blocked the stress-driven depressive behavior and comorbid cognitive deficits.

Furthermore, we evaluate neuronal structure in both hippocampus and PFC brain areas. As expected, stress exposure triggered neuronal atrophy in hippocampus of WT animals, as shown by reduction in dendritic length of DG granule cell (p = 0.005) and of CA3 pyramidal neurons (total dendritic length p = 0.02; Figure 3f, g). Importantly, this stress effect was not found in BACE-KO hippocampus [2-way ANOVA; DG ($F_{(1,83)}$ = 10.54, p=0.0017) and CA3 neurons ($F_{(1,99)}$ = 4.24, p = 0.04)]. Further, significant reduction was also found in total dendritic length of

pyramidal neurons of PFC cortex of stressed WT (p=0.02) when compared to control WTs; again, stress exhibited no effect on dendritic length of BACE-KO neurons in PFC (p=0.72) [2-way ANOVA; $F_{(1,95)} = 7.87$, $p = 0.006$] (Figure 3h). Additionally, the aforementioned dendritic atrophy was further confirmed by Sholl analysis that assessed the complexity of dendritic tree in different distances from the neuronal soma. As shown at Figure 3i, stress resulted in decreased number of intersections of dendritic arborization in WT stressed animals when compared with control WT, while stressed BACE-KO showed no significant differences suggesting that lack of BACE attenuate the stress-driven neuronal atrophy.

DISCUSSION

Despite the considerable progress in the understanding of pathophysiology and neurobiology of psychiatric disorders over the past three decades, depression remains a complex mood disorder whose ethiopathogenesis seems to involve interaction(s) between genetic and environmental factors. For example, stressful life events are considered to play a causal role in precipitating depressive pathology while epidemiological studies illustrate a high comorbidity between depression and AD with a prevalence rate of 30-50%[13], suggesting a link between the two disorders. It should be noted that, in many ways, the impairments found in depression resemble those seen in subjects suffering from mild cognitive impairment (MCI), a diagnosis that is highly predictive of risk for development of AD[14,15]. Attempts to unravel (inter-) relationships between depression, MCI and AD have revealed that depressed patients who display MCI are at greater risk to develop AD than non-depressed subjects who suffer from MCI[16]. Here, it is pertinent to reiterate the fact that depression may predispose individuals to develop AD[2,17]. All these observations argue strongly in favor of the existence of an intimate relationship between mood and cognitive disorders, while clinical data point to a common neurobiological basis between depression and AD most likely reflecting common pathogenic factors[16,18]. Furthermore, accumulating evidence has suggested the continuum between depression, impaired cognition and AD[2,17,19] raising stress and GC as potential triggering/connecting factors[20]. Thus, better identification and characterization of the role of environmental factors, such as stress, that may add to the cognitive burden of old age, as well as exploration of potential biological targets for preventative and curative intervention, are urgently needed.

In line with previous evidence suggesting the AD-related cellular pathway of APP misprocessing

as a shared neurobiological underpinning of depressive pathology, we hereby demonstrate that APP misprocessing is causally related to stress-driven depressive symptomatology and accompanying cognitive deficits as well as neuronal atrophy, while lack of BACE-1 and subsequent APP misprocessing blocked both behavioral and neurostructural deficits. Structural changes within the hippocampus and the PFC are increasingly recognized as key components of depressive pathology. Using a well-validated animal model of depression of chronic mild stress, we found that depressive state was accompanied by significant neuronal atrophy of the apical dendrites of neurons in the hippocampus (dentate gyrus and CA3 areas) and PFC (layers II/III). Interestingly, the observed neurostructural rearrangements by our stress-based depressive model corroborate studies that report reduced hippocampal and PFC volumes[21,22] in depressed patients, as well as their pharmacological reversibility by antidepressant treatment[23,24]. The spatial distribution of these dendrites and its structural reorganization after chronic stress and their recovery after antidepressant administration suggests that interruption of hippocampus-PFC connectivity may underlie the manifestation of depressive-like behavior[6,25]. Indeed, the increased depressive levels in chronically stressed animals assessed by three different behavior tests (forced swim, tail suspension and sucrose preference test) were accompanied by cognitive deficits, whose manifestation is causally related to impairment of medial PFC and hippocampus. For instance, reversal learning in MWM as well as spontaneous alterations in Y-maze, reflecting working memory, was affected by our stress model are thought to involve PFC-hippocampus communication. Accordingly, neuronal atrophy in PFC brain area is shown to disrupt PFC-Hippocampus connectivity, resulting in cognitive deficits assessed by these tasks. In addition, while spatial learning, a mainly hippocampus-dependent task, was not affected by stress, another type of short-term working memory, the recognition memory assessed by NOR was also affected by stress. Importantly, both depressive-like behavior and accompanying cognitive deficits induced by stress were blocked in animals lacking BACE-1 and subsequent APP misprocessing.

Importantly, stress-driven depressive-like phenotype was accompanied by APP misprocessing, one of the major pathogenic mechanism of AD exhibiting elevated levels of BACE-1 and Nicastrin, two enzymes that are essentially involved in the production of Aβ. BACE-1 is needed for C99 production, the intermediate APP cleavage product, while previous studies have shown that even slight increases in this enzyme boost Aβ generation[26-28]. The current results go along with previous work from our and others labs, demonstrating that chronic stress and GC accelerates APP misprocessing in different AD animal models, with concomitant impairments of memory[11,12,20,29].

Hereby, we now show that chronic stress can trigger elevation of BACE-1 levels and APP misprocessing in wild-type animals, thus relating BACE-1 and APP misprocessing with the establishment of depressive pathology and accompanying cognitive deficits. Previous studies have described a GC response element in the promoter region of the APP and BACE-1 genes[30], making GC and glucocorticoid receptors (GR) possible mediators of the regulatory actions of stress on APP and BACE-1 expression. Interestingly, chronic stress failed to trigger both depressive-like behavior and neuronal atrophy in absence of BACE-1, providing a solid proof of BACE-1 in the establishment of stress-driven pathology. Despite that the enzyme has also other functions in the adult brain that future studies must also be addressed in the context of depressive behavior. Additionaly, BACE ablation blocked the stress-driven neuronal atrophy in both hippocampus and PFC brain areas, introducing BACE-1 in the underlying mechanisms through which chronic stress exerts its neuroremodeling action.

Despite the suggested common neurobiological basis between depression and AD, until recently there was a lack of mechanistic evidence regarding the involvement of AD-related mechanism(s) both in the manifestation of depressive symptomatology and the beneficial antidepressant action. Interestingly, our findings demonstrate that the stress-driven APP misprocessing was counteracted by the use of two different classes of antidepressants, fluoxetine, a widely-used selective serotonin reuptake inhibitor (SSRI) antidepressant and imipramine, a classical tricyclic antidepressant drug; an effect that was also accompanied by restoration of dendritic atrophy and amelioration of depressive-like behavior. Recent studies have demonstrated that both acute and chronic antidepressant treatment increased α-secretase activity, which was responsible for lowering Aβ levels[31], probably through ERK signalling pathway, which has been shown to modulate APP processing and Aβ generation[32,33]. While present clinical practice use antidepressants (often SSRIs) as first-line treatment for depression in dementia, their effectiveness on improving cognitive performance and/or AD pathology remains controversial[34,35]. However, a study from Sheline and colleagues showed that chronic antidepressant administration prevented the growth of pre-existing Aβ plaques and reduced the appearance of new plaques in an AD mouse model, while it was recently shown by the same group that acute antidepressant administration decreased the concentration of Aβ in cerebrospinal fluid of healthy humans strongly suggesting that antidepressant effect on APP misprocessing[36].

In summary, our findings suggest that chronic stress trigger BACE-1 and APP misprocessing

towards the establishment of depressive pathology and accompanying cognitive deficits highlighting BACE-1 and probably APP misprocessing as a crucial mediator of the stress-driven neuronal atrophy and brain pathology.

MATERIALS AND METHODS

Animals and chronic stress

All experiments were carried out in accordance with European Union Council Directive 86/609/EEC and National Institutes of Health guidelines for animal care and experimentation. 4 months old male Wistar rats (Charles River Laboratories, Barcelona, Spain) and 4-5 months-old male BACE1-*Knockout* animals (Bace-KO) and their *Wildtype* littermates (WT) (C57BL/6J background) were used in this study. Animals were housed under standard laboratory conditions (12h light/dark cycle; room temperature 22°C; relative humidity of 55%, ad libitum access to food and water). Animals were divided into two main treatment groups (control and stress—see below).

A 6-week long protocol of chronic unpredictable stress used was previously described by Bessa and colleagues[8], consisting of several mild stressors: confinement to a restricted space for 2h, placement in a tilted cage for 3h, housing on damp bedding for 8h, overnight illumination, food deprivation for 18h followed by exposure to inaccessible food for 2h, water deprivation for 18h followed by exposure to an empty bottle for 1h, and reversed light/dark cycle for 48h every 7 days over 6 weeks. During the last 2 weeks of chronic stress protocol, animals were injected daily with saline, fluoxetine or imipramine. All animals were submitted to a behavioural test battery, at the end of the stress period while the sucrose preference test was performed weekly over the 6 weeks of chronic stress protocol. For mice experiments, the chronic unpredictable stress protocol also lasted 6 weeks as previously described while animals were receiving one stressor per day (vibration, restraint, overcrowding, exposure to a hot air stream); the stressors were presented in random order during the protocol. All animals were weekly weighted throughout the study as an indication of treatment efficacy.

Drugs

Antidepressant drugs, Fluoxetine (10mg/kg, Kemprotec, Middlesborough, UK) and Imipramine (10 mg/kg, Sigma-Aldrich, St Louis, MO, USA) were used in this study. Compounds were dissolved in 5% DMSO in 0.9% saline. Both antidepressants and the vehicle were administered intraperitoneally (1 ml/kg) during the last 2 weeks of the stress protocol, at 8p.m. The doses of antidepressant drugs administered daily were chosen based in their therapeutic effects in previous studies.

Behavioral testing

Sucrose consumption test (SCT): During the six weeks of stress protocol, SCT was performed in the rats experiment in order to assess anhedonia. To establish baseline preference levels, animals were allowed to habituate to the sucrose solution during 1 week before the stress protocol. To test sucrose preference, animals were food- and water deprived for 18h; then, they were presented with two bottles containing 1% sucrose solution or tap water for 1h. The 1h intake was measured by weighting the bottles before and after the test. Anhedonia was defined as a reduction in sucrose preference when compared to baseline levels.

Forced swimming test (FST): As previously described by Bessa and colleagues[8] , animals were placed in glass cylinders filled with water (24°C; depth 30 cm) for a period of 5min. Trials were recorded and the duration of swimming and immobility time was analyzed using the Kinoscope software (http://sourceforge.net/projects/kinoscope/). The increase in time of immobility and a decrease in latency to immobility were defined as learned helplessness behavior.

Tail Suspension Test: Mice were suspended by the tails to the edge of a shelf 80 cm above the floor. Trials were recorded and Immobility and climbing time was recorded during a 5-min test as previously described by Porsolt and colleagues (1977).

Morris water maze (MWM): Cognitive performance was assessed by MWM using spatial learning and reverse learning tasks (Bessa et al., 2009). The test was conducted in a circular black tank (diameter: 170 cm; depth: 50 cm) filled to a depth of 30 cm with water (22°C) and placed in a dimly lit room with extrinsic visual clues that were glued to the walls. The tank was divided in

quadrants by imaginary lines and, during the test, a transparent platform (12 × 12 cm; invisible to the animals) was placed in one of them. Data were collected using a video camera fixed to the ceiling and connected to a video-tracking system (Viewpoint, Champagne au mont d'or, France). For the spatial learning task, animals were tested for 3 consecutive days (four trials per day). The platform was placed at the centre of an arbitrarily defined quadrant, assigned to a specific test subject. At the beginning of each of the four daily trials, animals were placed facing the wall of the maze in a defined start position. A trial is considered complete when the escape platform had been reached; when this escape fails to occur within 120s, the animal is gently guided to the platform and an escape latency of 120s is recorded for that trial. For the reverse learning task (4th day), the escape platform was positioned in a new (opposite) quadrant and animals were tested in a four-trial paradigm, as described above. For this task, distance and time-spent swimming in each quadrant were recorded. The difference between distances travelled in the quadrant containing the newly positioned platform ("new") and the quadrant that previously contained the platform ("old") was calculated as a measure of reversal performance. The total distance swum was evaluated as a measure of locomotor activity.

Y-Maze: For monitoring PFC-dependent cognitive function, spontaneous alternations were monitored using the Y-maze apparatus (33cm x 7cm x 15cm). Animals were placed in the center of the Y-maze apparatus and were allowed to explore freely for 8 minutes; the number and order of arm entries was recorded. Spontaneous alternation was calculated as the ratio of number of triads (sequence of 3 consecutive arm entries) and total arm entries. The percentage of alternations is used as index of spatial working memory performance.

Novel Object Recognition: This memory test is based on the animal's natural propensity to explore novel objects. The test arena consisted of a black rectangular box (33cm x 33cm x 33cm). Animals were given 20 minutes in 3 consecutive days to habituate to the test environment with no objects to explore. On the next day, animals were placed in the test arena, which contained two identical objects equal distant for 10 min. After one-day delay, the animals were presented one new (novel) object and one old (familiar) object for a period of 10 min. Each animal was placed equidistant from the two identical objects and the time spent exploring each object was recorded. Memory was operationally defined by the discrimination index (DI) for the novel object as the proportion of time that animals spend exploring the novel object. The test was recorded and the videos were analyzed with the Kinoscope software

(http://sourceforge.net/projects/kinoscope/).

Structural analysis

For 3D morphometric analysis, animals were transcardially perfused with 0.9% saline under deep anesthesia [ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg)]. Brains were immersed in a Golgi-Cox solution for 14 days and then transferred to 30% sucrose. Vibratome coronal sessions (200μm thick) were collected in 6% sucrose, dried onto gelatin-coated microscope slides. Then, they were alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed and dehydrated and mounted with entellan. Dendritic arborization was analyzed in the dentate gyrus, CA3 region of the hippocampus and layer II/III of the prelimbic area of the mPFC. 25-30 neurons per experimental group were studied and measurements from individual neurons from each animal were averaged. For each selected neuron, all branches of the dendritic tree were reconstructed at 600 (oil) magnification using a motorized microscope (Axioplan 2; Carl Zeiss) and Neurolucida software (Microbrightfield). The 3D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). For Sholl analysis (index of dendritic complexity and degree of arborization), the number of dendritic intersections with concentric spheres positioned at radial intervals of 20μm from the soma was accessed using NeuroExplorer software (Microbrightfield, Williston, VT) as previously described[6,8].

Western Blot Analysis

After sacrifice, hippocampi were dissected and immediately frozen. Frozen hippocampal tissues were homogenized in lysis buffer (100mM Tris-HCl, 250mM NaCl, 1mM EDTA, 5mM MgCl2, 1% NP-40, Complete Protease Inhibitor (Roche, Mannheim, Germany) and Phosphatase Inhibitor Cocktails I and II (Sigma, St Louis, MO)) using a Dounce glass homogenizer; extracts were cleared by centrifugation (14 000 g) and their protein contents were estimated by Bradford assay. Lysates, in Laemmli buffer (250mM Tris-HCl, pH 6.8, containing 4% sodium dodecyl sulfate, 10% glycerol, 2% b-mercaptoethanol and 0.002% bromophenol blue), were thereafter electrophoresed on 8 or 10% acrylamide gels, and transferred onto nitrocellulose membranes

(Protran BA 85, Schleicher & Schuell, Dassel, Germany). Membranes were blocked in Tris-buffered saline containing 5% nonfat milk powder and 0.2% Tween-20 before incubation with the following antibodies: anti-APP369 (1:5000; kindly provided by Dr Sam Gandy), anti-BACE-1 (1:500; ProSci Inc., Poway, CA, USA), anti-nicastrin (1:5000; Sigma) and anti-actin (1:2000; Chemicon, Temecula, CA, USA).

Antigens were revealed by enhanced chemiluminescence (Santa Cruz, CA, USA) after incubation with appropriate horseradish peroxidase–immunoglobulin G conjugates (Santa Cruz); blots were scanned and quantified using TINA 3.0 bioimaging software (Raytest, Straubenhardt, Germany) after ascertaining linearity. All values were normalized and expressed as percentages of control.

Statistical Analysis

Numerical data are presented as group means ± SEM. All data sets were subjected to one-way (anti-depressant experiment) or two-way ANOVA (for BACE-KO experiment) before application of appropriate *post hoc* pair-wise comparisons (GraphPad). Values of $p<0.05$ were considered as significant differences.

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Conflict of Interest

The authors declare no conflict of interest.

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Figures

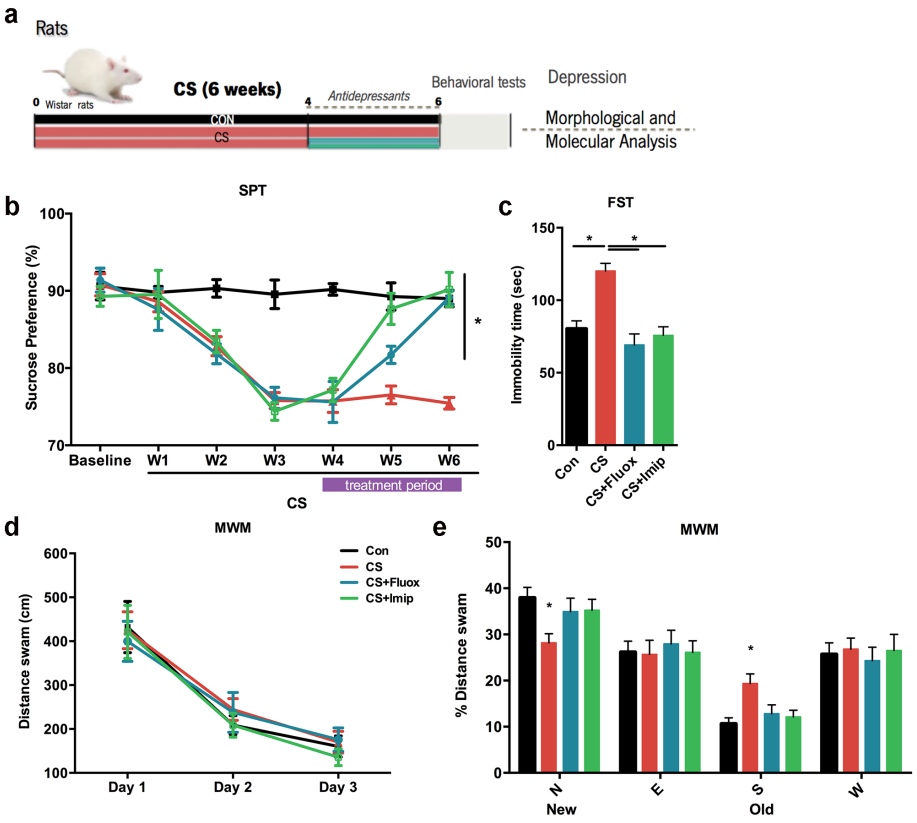

Figure 1. Stress-driven depressive symptomatology and accompaning cognitive deficits are rescued by antidepressants. Behavioral effects of CS and antidepressant treatment on mood and cognitive domains. (a) The experimental design is represented schematically. Control and stressed Wistar rats were used, (b) Anhedonia was assessed weekly in the sucrose consumption test performed during CS, (c) Learned helplessness was evaluated in the FST at the end of the chronic stress protocol, (d) Average distance swum in the spatial learning task and (e) the percentage of distance swum in each quadrant in the reverse learning task in the Morris watermaze. All numerical results represent mean ± SEM (* $p<0.05$).

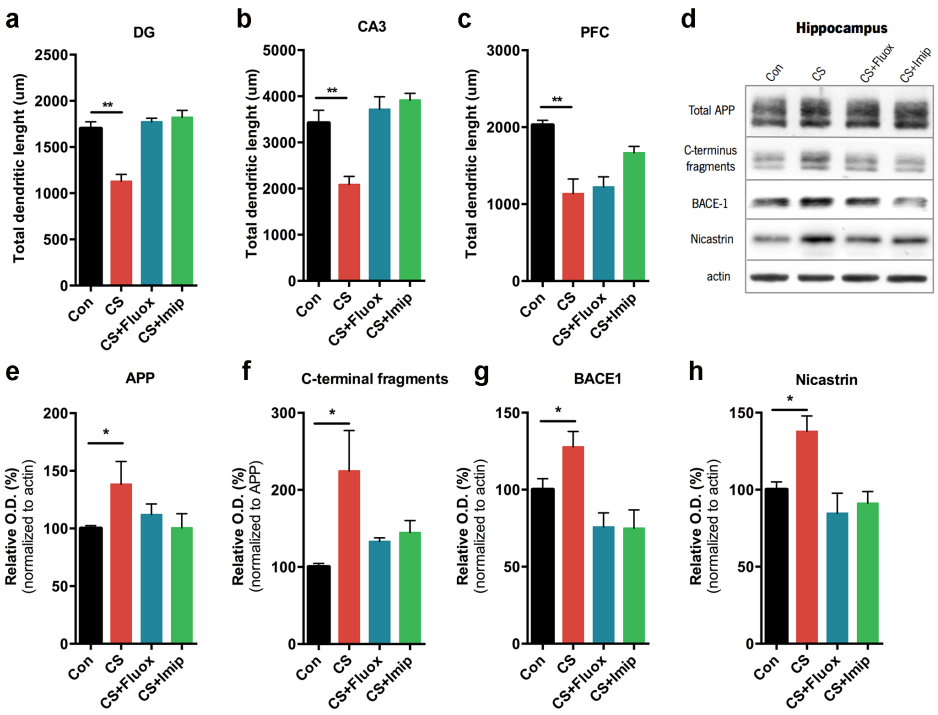

Figure 2. Stress-triggered neuronal atrophy and APP misprocessing is reversed by antidepressant treatment. Three-dimensional morphometric analysis of Golgi-impregnated neurons using computer-assisted reconstructions of hippocampal and mPFC neurons. Total dendritic length of (a) dentate granule neurons in the subgranular zone, (b) CA3 pyramidal neurons and (c) pyramidal neurons from layer II/III from the prelimbic area of the mPFC; (d-h) APP processing related-proteins changed levels with CS. CS induced APP misprocessing as confirmed by the increase of APP levels (e) and (f), together with an increase of the levels of the core enzymes responsible for the cleavage of APP molecule to generate Aβ, BACE1 (g) and Nicastrin (h). All these alterations were reversed by antidepressants action. All numerical results represent mean ± SEM (* $p<0.05$).

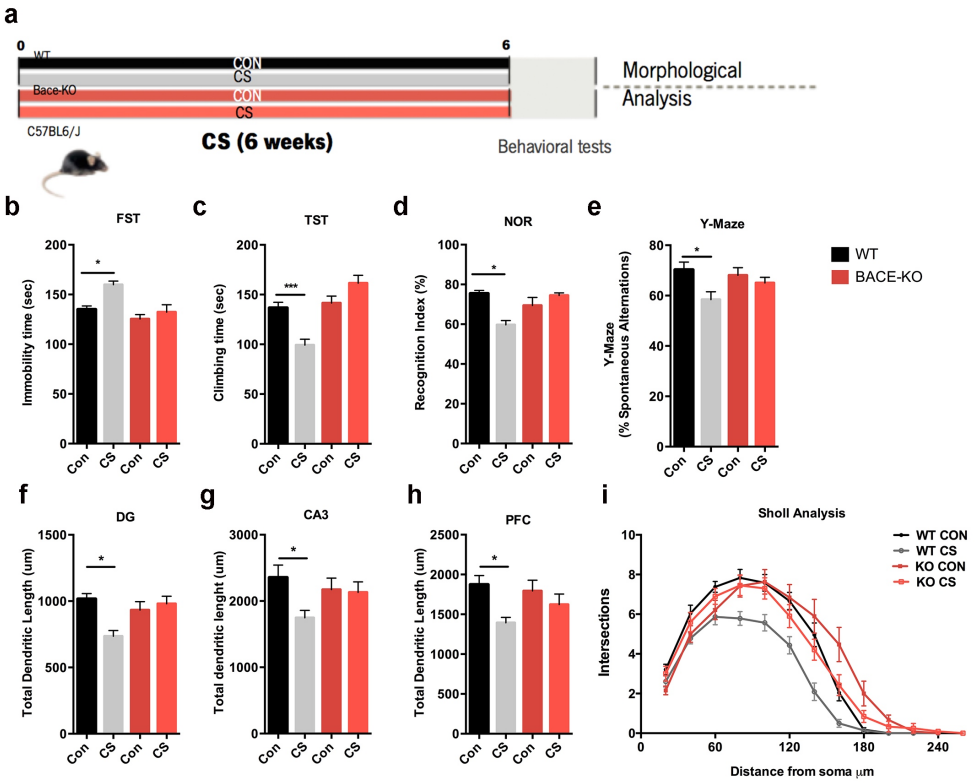

Figure 3. Lack of BACE1 blocked stress-driven neuronal atrophy, depressive behavior and cognitive deficits. (a) The experimental design is represented schematically. Control and stressed WT and BACE-KO animals were used. FST and TST revealed a stress-induced depressive-like phenotype in WT animals although lack of Bace1 revealed protective against this phenotype (b) and (c). In the same line, Bace-KO animals did not present cognitive deficts in NOR (d) and Y-Maze (e) in contrast to WT. Corroborating the behavioral data, structural analysis showed a neuronal atrophy by stress in WT animals and no change effect in Bace-KO animals in Hippocampal DG (f) and CA3 (g) and PFC neurons (h). All numerical results represent mean ± SEM (* $p<0.05$).

Chapter 2.4

Lopes S, Lopes A, Pinto V, Guimarães MR, Sardinha VM, Duarte-Silva S, Pinheiro S, Pizarro J, Oliveira JO, Sousa N, Leite-Almeida H, Sotiropoulos I

Absence of Tau triggers age-dependent sciatic nerve morphofunctional deficits and motor impairment

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Absence of Tau triggers age-dependent sciatic nerve morphofunctional deficits and motor impairment

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Summary

Dementia is the cardinal feature of Alzheimer's disease (AD), yet the clinical symptoms of this disorder also include a marked loss of motor function. Tau abnormal hyperphosphorylation and malfunction are well-established key events in AD neuropathology but the impact of the loss of normal Tau function in neuronal degeneration and subsequent behavioral deficits is still debated. While Tau reduction has been increasingly suggested as therapeutic strategy against neurodegeneration, particularly in AD, there is controversial evidence about whether loss of Tau progressively impacts on motor function arguing about damage of CNS motor components. Using a variety of motor-related tests, we herein provide evidence of an age-dependent motor impairment in *Tau−/−* animals that is accompanied by ultrastructural and functional impairments of the efferent fibers that convey motor-related information. Specifically, we show that the sciatic nerve of old (17–22-months) *Tau−/−* mice displays increased degenerating myelinated fibers and diminished conduction properties, as compared to age-matched wild-type (*Tau+/+*) littermates and younger (4–6 months) *Tau−/−* and *Tau+/+* mice. In addition, the sciatic nerves of *Tau−/−* mice exhibit a progressive hypomyelination (assessed by g-ratio) specifically affecting large-diameter, motor-related axons in old animals. These findings suggest that loss of Tau protein may progressively impact on peripheral motor system.

Key words: Tau; motor deficits; peripheral nerve; myelination; nerve conduction; knockout.

Introduction

Clinical presentation of Alzheimer's disease (AD) is complex and extends well beyond the cognitive impairments that characterize this disorder (Duker *et al.*, 2012). Alterations in facial expression, gait and posture, and manifestations of rigidity, bradykinesia, and tremor are found in late AD stages although mounting evidence suggests that motor problems emerge long before any recognizable sign of AD (Wilson *et al.*, 2000; Scarmeas *et al.*, 2004; Buchman & Bennett, 2011). Tau abnormal hyperphosphorylation and subsequent malfunction are postulated as crucial mechanisms in AD neuronal dysfunction where hyperphosphorylated and/or aggregated (insoluble) forms of Tau exhibit neurodegenerative action(s) that also interfere with normal Tau, sequestering and reducing soluble Tau forms (Ksiezak-Reding *et al.*, 1988; Zhukareva *et al.*, 2003). These lesions are mainly found at different areas of CNS such as hippocampus and cortex although some studies also demonstrate the presence of Tau deficits in peripheral nervous system (PNS; e.g., autonomic ganglia and sciatic nerves Bohl *et al.*, 1997; Holzer *et al.*, 1999). While evidence suggests that Tau reduction can block AD pathology progression (Roberson *et al.*, 2007), indicating that Tau-targeted strategies might be of interest for AD therapy (Gotz *et al.*, 2012), the safety and/or potential side effect(s) of these approaches is not well studied.

Tau genetic deletion seems to be well tolerated by young animals as the majority of *Tau−/−* models do not exhibit behavioral or microtubule alterations (Dawson *et al.*, 2001; reviewed by Ke *et al.*, 2012). However, chronic loss of Tau has been described to result in subtle or mild motor deficits in increasingly aged animals (reviewed by Gotz *et al.*, 2013). Indeed, one study revealed loss of substantia nigra (SN) dopaminergic neurons in middle-aged *Tau−/−* animals (Lei *et al.*, 2012), while in another study, using aged *Tau−/−* of the same strain, similar motor deficits were shown but in a SN-/dopamine-independent manner (Morris *et al.*, 2013), raising uncertainty on the underlying mechanisms of the motor deficits in *Tau−/−* animals. Surprisingly, the involvement of the PNS has not been assessed in any of the previous studies given the fact that Tau reduction is found in peripheral nerves (e.g., sciatic nerve) of patients with AD (Holzer *et al.*, 1999). This study aimed therefore to monitor the impact of chronic loss of Tau protein in PNS efferents, primary compartment of motor circuitry, and motor performance using a battery of behavioral tests analyzing motor function in both young (4–6 months) and old (17–22 months) *Tau−/−* mice combined with a systematic morphofunctional analysis of their sciatic nerve.

Results

Chronic lack of Tau results in motor deficits in old animals

To clarify the discrepancies of previous studies regarding the age-dependent motor deficits in *Tau−/−* animals (cf. Lei *et al.*, 2012 and Morris *et al.*, 2013), we performed a battery of motor-related behavioral tests in adult (4–6 months old) and old (17–22 months old) male *Tau−/−* and *Tau+/+* animals. Motor function/coordination and locomotor activity were assessed in the rotarod and open-field tests, respectively. As shown in Fig. 1A, the accelerating protocol of rotarod test showed that old *Tau−/−* animals presented reduced latency to fall, indicating an impairment in motor coordination. Two-way ANOVA rendered an overall genotype effect [$F_{1,37} = 4.050$, $p = 0.021$], where old *Tau−/−* exhibit a motor impairment when compared with old *Tau+/+* ($p = 0.019$). In the open-field arena, we found a *Genotype x Aging* interaction of total area travelled by the animals [$F_{1,31} = 4.339$, $p = 0.045$] (Fig. 1B). Further analysis revealed that old *Tau−/−* travelled significantly less than their

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age-matched *Tau*+/+ (p = 0.005) as well as than adult *Tau*−/− animals (p = 0.019). As potential body weight differences among groups could affect the assessment of the above motor-related parameters, we monitor body weight of these animals (adult *Tau*+/+: 27.876 ± 0.95; adult *Tau*−/−: 27.879 ± 0.53; old *Tau*+/+: 34.943 ± 0.60; old *Tau*−/−: 36.215 ± 1.67) finding no overall *Genotype* ([$F_{1,37}$ = 0.4187, p = 0.52]), but a significant *Aging* overall effect ([$F_{1,37}$ = 61.15, p < 0.0001]). Further analysis showed that both old *Tau*+/+ and *Tau*−/− animals exhibit higher body weight than their younger counterparts (p < 0.0001 for both genotypes). For monitoring muscle strength, we use wire-hanging test where a *Genotype x Aging* interaction [$F_{1,31}$ = 5.768, p = 0.022] was found; old *Tau*−/− present significantly less hanging time than old *Tau*+/+ (*P* = 0.008) as well as when compared with adult *Tau*−/− (p = 0.0001) suggesting compromised muscle strength (Fig. 1C). Furthermore, monitoring separately hindlimb and forelimb performance by hindlimb tonus and forelimb grid strength tests, we found a *Genotype x Aging* interaction (for tonus $F_{1,38}$ = 11.97, p = 0.001; for strength $F_{1,38}$ = 12.14, p = 0.001) represented by diminished hindlimb tonus resistance and reduced forelimb strength in old *Tau*−/− animals when compared to old *Tau*+/+ (p_{tonus} = 0.0003 and $p_{strength}$ = 0.004, respectively; Fig. 1D,E); note that this difference was not found in adult animals (p_{tonus} = 0.98 and $p_{strength}$ = 0.80, respectively) in line with the findings of the above tests used in this study. In addition, no significant changes among groups were found in hindlimb clasping score (Fig. 1F). Moreover, for extra monitoring of motor coordination and gait, we performed footprinting-based analysis showing a *Genotype x Aging* interaction in the stride length of animals ($F_{1,23}$ = 4.69, p = 0.04) where, in contrast to adult animals, old *Tau*−/− presented a decreased stride length when compared to old *Tau*+/+ (p = 0.03) (Fig. 1G). In addition, we found an *Aging* main effect on forepaw base ($F_{1,23}$ = 13.01, p = 0.001) where old *Tau*−/− animals presented an increase in this parameter compared to adult *Tau*−/− animals (p = 0.04) (Fig. 1H). No significant differences were found in other parameters of gait analysis such as hindpaw base and forepaw–hindpaw overlap.

Tau ablation alters sciatic nerve structure and function by aging

As *Tau*−/− mice are shown to exhibit motor deficits, it was of interest to monitor the impact of Tau ablation on the peripheral component of motor circuit; for that purpose, we studied the sciatic nerve, in the same mouse line and background (C57BL/6; Dawson *et al.*, 2001) used in previous studies describing motor deficits after Tau ablation (Morris *et al.*, 2013; Lei *et al.*, 2014). It is important to note that the sciatic nerve is known to exhibit important morphological and functional changes with aging (Melcangi *et al.*, 2000). Thus, we monitored myelinated (A-fibers) density in p-phenylene diamine (PPD)-stained sections. In agreement with previous studies (Ceballos *et al.*, 1999), two-way ANOVA showed an aging effect [$F_{1,16}$ = 11.71, p = 0.0038] with aged animals exhibiting reduced density of myelinated fibers independently of genotype (p < 0.05 for both genotypes) (Fig. 2A,B). Furthermore, electron micrographs of the sciatic nerves revealed, in addition to normal myelinated axons (Fig. 2C*i*), the presence of dysmorphic myelin sheaths as shown in Fig. 2C*ii*–*iv*. Thus, we also evaluated the percentage of myelinated fibers that exhibit the above abnormalities in their myelin sheath finding an overall aging effect [$F_{1,16}$ = 7.41, p = 0.015] and an overall genotype effect [$F_{1,16}$ = 5.58, p = 0.031]. Additional analysis showed a significant increase in the percentage of degenerating myelinated fibers in old *Tau*−/− animals when compared with old Tau+/+ animals (p = 0.049) and adult *Tau*−/− (p = 0.0289) (Fig. 2D). In the next step of our analysis, we compared myelin sheath thickness of fibers of sciatic nerves by electron microscopy determining the g-ratio, an index of myelination independent of axonal diameter (Michailov *et al.*, 2004). A total of 2885 axons were sampled and classified according to axon diameter. *Tau*−/− axons exhibited increased g-ratio indicating reduced myelination while two-way ANOVA per category exhibited an overall *Genotype* effect in all categories [$F_{1,270}$ = 23.891, p < 0.001 (0–2 μm); $F_{1,1390}$ = 99.885, p < 0.001 (2–4 μm); $F_{1,705}$ = 132.891, p < 0.001 (4–6 μm); $F_{1,372}$ = 39.379, p < 0.001 (6–8 μm); $F_{1,143}$ = 9.060, p = 0.02 (8–10 μm)] and an age effect [$F_{1,1390}$ = 31.672 (2–4 μm); $F_{1,705}$ = 74.302 (4–6 μm); $F_{1,372}$ = 100.001 (6–8 μm); $F_{1,143}$ = 30.694 (8–10 μm); p < 0.001] in all categories except 0–2 μm class of axons ($F_{1,270}$ = 3.256; p = 0.072) (Fig. 2E,F). Large-diameter (8–10 μm) axons g-ratio (motor-related) was different between old (p < 0.001), but not adult, *Tau*+/+ and *Tau*−/−. This g-ratio variation in large-diameter axons between *Tau*−/− and *Tau*+/+ of the two ages is also depicted in the regression lines of g-ratio growth in *Tau*−/− (grey) and *Tau*+/+ (black) of adult and old animals (inserted micrographs in Fig. 2E and 2F, respectively), where the regression lines for adult, but not old, animals are converging at the large-diameter axons indicating no difference between g-ratios of *Tau*−/− and *Tau*+/+ of this fiber category. The above ultrastructural hypomyelination findings in sciatic nerves of *Tau*−/− animals were further confirmed by reduced protein levels of myelin basic protein (MBP) (Fig. 2G).

Next, we monitored *in vivo* compound muscle (M) and reflex (H) nerve action potentials in hindpaw of anesthetized animals (Fig. 3A–E). As shown in Fig. 3B,C, there were no differences between amplitudes or total response area of direct muscle action potentials—M-wave—between *Tau*+/+ and *Tau*−/− on both ages. Regarding the H-waves which represent the reflex response that travels *via* the sciatic nerve (sensitive to lidocaine local sciatic application; Fig. 3A), two-way ANOVA showed an *Aging* main effect [$F_{1,17}$ = 6.701, p = 0.018] on the amplitude but no changes between *Tau*−/− and *Tau*+/+ animals of both ages (Fig. 3D). Moreover, assessment of the total response area exhibited a significant reduction in old *Tau*−/− animals when compared to old *Tau*+/+ (p = 0.02; Fig. 3E); note that this difference was not found in adult animals (p = 0.64). We also found a *Genotype x Aging* interaction effect [$F_{1,17}$ = 9.40, p = 0.007] as well as an aging main effect [$F_{1,17}$ = 49.78, p < 0.0001]. Based on the morphological alterations of sciatic nerve described above, we next monitored nerve function by *ex vivo* recordings in isolated sciatic nerves, as previously described (Pinto *et al.*, 2008) (Fig. 3F). Two-way ANOVA revealed no differences in conduction velocity of A-fibers among both ages and genotypes (Fig. 3G). However, assessment of the total response area revealed a significant *Genotype x Aging* interaction [$F_{1,31}$ = 6.715, *P* = 0.0144]. Particularly, we observed that sciatic nerves from old *Tau*−/− animals presented a decreased conduction capacity when compared with old *Tau*+/+ animals (*P* = 0.03) (Fig. 3H), indicating diminished overall response ability for old *Tau*−/− sciatic nerves; the same was not valid for adult animals again providing further evidence about the age-dependent impact of Tau loss on sciatic nerve.

Discussion

Tau is a soluble cytoskeletal protein that is expressed mainly in neurons and in glial cells, of both CNS and PNS; it is implicated in various cellular processes such as microtubule stabilization, axonal maintenance, and transport. Tau malfunction is also causally related to AD pathology in

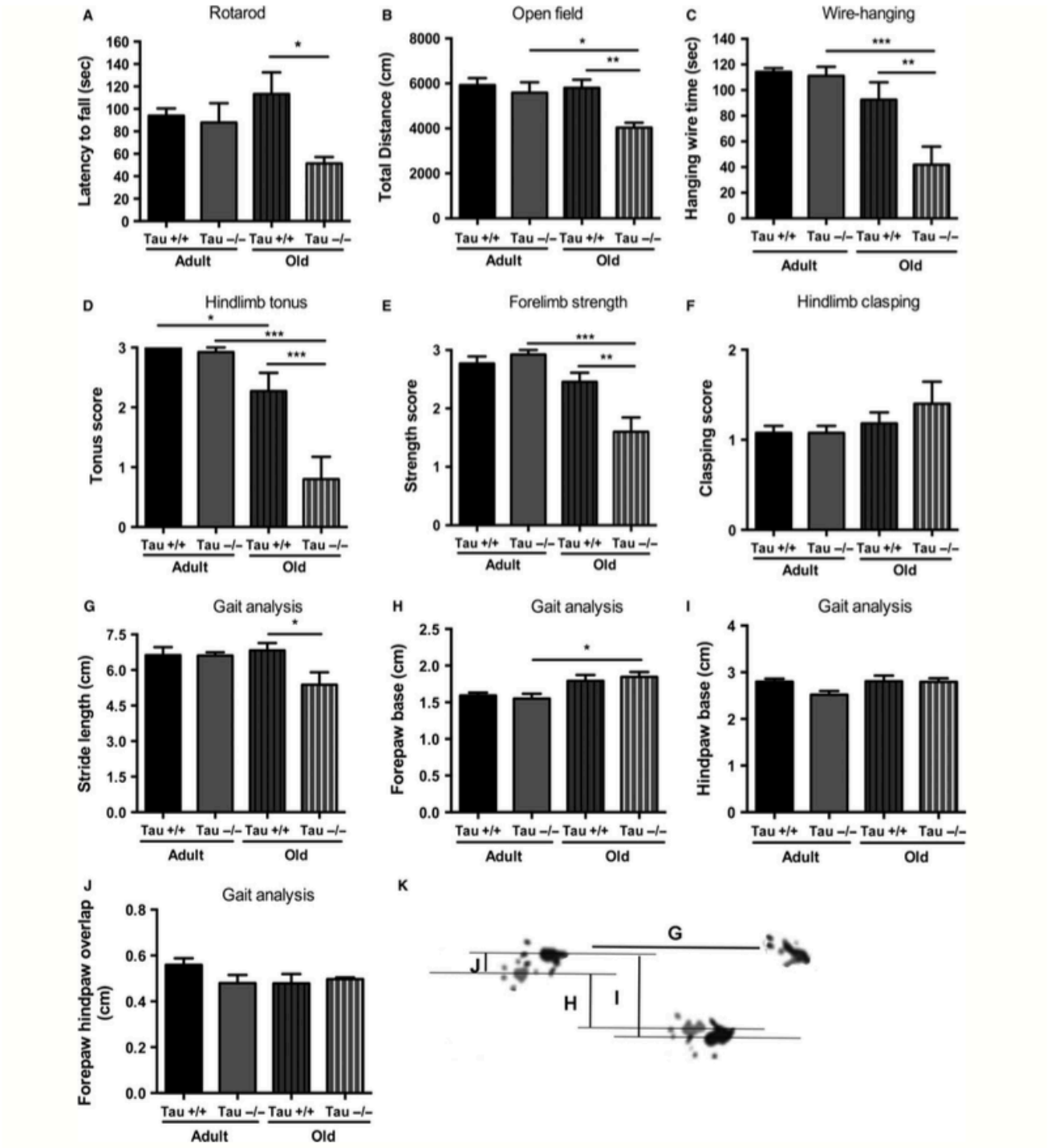

Fig. 1 Impaired motor behavior of old *Tau−/−* animals. Behavioral screening of motor functions revealed that lack of Tau protein led to an age-dependent motor dysfunction. (A) Rotarod test showed that old, but not adult, *Tau−/−* animals exhibited reduced latency to fall when compared with *Tau+/+* reflecting motor impairment. (B) Total distance travelled in open field was reduced in old *Tau−/−* when compared with old *Tau+/+* and adult *Tau−/−* animals indicating reduced locomotor activity. (C) Wire-hanging test assessments showed that old *Tau−/−* animals differed from old *Tau+/+* and adult *Tau−/−* presenting significantly less hanging time. D-E) Hindlimb tonus resistance (D) and forelimb strength (E) were severely reduced in old *Tau−/−* animals compared with old and adult *Tau+/+*. (F) No changes in hindlimb clasping score between animals of both ages and genotypes. (G–J) Footprinting-based gait analysis showed that, in contrast to adult animals, old *Tau−/−* present reduced stride length when compared to old *Tau+/+* (G), while no other changes were found between animals of two genotypes. (K) Representative image of animal footprints which were used to calculate the different gait parameters, for example, stride length *(G), forepaw base (H), hindpaw base (I), and forepaw/hindpaw overlap (J). All numeric data are presented as mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001.

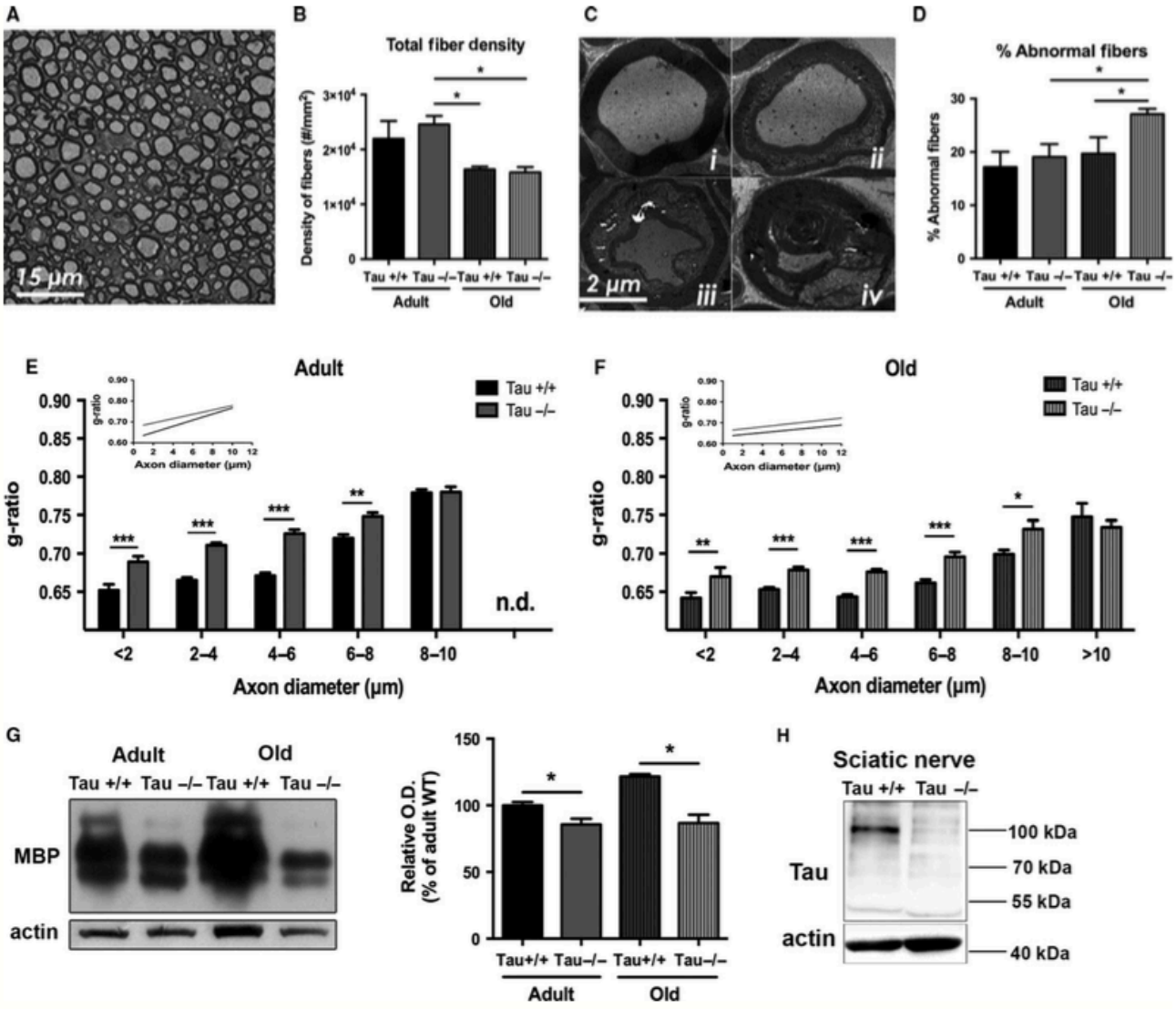

Fig. 2 Ultrastructural analysis of *Tau−/−* and *Tau+/+* sciatic nerves. (A, B) Representative light microscopy photograph of ppd staining of sciatic nerve (A) and quantification of fibers density (B) showing an age-dependent reduction in both *Tau−/−* and *Tau+/+*. (C) TEM analysis images of sciatic nerve showing normal (i) and degenerating (ii, iii) myelinated fibers with obvious myelin anomalies while severely damaged axons (iv) were very rarely found in all groups. (D) Old *Tau−/−* animals present a significant increase in percentage of degenerating fibers when compared with old *Tau+/+* animals. (E, F) Detailed evaluation of g-ratio [axon diameter/(axon diameter + myelin thickness)] per axon diameter category in adult (E) and old (F) animals. There is an age-dependent g-ratio reduction (hypomyelination) in Tau−/− vs. *Tau+/+* starting from axons of small and middle diameter (nonmotor related) in adult animals (E) and extending to large-diameter (8–10 µm), motor-related axons in old Tau−/− (F). Specifically, compared to adult *Tau+/+* ones, adult *Tau−/−* animals exhibited increased g-ratio in all fiber categories except that of 8–10 µm, while in old *Tau−/−*, this category g-ratio was also affected (E). Insets represent regression lines of g-ratio growth in *Tau−/−* (red) and *Tau+/+* (black) of adult (D) and old (E) animals where the regression lines for adult, but not old, animals are converging at the large-diameter axons, indicating no difference between g-ratio of *Tau−/−* and *Tau+/+* of this fiber category. G-H) Western blot analysis showed reduced myelin basic protein (MBP) levels in Tau−/− sciatic nerves (G) in line with the g-ratio analysis as well as absence of the characteristic high molecular weight Tau (HMW-Tau; 110 kDa) (H) in sciatic nerves of Tau−/− animals. Data are presented as mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001.

where aberrant, hyperphosphorylated and/or aggregated, forms of Tau are found in the CNS and in the PNS (Holzer *et al.*, 1999). Accordingly, recent evidence suggests Tau protein as the final executor of AD neuronal dysfunction (Roberson *et al.*, 2007; Ittner & Gotz, 2011) and, therefore, anti-Tau therapies emerged as potential strategies against AD (Frost *et al.*, 2015); however, their safety or potential risks have not been defined yet.

Evidence from different *Tau−/−* models suggests that absence of Tau is well tolerated by young/adult animals probably due to the occurrence of compensatory mechanisms (e.g., increase in MAP1A, another microtubule-associated protein) (Dawson *et al.*, 2001; Ma *et al.*, 2014). In contrast, aged *Tau−/−* seem to exhibit mild motor deficits albeit the effect of Tau depletion on motor performance by aging remains controversial and unclear. A study from Ikegami *et al.* (2000) reports signs of muscle weakness (tested by wire-hanging) and reduced balance coordination in 2-month-old *Tau−/−*, but Morris *et al.* (2011) showed normal motor function of young (3–5 months old) and adult animals of another Tau−/− mouse line in a variety of motor tests (B6 background; Dawson *et al.* (2001)) in agreement with this study

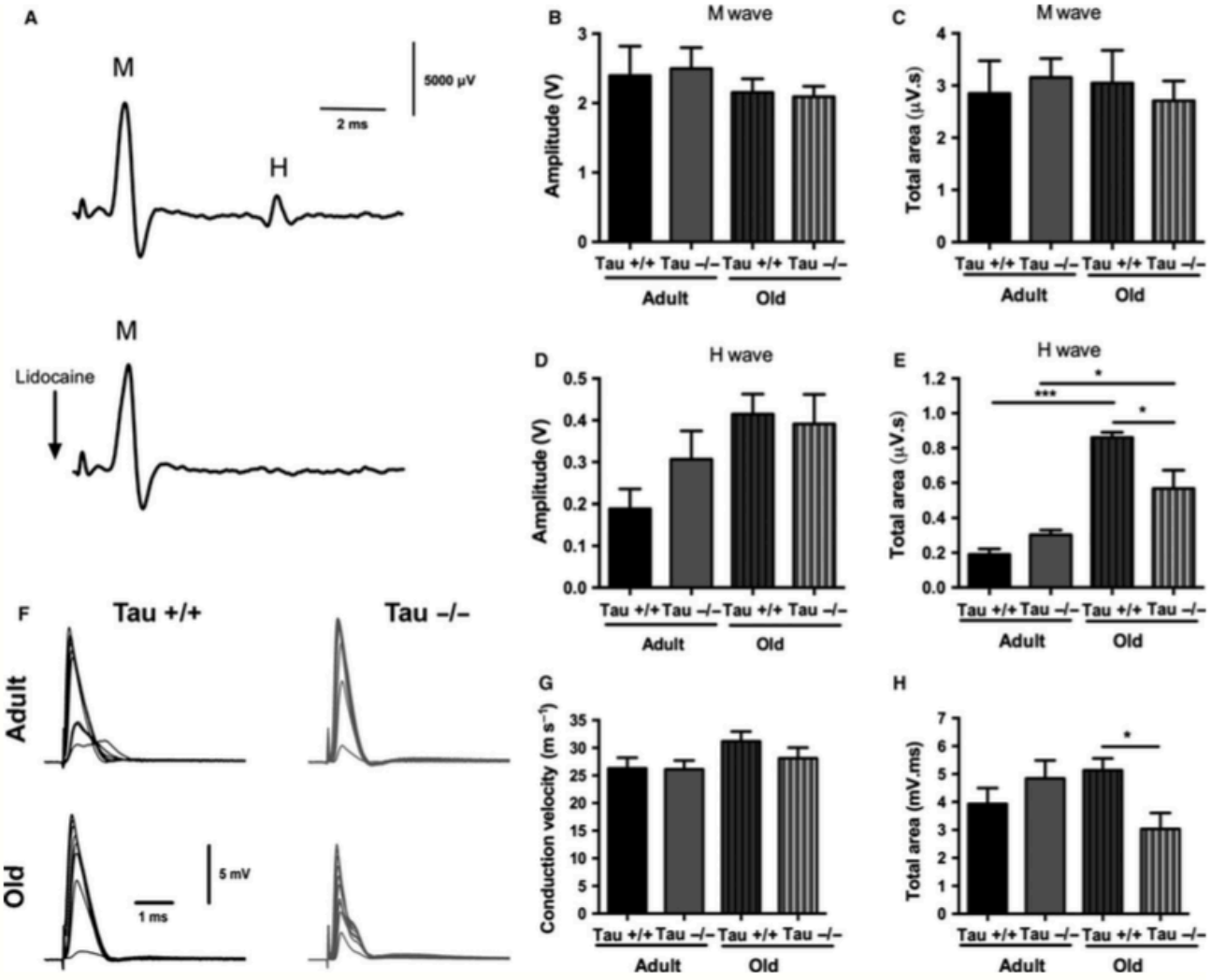

Fig. 3 Tau ablation results in age-dependent sciatic nerve malfunction. (A) Representative traces of *in vivo* electrophysiological recordings of compound muscle (M) and reflex (H) nerve action potentials (upper panel); lidocaine injection locally at the sciatic resulted in loss of H-wave without affecting the muscle M-wave (lower panel). (B, C) No differences in both amplitude (B) and total response area (C) of M-waves between animals of both genotypes and ages. (D) Age-dependent increase in amplitude of H-waves (*Aging* main effect, see also results), but no difference between *Tau−/−* and *Tau+/+* animals. (E) Old *Tau−/−* exhibited significantly reduced total response area compared to old *Tau+/+* animals, while this difference was not found in adult animals. In addition, there was an age-dependent difference in both *Tau+/+* and *Tau−/−* animals. (F) Representative traces of current-clamp recordings of sciatic nerve compound action potentials in adult and old *Tau+/+* and *Tau−/−* mice. Sciatic nerves received repeated stimuli with increasing amplitudes pulses until saturation. Stimulation artifacts were truncated (conduction distance: adult *Tau+/+*: 5.5 mm; adult *Tau−/−*: 9.6 mm; old *Tau+/+*: 8.0 mm; old *Tau−/−*: 9.8 mm); traces are averages of five consecutive recordings. (G, H) While no differences in conduction velocity (m s^{-1}) between *Tau+/+* and *Tau−/−* were found (G), total area under the curve (mV.ms) of old *Tau−/−* was significantly reduced compared to old *Tau+/+* animals indicating sciatic nerve malfunction (H). Data presented as mean ± SEM; $p < 0.05$.

findings. Moreover, the same team found mild motor deficits in middle-aged (12–15 months old) *Tau−/−* animals using rotarod and pole tests but, unexpectedly, these motor deficits were not clearly evident in old (21–22 months old) *Tau−/−* (Morris *et al.*, 2013). In contrast, we observed clear motor deficits in old (17–22 months old) *Tau−/−* using the rotarod, wire-hanging, and open-field tests and these deficits were not correlated with body weight. In addition, our study offers additional evidence of motor dysfunction showing that, in line with our wire-hanging data, old *Tau−/−* animals also exhibit reduced forelimb (grip) strength and diminished hindlimb tonus resistance compared to old wild-types but they exhibit no deficits in gross aspects of motor function/balance as assessed by hindlimb clasping tests (see Fig. 1) and negative geotaxis (data not shown). Interestingly, reduced motor strength and coordination was also shown in 13- to 17-month-old animals lacking 4R-Tau, suggesting the potential role of 4RTau in the age-dependent development of motor deficits (Gumucio *et al.*, 2013).

The mechanisms underlying the impaired motor behavior in the absence of Tau in aged individuals are not entirely understood with contradictory findings being reported. Lei *et al.* (2012), for instance, reported an age-related loss of SN dopaminergic neurons in middle-aged *Tau−/−* animals. In contrast, a recent study on the same *Tau−/−* line failed to observe any major dopaminergic loss in different CNS motor components of old *Tau−/−* mice (Morris *et al.*, 2013). While both studies showed motor deficits, Lei *et al.* (2012) showed nigrostriatal loss

in middle-aged Tau−/− mice of C57BL/6/SV129 background, while our study and the study of Morris *et al.* (2013) found no nigrostriatal neuronal loss in old mice with C57BL/6 background (data not shown). Furthermore, in a later study, Lei *et al.* (2014) showed that motor deficits of old *Tau−/−* animals do not depend on background or have a sex-dependent profile. In line with it, despite the fact that our study has used male animals and Morris study (2013) used a mixed (male and female) cohort, both studies exhibit motor deficits providing further support of no gender influence in the old *Tau−/−* motor deficits.

While a number of CNS areas have been implicated in the emergence of AD-associated motor deficits, little attention has been devoted to PNS primary efferents. Previous evidence suggests that the sciatic nerves of patients with AD, but not of age-matched healthy individuals, exhibit reduced Tau levels (Holzer *et al.*, 1999). In our study, we demonstrate that chronic loss of Tau protein results in sciatic nerve morphofunctional deficits which includes increased percentage of degenerating fibers, hypomyelination of large-diameter, motor-related fibers, and diminished conduction properties in old, but not young, *Tau−/−* sciatic nerve. While other mechanisms cannot be excluded, the aforementioned sciatic nerve deficits may critically contribute to motor deficits found in old *Tau−/−* as fine-tuning of myelin sheath thickness and formation is important for maintenance and proper function of motor fibers. In addition, while no amplitude differences in muscle action potentials of *Tau−/−* were found, further studies are needed to clarify the impact of loss of Tau on neuromuscular junction by aging as Tau-related pathology in motor neurons has been shown to have neuromuscular junction malfunction and motor deficits in AD Tg models (Zhang *et al.*, 2005; Ubhi *et al.*, 2007).

Despite the fact that axon–Schwann cell interactions are critical for nerve function and maintenance, the underlying mechanisms of maintaining normal nerve and Schwann cell structure and function remain poorly understood. Tau is expressed in both CNS and PNS, but the low molecular weight isoforms of Tau protein that are expressed in adult CNS differ from high molecular weight (HMW) Tau isoforms mainly found in PNS (e.g., sciatic nerves) but also in optical nerves (Sato-Yoshitake *et al.*, 1989; Georgieff *et al.*, 1991; Nothias *et al.*, 1995). The expression of HMW tau isoforms may confer increased stabilization and spacing of microtubules (Frappier *et al.*, 1994; Boyne *et al.*, 1995) but yet, our knowledge about Tau function in PNS is very limited. While previous cell-based evidence suggests the involvement of Tau in myelination of oligodendrocytes, the current study provides ultrastructural and molecular support of Tau role in myelination of PNS. Neuronal axon–Schwann cell interaction(s) are essential for myelination, and myelin maintenance is a dynamic process tightly controlled by axon-dependent transcription factors such as Krox20 and Oct6 (Murphy *et al.*, 1996; Decker *et al.*, 2006). In line with previous studies demonstrating an absence of axonal deficits in neurons of *Tau−/−* animals (Yuan *et al.*, 2008; Vossel *et al.*, 2010), there were no differences in mRNA levels of transcription factors that critically regulate myelination process and/or maintenance, for example, Krox20 and Oct6 (data not shown), suggesting no gross changes in myelin gene regulation of *Tau−/−* Schwann cells. Furthermore, supporting the involvement of Tau and other cytoskeletal elements in myelination process, previous evidence suggests that Tau strongly colocalizes with MBP in distal tips of oligodendrocytes (LoPresti *et al.*, 1995; Muller *et al.*, 1997), suggesting that transportation and/or local MBP translation may require microtubule cytoskeleton and might be controlled by Tau–Fyn interaction (Klein *et al.*, 2002). As recent evidence suggests that Tau is responsible to locate Fyn in spines and Fyn has a well-described role in myelination of both CNS and PNS (Kramer-Albers & White, 2011), the above Tau–Fyn–MBP complex could be disrupted in the absence of Tau protein, affecting myelination signaling and process. Indeed, Taiep myelin-mutant animals exhibit irregular microtubule polarity and display abnormal Tau accumulation and intracellular accumulation of myelin proteins in oligodendrocytes (Song *et al.*, 2001), while PNS myelin-deficient mice also exhibited reduction in Tau and other cytoskeletal protein in their sciatic nerves (Kirkpatrick & Brady, 1994). The above findings suggest that the absence of Tau may impact on Schwann cells' myelination process/signaling, opening a novel window for further research on role of Tau protein in PNS. Furthermore, in light of the suggested therapeutic potential of Tau reduction against AD, a better understanding of the impact of Tau ablation in both CNS and PNS is of great importance. Our findings highlight the morphological and functional implication of peripheral nerves induced by Tau loss in the precipitation of motor deficits with increasing aging and should be taken into account in the development of future AD therapies.

Experimental procedures

Animals

In this study, 4- to 6- and 17- to 22-month-old male *Tau+/+* and *Tau−/−* (Dawson *et al.* (2001); C57BL/6 background) were used. Mice were housed 4–5 animals per cage under standard environmental conditions with *ad libitum* access to food and water. All experimental procedures were approved by the local ethical committee and national authority for animal experimentation and were in accordance with the guidelines for the care and handling of laboratory animals, as described in the Directive 2010/63/EU.

Behavioral tests

Rotarod, wire-hanging, and open-field tests were performed. Animals were acclimated to the testing conditions for 60 min before testing. For rotarod test (TSE systems, Bad Homburg, Germany), mice were first trained a constant speed (15 rpm) for 3 days (4 trials per day; 60-s max trial time; 10-min resting interval among trials). At fourth day, mice (N = 9–11 for each genotype) were tested on an accelerating rod (4–40 rpm, 5 min; four trials); in this case, latency to fall was automatically recorded. For wire-hanging test, animals were placed on a standard wire cage grid, which was then inverted and at 30 cm height from the table surface. The latency to animal fall was manually recorded (two trials; maximum time of each trial: 120 s). The open field was performed in a square arena (43.2 × 43.2 cm) surrounded by tall perspex walls (Med Associates Inc., St. Albans, VT, USA). Mice were placed in the center of the arena allowed to explore during 10 min. Infrared beams were used to automatically register animals' movements. We next monitored forelimb strength, hindlimb tonus, hindlimb clasping, negative geotaxis, and gait analysis as previously described (Silva-Fernandes *et al.*, 2014). For all tests, scoring was carried out manually by an experimenter blind to the genotype and age of the animals. Briefly, for the assessment of forelimb (grid) strength, animal was positioned on a metal grid and gently pulled off by its tail (hindlimbs were suspended slightly above the grid), while the animal was gripping a wire using its forelimbs. A scale of four different scores was used by the experimenter evaluating forelimb strength of the animal: 0 (no strength), 1 (light/semi-effective), 2 (moderate/effective), and 3 (active/effective). Hindlimb tonus was tested by pressing animals' hindlimb paw (by experimenter index finger) evaluating muscular resistance and classifying into four different categories: 0 (no resistance), 1 (slight resistance), 2 (moderate resis-

tance), and 3 (pronounced resistance). Assessment of hindlimb clasping was performed suspending animals by the tail during 30 s. The hindlimbs were observed, and each mouse was given a score for each trial. Hindlimb clasping was rated based on two classes: 1 (hindlimb splayed outward and away of the abdomen) and 2 (hindlimbs retracted inward, toward the abdomen for at least more than 50% of the testing time). Next, we also performed gait analysis based on footprinting. Briefly, the hind- and forepaws of the mice were coated with black and red nontoxic paints, respectively. The animals were allowed to walk along 100-cm-long × 4.2-cm-width × 10-cm-height corridor, with ordinary white paper on the floor of the runway for each run. Each animal was tested to achieve one valid trial. The footprint patterns were analyzed manually for four step parameters (measured in cm): the front- and hind-base width, the footstep uniformity, and the stride length. For the analysis of each step parameter, three values were used based on three consecutive steps.

Sciatic nerve ultrastructure analysis

Under deep anesthesia [ketamine hydrochloride (75 mg kg^{-1}) and medetomidine (1 mg kg^{-1})], sciatic nerves were collected and immediately fixed in 4% glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.4) for 7 days and room temperature and then postfixed in 1% OsO_4, dehydrated. Finally, the tissue was embedded in epon resin (Electron Microscopy Sciences) and sectioned according to the objective (see below). One-micrometer transverse sections covering the complete cross-sectional nerve area were stained with 1% p-phenylene diamine and mounted on Entellan (Merk). Light microscope (Olympus DP70, Hamburg, Germany) images were then mounted on Photoshop and used for manual calculation of number and density of myelinated fibers per transverse section (4–5 animals per group). For the assessment of degenerating fibers, 16 nonoverlapping TEM photographs (3000×) of counterstained ultrathin sections (60 nm) were used (obtained by JEM-1400 TEM). The same TEM images were also used for g-ratio [axon diameter/(axon diameter + myelin thickness)] calculation; more than one hundred fibers were measured per animal. Morphometric analysis was performed by an experimenter blind to the samples provenience.

Ex vivo measurement of compound action potentials

Acutely isolated sciatic nerves from each group (6–8 animals per group) were used for the assessment of A- and C-fibers conduction velocity and compound action potentials as previously described (Pinto *et al.*, 2008). Briefly, sciatic nerves were dissected and cleaned from the connective tissue sheath in artificial cerebrospinal fluid. Compound action potentials recordings were made with a Multiclamp 700B amplifier in CC mode and digitized with the Digidata 1440a digitizer using PCLAMP 10 software (Axon Instruments, Sunnyvale, CA, USA). Signals were low-pass-filtered at an effective corner frequency of 16 KHz and sampled at 50 KHz. Fibers were stimulated at 60 μs, and conduction velocities were calculated for the first compound action potentials peak; total areas were calculated using CLAMPFIT software (Axon Instruments). Electrophysiological recordings and analyses were performed by an experimenter blind to the provenience of the tissue.

In vivo electrophysiological measurements

The *in vivo* approach allowed the acquisition of electroneuromyographic recordings in a noninvasive manner as described previously (Petit *et al.*, 2014). Briefly, 5–6 mice per group were anesthetized with a mixture of ketamine (75 mg kg^{-1}; Imalgene®, Merial, France) and medetomidine (1 mg kg^{-1}; Dormitor®, Pfizer, New York, NY, USA) i.p. administered. Temperature was monitored and maintained at 37 °C by a homeothermic blanket (Stoelting, Dublin, Ireland). A concentric bipolar platinum/iridium–stainless steel stimulation electrode (WPI, Worcester, MA, USA) was used to deliver 0.1 ms square pulses to the tibial nerve at the ankle, through a small skin incision. The ground stainless steel electrode was inserted at the base of the tail. Compound muscle action potentials (CMAPs) were recorded from the plantar muscles of both hindpaws individually through stainless steel electrodes (diameter: 0.28 mm; Science Products, Hofheim, Germany) inserted in the plantar muscle and subcutaneously in the third toe. Signals evoked by 1-mA electrical stimulation at 0.1 Hz were amplified, filtered (3–3000 Hz, LP511 Grass Amplifier, Astro-Med, Rodgau, Germany), acquired (Micro 1401 mkII, CED, Cambridge, UK), and recorded using SIGNAL Software (CED). This setup allowed the recording of CMAPs containing (1) stimulus artifact; (2) early nerve action potential; (3) direct muscle response (M-wave); and (4) monosynaptic reflex response (H-wave; Fig. 3A, upper panel); the latest was specifically abolished by lidocaine (2%; B Brown, Germany) injected in the vicinity of the sciatic nerve in the mouse thigh in a control experiment (Fig. 3A, lower panel). Averages of five consecutive responses were analyzed to measure amplitudes and area under curve of M- and H-waves to the baseline. The values obtained for each paw were averaged per animal.

Western blot analysis

Tau−/− and *Tau+/+* sciatic nerves were homogenized [10 mM HEPES pH 7.9, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10% glycerol, 1% NP-40, Complete Protease Inhibitor (Roche, Mannheim, Germany) and Phosphatase Inhibitor Cocktails II and III (Sigma, St Louis, MO, USA)]. After sonication and centrifugation (15 000 g; 10 min; 4 °C), protein contents were estimated by Bradford assay and lysates were electrophoresed on 10% acrylamide gels, and transferred onto nitrocellulose membranes (BIORAD Turbo, Munich, Germany). For detecting MBP levels, membranes were blocked in Tris-buffered saline containing 5% nonfat milk in TBS-T before incubation with antibodies against MBP (Serotec, Oxford, UK; 1:500), Tau (abcam, Cambridge, UK; 1:1000), and actin (DSHB, University of Iowa, IA, USA; 1:2000). Antigens were revealed by enhanced chemiluminescence (BIORAD) after incubation with appropriate horseradish peroxidase–immunoglobulin G conjugates (BIORAD). Blots were scanned and quantified using TINA 3.0 bioimaging software (Raytest, Straubenhardt, Germany). All values were normalized against actin.

Statistical analysis

Unless otherwise specified, two-way ANOVA was used having genotype (*Tau+/+* vs. *Tau−/−*) and aging (adult vs. old) as factors and followed by Tukey post hoc analysis (SPSS, Aspire Software, Armonk, NY, USA). Differences were considered to be significant if $p < 0.05$.

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Authors' contribution

SL, AL, VP, VMS, SP, JP, JFO, HLA, and IS performed experiments; SL, AL, MG, VMS, JFO, HLA, and IS analyzed data and contributed with

reagents; and IS, NS, and HLA designed the overall study and wrote the manuscript.

Conflict of interests

None of the authors report competing interests.

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Chapter 3

3.1 General Discussion

Current lifestyle places individuals under increasingly greater loads of stress. Although the mechanisms that are triggered by stress are primarily adaptive, it is now thoroughly established that exposure to chronic stress and/or main stress hormones, glucocorticoids (GC) cause a profound impact of neuronal structure and function, which culminates in impairments of mood and cognition (Lucassen et al., 2014). Interestingly, clinical and experimental studies suggest a strong causal role for chronic stress and GC in the development of both depressive pathology and Alzheimer's disease (AD) (Lucassen et al., 2014), whereas a growing body of evidence supports a pathological link between depressive pathology, dementia and AD pointing to shared neurobiological substrates (Ownby et al., 2006); yet, the appealing conceptual framework that links depression to AD neuropathology is still waiting for mechanistic evidence. Supporting the hypothesis that stress may serve as the connecting parameter between both disease states (Sotiropoulos et al., 2008b), this PhD thesis provides novel evidence about the essential role of AD cellular mechanisms, APP misprocessing and Tau hyperphosphorylation, in the stress-directed orchestration of cellular cascades involved in dendritic atrophy and synaptic loss leading to depressive pathology and cognitive deficits (**chapters 2.1, 2.2, 2.3**).

In addition, the current findings (shown at **chapters 2.1, 2.2**) as well as previous studies in AD field suggest that Tau hyperphosphorylation and the related malfunction are key events in neuronal damage and neurodegeneration (Gotz et al., 2013). However, it is not clear yet whether this Tau-related neuronal damage is attributed to gain of (novel) toxic function for Tau (e.g. synaptic damage; see Chapter 2.3 and (Ittner et al., 2010)) or loss of normal Tau function (e.g. loss of microtubule binding capacity; (Gotz et al., 2013)). Indeed, the impact of loss of normal Tau function in neuronal degeneration and subsequent behavioral deficits are still under intense debate. This PhD thesis shows that loss of Tau precipitates an age-dependent motor impairment that is followed by ultrastructural, structural and functional deficits in the efferent fibers conveying motor-related information (**chapter 2.4**). These findings suggest that loss of Tau protein may progressively impact on peripheral motor system adding to our understanding of peripheral neurological deficits in AD.

AD cellular mechanisms in stress-driven depressive pathology and cognitive impairment

Neuronal atrophy and synaptic loss within the hippocampus and the prefrontal cortex (PFC) are widely recognized as key components of both depressive and AD pathologies, while stress and GC are considered important disruptors of neuroplasticity, by triggering dendritic atrophy and synaptic loss in these brain areas (Cerqueira et al., 2005; Magarinos and McEwen, 1995a; Sousa et al., 2000). Indeed, elevated GC levels are suggested to play a causal role in hippocampal volume (Sheline et al., 2003) and PFC atrophy (Cerqueira et al., 2007b) in stress-based animal models of depression and cognitive impairment. In humans, therapeutic reversal of hypercorticism reverses hippocampal atrophy and associated depressive and cognitive symptoms (Green et al., 2006; Modrego and Ferrandez, 2004; Sheline et al., 2003). This PhD thesis suggests that chronic stress triggers the two main AD-related cellular pathways, APP misprocessing and Tau hyperphosphorylation resulting in neuronal atrophy and synaptic loss in hippocampus and PFC leading to depressive behavior and cognitive deficits. Importantly, both stress-driven neurostructural and behavioral deficits were attenuated by ablation of BACE-1, the first enzyme responsible for APP misprocessing, (**chapter 2.3**) or Tau deletion (**chapters 2.1 & 2.3**). Indeed, APP misprocessing and the subsequent Aβ generation are causally associated to neuronal atrophy and malfunction in the AD brain. Several studies showed that elevated Aβ levels cause synaptic damage and loss directly modulating both pre- and postsynaptic structures and function in a dose- and assembly-dependent manner (for reviews, see Selkoe 2002; Palop and Mucke 2010). In line with the current findings showing that BACE-KO animals are rescued from stress-driven neuronal atrophy as well as depressive behavior and cognitive deficits, genetic deletion of BACE-1 in an AD Tg model was found to reverse Aβ overproduction and synaptic loss (McConlogue et al., 2007) supporting the idea that APP misprocessing and the related Aβ overproduction underlie cognitive deficits at least partly through hampering synaptic structure function. Further studies are necessary to deeply clarify the downstream pathways in which lack of BACE-1 and subsequent APP misprocessing offers the aforementioned neuronal protection against chronic stress.

Our knowledge in AD research field suggests that the Aβ detrimental actions are mediated by Tau protein as Tau ablation is shown to be neuroprotective against Aβ-driven AD brain pathology and neurotoxicity (Rapoport et al., 2002; Roberson et al., 2007). Accordingly, the

current PhD findings demonstrate that deletion of Tau block the neurostructural and behavioral deficits induced by chronic stress showing the essential role of Tau in stress-induced dendritic remodeling, synaptic loss as well as cognitive impairment and depressive behavior (**chapters 2.1 & 2.2**). Recently, Tau protein is also found at dendritic spines and synapses where it is suggested to be involved in the GluN2B-related NMDA signaling (Gotz et al., 2013; Ittner et al., 2010). These new data on the relationship between Tau protein and NMDA receptors support the results of earlier work suggesting the essential role of NMDA, receptors in stress-triggered morphofunctional alterations in neurons where blockage of NMDA, but not AMPA, receptors attenuate stress-induced atrophy (Magarinos and McEwen, 1995a, b; Martin and Wellman, 2011; Pawlak et al., 2005). Indeed, apical dendrites, which display stress-evoked atrophy (see **chapters 2.1 & 2.2**), are enriched in GluN2B-contaning NMDA receptors (Rudolf et al., 1996) whereas AMPA receptors, known to offer neuroprotection against glutamate (Wu et al., 2004), are clustered in basal dendrites and soma (Vickers et al., 1993). The above suggested enrichment of NMDA receptors in apical dendritic tree together with the selective atrophy of apical, but not basal, dendrites by stress or GC [Chapter 2.1 & 2.2; see also (Cerqueira et al., 2007b)] support a potential interplay between Tau and NMDA receptors underlying stress-driven neuronal atrophy and damage.

With the exception of a few studies (Cereseto et al., 2006), the effects of stress on cytoskeleton proteins have received little attention, despite the fact that the cytoskeleton is important for maintaining neuronal architecture and function (Morris et al., 2011). Previous work from the supervisors of this thesis showed that stress and/or GC lead to the abnormal hyperphosphorylation of Tau (Sotiropoulos et al., 2011; Sotiropoulos et al., 2008a) through the induction of two well-known Tau kinases (GSK3β, cdk5). The current PhD studies demonstrate that chronic stress leads to an accumulation of Tau and different isoforms of hyperphosphorylated Tau in the cytosolic and synaptic compartments of hippocampal neurons (**chapter 2.2**). Notably, two of these isoforms, pThr231 and pSer262, reduce the microtubule-binding capacity of Tau which subsequently results in destabilization of the neuronal cytoskeleton, disrupted intracellular trafficking and hippocampal atrophy in AD (Hall et al., 2000; Kimura et al., 2007; Lauckner et al., 2003; Mairet-Coello et al., 2013; Sengupta et al., 1998); this fits with the neuronal atrophy and synaptic loss that accompanies Tau hyperphosphorylation in stressed animals.

Novel mechanisms of stress-triggered synaptic deficits; lessons from AD

Tau hyperphosphorylation and subsequent malfunction has an essential role in the establishment of AD neuropathology affecting many cellular processes essential for neuronal maintenance and survival (Gendron and Petrucelli, 2009). For instance, Tau hyperphosphorylation has been recently reported to damage mitochondria mobility (Shahpasand et al., 2012). Interestingly, Tau ablation was shown to prevent Aβ-induced defects of mitochondrial motility in dendrites (Zempel et al., 2013) and axons (Vossel et al., 2015; Vossel et al., 2010), as mitochondria movement in neurons lacking Tau remained normal after Aβ treatment. The above work suggests that Tau has an essential role in Aβ-induced mitochondria trafficking. Accordingly, findings described at **chapter 2.1** of this PhD thesis suggest that exposure to stress in Tau-KO resulted in structural neuroprotection (absence of the classical stress-evoked neuronal atrophy and synaptic loss) followed by enriched synaptic localization of mitochondria; the latter was attenuated in WT animals (see also Figure 1). As previous work showed that stress increases the generation of Aβ and results in Tau hyperphosphorylation (Catania et al., 2009; Green et al., 2006; Sotiropoulos et al., 2011), it is highly plausible that stress-induced Tau hyperphosphorylation attenuates synaptic mitochondrial localization and subsequent proteome changes that was detected in Tau-KO animals under stress (MacAskill et al., 2009).

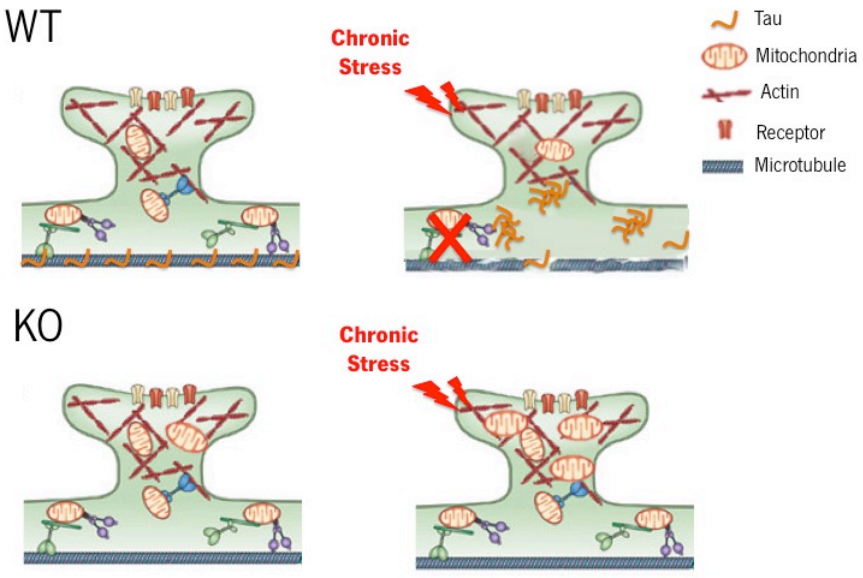

Figure 1: Working model showing the Tau-dependent impact of stress on motility of synaptic mitochondria. The scheme describes a hypothetical model where chronic stress triggers the hyperphosphorylation of the cytoskeletal protein Tau that dissociates from microtubules resulting in an impairment of mitochondria transport to synapse. In contrast, absence of Tau blocked the above transport failure providing neuroprotection against chronic stress (Adapted from Zu-Hang Sheng & Qian Cai).

Synapses are highly vulnerable to impairments in intracellular transport and motility perturbations could cause malfunctions in neurotransmission and signal propagation, leading to synaptic degeneration (Gendron and Petrucelli, 2009). Accordingly, disruption of mitochondrial proteins has been shown to reduce dendritic arborization (without major changes in axon morphology) (Chihara et al., 2007), indicating a critical role of mitochondria in neuronal architecture (Cho et al., 2009). Furthermore, impairment of mitochondrial transport to pre- and postsynaptic terminals is thought to cause synaptic loss due to the essential roles of mitochondria in ATP production providing the energy necessary for synaptic maintenance and activity as well as for calcium homeostasis and buffering (Kopeikina et al., 2012; Sheng and Cai, 2012). These findings provide the first experimental evidence supporting the involvement of Aβ – Tau – synaptic mitochondria "triangle" in the underlying mechanisms of vulnerability and resilience to stress-driven dendritic damage and synaptic loss. GC are shown to dynamically regulate mitochondria, playing an essential role in cytoprotection and neuronal plasticity (Cheng et al., 2010; Toescu and Verkhratsky, 2004; Du et al., 2009). Future studies should focus on the identification of the Tau-dependent mechanism through which stress impacts on mitochondria motility.

As mentioned above, **chapter 2.2** results show that chronic stress, probably through GC, triggers Tau mislocation at synapses, so called missorting, while specific phosphorylated Tau isoforms participate in this mislocation. Indeed, this is in line with previous findings suggesting that synaptic missorting of Tau requires hyperphosphorylation. For instance, only pseudophosphorylated Tau (which mimics hyperphosphorylated Tau), but not phosphorylation-deficient Tau, is mislocalised and accumulated in dendritic spines (Hoover et al., 2010). Previous studies have shown that Aβ triggers both Tau hyperphosphorylation and missorting at synapses resulting to dendritic collapse and synaptic malfunction and loss (Zempel et al., 2010; Tsushima et al., 2015). The above findings offer a mechanistic explanation of the attenuation of stress-evoked neuronal atrophy and synaptic loss in animals lacking BACE-1 and subsequently Aβ (**chapter 2.3**) as well as in animals whose Tau gene is deleted (Tau-KO; **chapter 2.2**).

Furthermore, Aβ-driven Tau missorting at dendritic spines is suggested to represent an early event in AD, preceding the manifestation of detectable neurodegenerative processes (Hoover et al., 2010; Kimura et al., 2007). Based on this model of AD synaptic toxicity, Aβ evokes

missorting of Tau in a phosphorylation-dependent manner (Hoover et al., 2010; Miller et al., 2014; Mondragon-Rodriguez et al., 2012) leading 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 (Ittner et al., 2010; Salter and Kalia, 2004). This phosphorylation is known to stabilize GluN2B at postsynaptic density linking NMDARs to downstream excitotoxic signalling due their over-excitation (Ittner et al., 2010; Salter and Kalia, 2004). Accordingly, the results reported at **chapter 2.2** of this PhD thesis demonstrate that chronic stress triggers synaptic missorting of Tau, increased postsynaptic targeting of Fyn and elevated pGluN2B levels at postsynaptic density representing a potential mechanism of stress-driven neurotoxicity, which could be abrogated by the ablation of Tau; notably, the above stress-triggered cellular cascade was not active in Tau-KO animals under stress (see also Figure 2). Indeed, previous studies have shown that reduction of Tau or Fyn proteins, as well as disruption of the Fyn/GluN2B/PSD interactions prevents excitotoxic damage in AD Tg animal models and in stroke models suggesting the involvement of the above mechanism in different pathologies (Aarts et al., 2002; Ittner et al., 2010). Beyond AD (Hu et al., 2009), NMDARs are also shown to be involved in stress-driven neurotoxicity (Yang et al., 2005), as blockage of NMDARs, but not AMPARs, attenuates neuroremodeling actions of stress (Magarinos and McEwen, 1995a, b). In addition, extra findings of these PhD thesis show that chronic stress also elevates the extrasynaptic levels of GluN2B, previously suggested to contribute to neuronal deficits and atrophy in AD (Hu et al., 2009; Parsons and Raymond, 2014; Talantova et al., 2013) as well as in stress (Yang et al., 2005), Interestingly, Aβ-evoked extrasynaptic NMDAR activation is recently shown to trigger Tau hyperphosphorylation and synaptic atrophy (Talantova et al., 2013). Providing further support to common neurobiological substrates between stress and AD pathologies (Sotiropoulos et al., 2008b), we propose here a working model where stress-triggered Tau hyperphosphorylation and missorting leads to the induction of GluN2B-mediated neurodamaging cascades, introducing Tau and its missorting in the neuronal mechanisms through which stress impairs neuronal connectivity and hippocampal integrity.

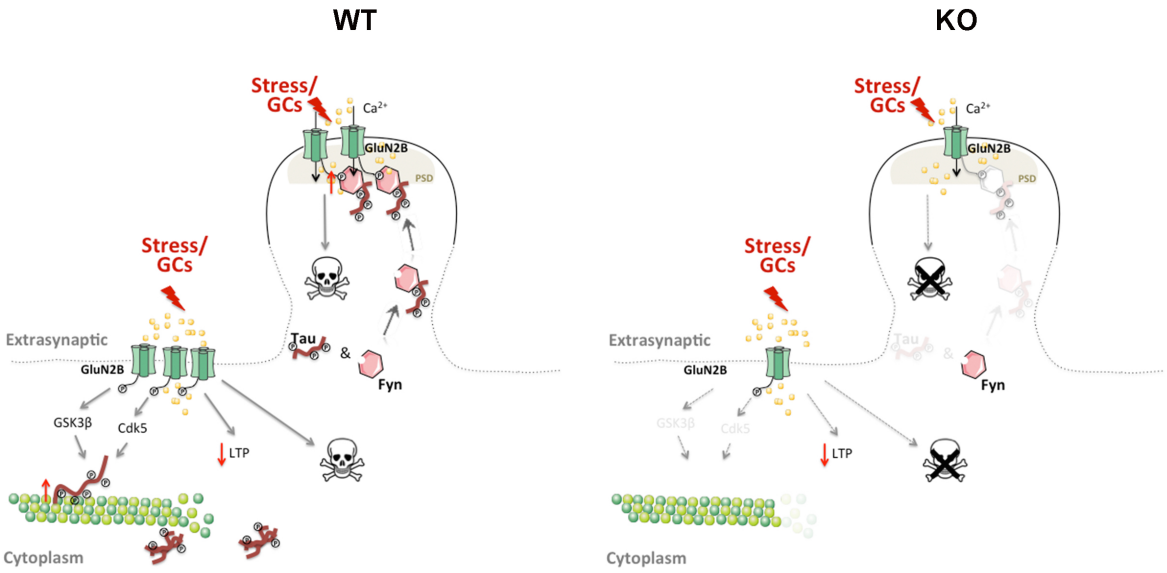

Figure 2. Working model of Tau-mediated cellular processes underlying stress-driven neuronal malfunction. The diagram describes a hypothetical model where chronic stress triggers the hyperphosphorylation of the cytoskeletal protein Tau that dissociates from microtubules (MT) resulting to its cytoplasmic accumulation as well as its missorting at dendritic spines. It leads to increase of Tau-dependent targeting of Fyn at postsynaptic density (PSD; grey box) where Fyn phosphorylates the NMDA receptor subunit GluN2B at Y1472 epitope. This phosphorylation increases the stability of NMDA receptors within the PSD coupling them to downstream excitotoxic signalling. Additionally, chronic stress elevates the levels of extrasynaptic GluN2B subunit known to be responsible for activation of kinases, damage of LTP and neurodamaging cascades. In contrast, absence of Tau blocked the above stress-driven pathway providing neuroprotection against the detrimental effects of chronic stress. (*drawn by Lopes S and Sotiropoulos I*)

Importantly, previous studies have shown that the detrimental effects of chronic stress and prolonged exposure to elevated GC levels on brain structure and function exhibit a cumulative profile with specific temporal dynamics. Specifically, the sequential and cumulative pattern of stress-induced neurostructural and behavioral deficits as well as of Tau hyperphosphorylation starts from hippocampus gradually spreading to PFC and other cortical areas following the increasing time of stressful challenges and stimuli (Cerqueira et al., 2007a; Sotiropoulos et al., 2011). Interestingly, similar spatiotemporal pattern of Tau pathology and propagation is found in AD patients providing extra support to the shared neurobiological mechanisms between chronic stress and AD. Unfortunately, these PhD studies did not address the temporal dynamics of the Tau-dependent detrimental effects of chronic stress on neuronal structure and related behavioral deficits. Whereas the current studies used broad and multiscale ways of analysis combining neurostructural, proteomic, neurochemical, TEM, molecular, electrophysiological, MRI and behavioural analysis), the provided evidence about the stress-

driven damage is only based in one (narrow) time window (end of 6 weeks of stress). Future studies should monitor the role of APP misprocessing and Tau during the progression and propagation of stress-driven brain pathology. Moreover, it is known that stress-induced neuronal atrophy, especially in young/adult animals, can be partly recovered after the termination of stressful stimuli/period (Sousa et al., 2000), while part of Tau hyperphosphorylation is also reversible. Accordingly, it is possible that, when the stress-driven molecular and neurostructural changes exceed a critical threshold, brain pathology could not be easily reversed (Sotiropoulos et al., 2008b).

In addition, while the ability of stress to interfere with cognitive and affective functions as well as neuronal structure is mainly attributed to the actions of GC in the brain (de Kloet et al., 2005), previous studies have also suggest the contribution of other stress-related molecules, e.g. corticotrophin-releasing hormone, in establishment of stress-induced brain damage (Carroll et al., 2011; Rissman et al., 2007). Furthermore, while the findings of **chapter 2.2** show that both chronic stress and GC triggered synaptic missorting of Tau, specific Tau phosphoepitopes may be differentially regulated by chronic stress and prolonged GC treatment. Future studies should dissect the contribution of GC and the subsequent activation of their receptors (GR) in the development of stress-related deficits using animals lacking GR.

Conclusively, the current PhD studies reveal the neuroprotective role of Tau reduction against the establishment of stress-driven depressive pathology and cognitive deficits. This observation is in line with other approaches using Tau-lowering strategies to tackle neuropathologies with diverse etiology (Gheyara et al., 2014; Holth et al., 2013; Ittner et al., 2010; Roberson et al., 2007). Furthermore, these findings provide the first solid proof of the involvement of Tau and its missorting in the mechanisms of synaptic damage, beyond AD pathology adding to our limited knowledge about the underlying mechanisms of stress and GC-evoked synaptic atrophy and neuronal disconnection.

Novel aspects of Tau loss in neuronal degeneration

Tau is a cytoskeletal protein implicated in various neuronal processes such as microtubule stabilization, axonal maintenance and transport, while recent evidence suggest novel function for Tau in synaptic signaling and structure (Gotz et al., 2013). Despite the aforementioned role

of Tau, evidence based on conventional Tau-KO models suggests that young/adult Tau-KO animals has no obvious behavioral or neuronal alterations (Dawson et al., 2001; Ke et al., 2012; Ma et al., 2014; Morris et al., 2013) and neurons of animals lacking Tau do not exhibit axonal abnormalities (Vossel et al., 2010; Yuan et al., 2008). Accordingly, this thesis findings (see **chapters 2.1, 2.2 and 2.4**) demonstrate no cognitive, mood or locomotive deficits in adult (4-5 months old) Tau-KO mice compared to WTs accompanied by absence of structural differences of PFC neurons as assessed by 3D neuronal reconstruction and TEM analyses. However, this lack of obvious phenotype in Tau-KO animals may be attributed to the occurrence of compensatory mechanisms due to altered expression of other cytoskeletal proteins (e.g. MAPs) (Dawson et al., 2001; Harada et al., 1994; Ma et al., 2014). Accordingly, our proteomic data provide novel evidence about alterations in cytoskeletal proteins of adult Tau-KO animals (e.g. tubulin 4A) adding further support to the close interrelationship between Tau and other proteins on cytoskeletal integrity as previously suggested (Takei et al., 2000).

Furthermore, despite the absence of behavioral differences due to Tau ablation, both MEMRI-based hippocampal activity and LTP levels in hippocampus of adult Tau-KO under resting (control) conditions were slightly but significantly different when compared to their (control) WT littermates (**chapter 2.2**). While a mechanistic explanation for these differences can not be offered based on the current literature, it is possible that overall hippocampal activity is probably dependent on neuronal connectivity/input from other brain areas reflecting different neuronal/brain network balance in animals lacking Tau; this notion is supported by previous studies showing alterations in LTD mechanisms of synaptic plasticity in hippocampus of Tau-KO animals (Kimura et al., 2014). Further studies should focus on the network coherence and communication using simultaneous recordings from different brain areas of (conventional) Tau-KO animals. In addition, the future use of conditional Tau-KO animals will provide further answers about the above, unanswered questions avoiding potential developmental compensation mechanisms that the lack of Tau could trigger.

In contrast to young/adult animals, aged Tau-KO animals exhibit mild motor deficits albeit the effect of Tau depletion on motor performance as well as the underlying mechanism(s) remain controversial and unclear. For instance, while one study showed that Tau-KO at middle age exhibit motor deficits accompanied by Substantia Nigra (SN) neuronal loss (Lei et al., 2012), another study found mild motor deficits in middle-aged (12-15 months old) Tau-KO but,

unexpectedly, these motor deficits were not clearly evident in old (21-22 months old) animals (Morris et al., 2013); note that this study found no neuronal deficits in SN of Tau-KO raising uncertainty about the source of the motor deficits. Thus, the results presented at **chapter 2.4** show clear motor deficits in old (17-22 months old) Tau-KO animals using a detailed and well-characterized battery of motor-related behavioral tests. Interestingly, reduced motor strength and coordination was also shown in 13-17 months old animals lacking 4R-Tau, suggesting the potential role of 4RTau in the age-dependent development of motor deficits (Gumucio et al., 2013).

Although a number of Central Nervous System (CNS) areas have been implicated in the emergence of AD-associated motor deficits, little attention has been devoted to (Peripheral Nervous System) PNS primary efferents. Previous evidence suggests that the sciatic nerves of AD patients, but not of age-matched healthy individuals, exhibit reduced Tau levels (Holzer et al., 1999). These PhD studies demonstrate that chronic loss of Tau protein results in sciatic nerve morphofunctional deficits which includes increased percentage of degenerating fibers, hypomyelination of large-diameter, motor-related, fibers and diminished conduction properties in old, but not young, Tau-KO sciatic nerve. While other mechanisms cannot be excluded, the aforementioned sciatic nerve deficits may critically contribute to motor deficits found in old Tau-KO as fine-tuning of myelin sheath thickness and formation is important for maintenance and proper function of motor fibers.

Tau is expressed in both CNS and PNS but the low molecular weight (LMW) isoforms of Tau protein that are expressed in adult CNS differ from high molecular weight (HMW) Tau isoforms mainly found in PNS (e.g. sciatic nerves) but also in optical nerves (Georgieff et al., 1991; Nothias et al., 1995; Sato-Yoshitake et al., 1989). Despite our knowledge about Tau function in PNS is very limited, previous cell-based evidence suggests the involvement of Tau in myelination of oligodendrocytes (CNS) (Klein et al., 2002). Accordingly, the current findings (**chapter 2.4**) provide support of Tau role in myelination of PNS. Supporting the involvement of Tau and other cytoskeletal elements in myelination process, previous evidence suggest that Tau strongly co-localizes with MBP in distal tips of oligodendrocytes suggesting that transportation and/or local MBP translation may require microtubule cytoskeleton and might be controlled by Tau-Fyn interaction (LoPresti et al., 1995; Muller et al., 1997). As Tau binds to Fyn (Klein et al., 2002) and Fyn has a well-described role in myelination of both CNS and PNS

(Kramer-Albers and White, 2011), the above Tau-Fyn-MBP complex could be disrupted in absence of Tau protein affecting myelination signalling and process. Indeed, Taiep myelin-mutant animals exhibit irregular microtubule polarity, and display abnormal Tau accumulation and intracellular accumulation of myelin proteins in oligodendrocytes (Song *et al.* 2001) while PNS myelin-deficient mice also exhibited reduction of Tau and other cytoskeletal protein in their sciatic nerves (Kirkpatrick and Brady, 1994). The above findings suggest that absence of Tau may impact on PNS myelination process/signalling opening a novel window for further research on role of Tau protein in PNS. Furthermore, in light of the suggested therapeutic potential of Tau reduction against AD, a better understanding of the impact of Tau ablation in both CNS and PNS are of great importance. Our findings highlight the morphological and functional implication of peripheral nerves induced by Tau loss in the precipitation of motor deficits with aging and should be taken into account in the development of future AD therapies.

3.2 Conclusions

Based on the suggested common neurobiological substrates between depression and AD and the fact that chronic stress is a risk factor for both disorders, this PhD thesis provides novel experimental evidence that introduces for the first time the two main AD-related cellular mechanisms, namely APP misprocessing and Tau hyperphosphorylation, in the establishment of stress-driven neuronal atrophy and synaptic deficits related to depressive pathology and cognitive impairments. Given the critical role of chronic stressful events as a trigger for several neurological and psychiatric disorders, the present results are of relevance to open new pathways of intervention for several stress-associated conditions highlighting the potential usage of Tau reduction strategies that have been proposed for AD (Iqbal, Gong, & Liu, 2014).

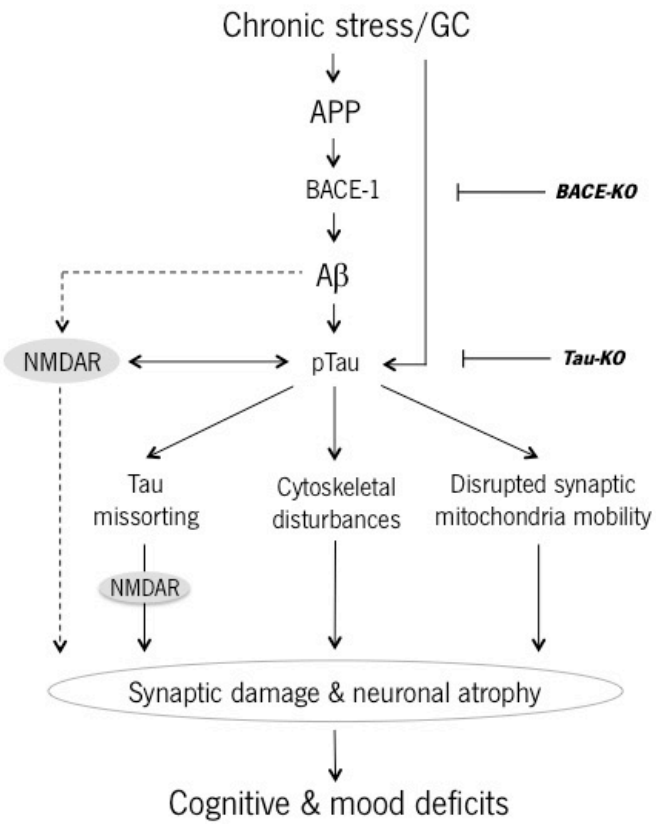

Figure 3. Hypothetical model summarizing the stress-driven cellular cascades identified by this PhD thesis. The above scheme summarizes some of the AD-related cellular pathways which chronic stress may act on to precipitate neuronal atrophy and malfunction leading to mood and cognitive deficits (*drawn by Lopes S and Sotiropoulos I*).

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Annexes

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Regular Article

Selective impact of Tau loss on nociceptive primary afferents and pain sensation

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ABSTRACT

Tau protein hyperphosphorylation and consequent malfunction are hallmarks of Alzheimer's disease pathology; importantly, pain perception is diminished in these patients. In physiological conditions, Tau contributes to cytoskeletal dynamics and in this way, influences a number of cellular mechanisms including axonal trafficking, myelination and synaptic plasticity, processes that are also implicated in pain perception. However, there is no in vivo evidence clarifying the role of Tau in nociception. Thus, we tested Tau-null (Tau −/−) and Tau +/+ mice for acute thermal pain (Hargreaves' test), acute and tonic inflammatory pain (formalin test) and mechanical allodynia (Von Frey test). We report that Tau −/− animals presented a decreased response to acute noxious stimuli when compared to Tau +/+ while their pain-related behavior is augmented under tonic painful stimuli. This increased reactivity to tonic pain was accompanied by enhanced formalin-evoked c-fos staining of second order nociceptive neurons at Tau-null dorsal horn. In addition, we analyzed the primary afferents conveying nociceptive signals, estimating sciatic nerve fiber density, myelination and nerve conduction. Ultrastructural analysis revealed a decreased C-fiber density in the sciatic nerve of Tau-null mice and a hypomyelination of myelinated fibers (Aδ-fibers) – also confirmed by western blot analysis – followed by altered conduction properties of Tau-null sciatic nerves. To our knowledge, this is the first in vivo study that demonstrates that Tau depletion negatively affects the main systems conveying nociceptive information to the CNS, adding to our knowledge about Tau function(s) that might also be relevant for understanding peripheral neurological deficits in different Tauopathies.

Introduction

Tau malfunction, through its abnormal hyperphosphorylation, is postulated as a crucial mechanism of Alzheimer's disease (AD) neuronal dysfunction but it remains unknown whether Tau protein could be a direct or indirect contributor to altered pain processing found in AD patients (Corbett et al., 2012). The predominant view about Tau function focuses on its key cytoskeletal role based on its ability to bind to microtubules (MT) and other cytoskeletal elements (Mandelkow and Mandelkow, 2012). Interestingly, neuropathic pain is a common side effect of MT-targeting agents commonly used in the clinics as chemotherapeutic drugs (e.g. vincristine, paclitaxel) (Jaggi and Singh, 2012). In addition, a growing body of evidence suggests that Tau has an important role in the dendritic compartment where, via interaction with Fyn, contributes to NMDA receptor/PSD95 complex formation (Frandemiche et al., 2014; Ittner and Gotz, 2011) that has an established role in pain-related sensitization (D'Mello et al., 2011). There is also direct evidence of Fyn involvement on pain sensitization through phosphorylation of the NMDA NR2B subunit (Abe et al., 2005). Furthermore, Fyn constitutional activation results in hyperalgesia in naive mice (Liu et al., 2014). Moreover, a cell culture-based study suggests that Tau–Fyn interaction may be involved in myelination regulation (Klein et al., 2002), which is of relevance for optimal axonal function and, consequently, for nociceptive transmission. Furthermore, there is also evidence that other cytoskeletal elements may be involved in pain as disruptors of microtubules, actin filaments and neurofilaments in primary afferent nociceptors interfere with normal pain sensation (Reichling and Levine, 2011) reinforcing the importance of understanding the relation between cytoskeletal proteins and pain.

Abbreviations: AD, Alzheimer's disease; NMDA, N-methyl-D-aspartate; CNS, central nervous system; PNS, peripheral nervous system; MT, microtubules; MBP, myelin basic protein.

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An initial step for pain perception is the activation of peripheral nociceptors which generates a local depolarization conveyed by specific fibers to secondary afferent neurons in the dorsal horn. Responding to mechanical, thermal and/or chemical stimuli, Aδ fibers are the small-diameter myelinated fibers that participate in the transmission of noxious stimuli together with the unmyelinated C-fibers. Myelination problems as well as reduced number of fibers in sciatic nerve have been shown to affect pain perception and pain circuitry function (Chen et al., 2003). While previous findings described alterations in small-caliber axons of animals lacking Tau protein (Harada et al., 1994), and despite the suggestion that Tau protein is implicated in myelination processes (Klein et al., 2002), yet, surprisingly, to the best of our knowledge, there is no in vivo evidence clarifying the role of Tau in nociception. Thus, herein, we assessed pain behavior in animals lacking Tau followed by ultrastructure, molecular and electrophysiological analysis of their sciatic nerve.

Materials and methods

Animals

This study used 4–6 month-old male Tau-null (Tau −/−) and Tau +/+ (C57BL/6) animals (Dawson et al., 2001). Adult Tau-null animals of this strain exhibited no cognitive or locomotor deficits as previously described (Dawson et al., 2010; Gotz et al., 2013). All animals were used in accordance with European Union Council Directive 86/609/EEC and local welfare regulations. Mice were housed in groups of 4 to 5 animals per cage under standard environmental conditions (ambient temperature of 21 ± 1 °C and a relative humidity of 50–60%; 12 h light/dark cycle (lights on at 8:00 A.M.) ad libitum access to food and water).

Pain-related behaviors

Hargreaves' test (acute thermal noxious stimulus)

Animals (N = 18–20 per genotype) were submitted to Hargreaves' test which consists of a radiant heat applied to the plantar skin of the hind limb and withdrawal latency was automatically registered using radiant heat equipment (Plantar Test Device Model 7370, Ugo Basile, Comerio, Italy) as previously described (Pinto-Ribeiro et al., 2013). After acclimatization period, the measurement was repeated 4 times with 60 second intervals. Cut-off time was 10 s. The experimenter was blind to the animals' genotype.

Formalin test (inflammatory stimulus)

The formalin test was performed as previously described (Almeida et al., 1999). Briefly, 50 μL of 5% formalin was injected subcutaneously in the dorsal surface of the right hind-paw. Animals were then placed over a transparent acrylic surface within a 10 cm^3 transparent acrylic box with perforations in all walls for proper air ventilation and sufficient space for animals to freely move. Animals were filmed from below for 60 min and pain-related behaviors (paw protection/licking periods and paw jerks) in the acute (0–5 min) and tonic (15–60 min) phases were posteriorly quantified by two independent observers using EthoLog software (Ottoni, 2000). Prior to the experiment, animals were acclimatized to the experimental conditions to minimize potential stress effects. Experimental groups for Tau-null and Tau +/+ were divided in: saline-injected (control) animals (Sal; N = 10) and formalin-injected animals (Form; N = 13–15). The experimenter was blind to the animals' genotype and drug.

Von Frey test (up-and-down method)

Tactile allodynia (pain evoked by a normally innocuous stimulus) was accessed by the up-and-down method (Chaplan et al., 1994). Animals (10–11 animals per group) were placed in an elevated grid and the glabrous skin of the hind paw was probed with a series of Von Frey calibrated monofilaments: 2.0 g, 1.0 g, 0.6 g, 0.4 g, 0.16 g, 0.07 g, and 0.04 g (North Coast Medical Inc., USA). Starting with the 0.4 g filament, the test would advance upward if no response was elicited (=O) or downward if a brisk withdrawn of the limb was produced (=X) until 6 measurements were obtained around the threshold point according to the model developed by Dixon (1980). The 50% response threshold was then calculated using the formula 50% g threshold = (10^[X_f + K.δ]) / 10.000, where X_f = value (in log units) of the final Von Frey filament; k = tabular value corresponding to pattern of positive and negative responses (X and O sequences; consult (Chaplan et al., 1994)); δ = mean difference (in log units) between stimuli (0.267). If no response (=O) was obtained up to maximal force (2.0 g) or conversely, if all filaments elicited a response (=X) down to the minimal force (0.04 g), these values were assumed as the 50% withdrawal threshold. The experimenter was blind to the animals' genotype while animals were habituated to the experimental conditions for 2 days before the experiment to minimize potential stress effects.

c-fos immunohistochemistry and density estimation

90 min after formalin test completion, animals were deeply anesthetized with sodium pentobarbital (CEVA Saúde Animal, Portugal) and then perfused through the ascending aorta with PBS followed with 4% paraformaldehyde in PBS. The spinal cord was removed and immersed in the same fixative for 24 h at room temperature (RT) and then, moved to an 8% sucrose solution and kept at 4 °C until use. 50 μm coronal sections of the lumbar area were then obtained in a vibratome (Leica 1000VTS) and serially collected in PBS. For c-fos IHC, sections were incubated in 3.3% H_2O_2 (in PBS, pH 7.2) for 30 min, RT and then, after several washes in PBS and 0.3% PBS-T (Triton-X-100; Sigma), incubated in 2.5% FBS (fetal bovine serum; Stem Cell, 062-00) in PBS-T 2 hour RT, followed by a solution of 1:2000 anti-Fos (Calbiochem, PC38-100) and 2.0% FBS in PBS-T overnight at RT. Sections were then washed several times in PBS-T and incubated for 1 h, RT in a solution of 1:200 biotinylated swine anti-rabbit secondary antibody (Dako, E0353). Sections were then washed in PBS-T and incubated for 1 h, RT in a solution of 1:200 avidin–biotin complex (ABC, Vector Laboratories). Sections were again washed with PBS-T, PBS and Tris–HCL (0.05 M, pH 7.6) and briefly incubated in 0.0005% (w/v) diaminobenzidine tetrahydrochloride (DAB; Sigma Immunochemicals, St. Louis, USA), 0.0002% H_2O_2 (v/v) in Tris–HCl. Sections were then serially placed in Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany), dehydrated and mounted in Entellan (Merck, Darmstadt, Germany). An Olympus BX51 microscope equipped with U-MAD3 camera was used to acquire images from the left and right dorsal horn of spinal cord. c-fos density was manually determined in specific outer (laminae I–II) and deeper (laminae III–V) regions by an experimenter (AL) blind to the samples provenience (i.e. saline or formalin of both genotypes; 5–6 animals per group).

Sciatic nerve ultrastructure analysis

Sciatic nerves of undisturbed Tau-null and Tau +/+ animals were collected and fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 1 week. Then, they were postfixed in 1% OsO_4, dehydrated and embedded in epon (Electron Microscopy Sciences). 1 μm thick transverse sections covering the complete cross-sectional nerve area were stained with 1% p-phenylene diamine (PPD) for 10 min and mounted on Entellan (Merck). An Olympus DP70 was used to photograph the transverse sections of the nerve and images were mounted on Photoshop. For each animal (N = 5–6 per genotype), the total number of myelinated fibers in one transverse section was counted. The g-ratio [axon diameter / (axon diameter + myelin thickness)] was determined through the analysis of at least 100 fibers. For electron microscopy, ultrathin-sections (60 nm) were counterstained with alcoholic uranyl acetate solution, followed by aqueous uranyl acetate solution and lead citrate. Grids were observed on a JEOL JEM-1400 transmission electron microscope equipped with an Orius Sc1000 digital camera. 16 non-overlapping images were obtained and used for all

determinations (number axons/bundle and density of unmyelinated fibers) by an experimenter in a manual way; experimenter was blind to the samples' provenience (*Tau-null* and *Tau*+/+; 5 animals per genotype).

Ex-vivo measurement of compound action potentials

Acutely isolated sciatic nerves from Tau-null and Tau+/+ animals (5–6 animals per group) were used for assessment of A- and C-fiber conduction velocity and compound action potential (CAP) area as previously described (Pinto et al., 2008). Briefly, sciatic nerves were dissected and cleaned from the connective tissue sheath in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 2.5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 Glucose, bubbled with carbogen gas (95% O_2, 5% CO_2). After isolation, the nerves were used at room temperature of 22–24 °C. CAP recordings were made with a Multiclamp 700B amplifier in VC mode and digitized with the Digidata 1440A digitizer using pClamp 10 software (Axon Instruments). Signals were low-pass filtered at an effective corner frequency of 16 kHz and sampled at 50 kHz. Fibers were stimulated through suction electrodes using an isolated pulse stimulator (STG4002, multichannel systems). Short pulses of 50 μs were applied to activate CAPs in Aαβ- and Aδ-fibers. C-fibers were stimulated with 1 ms current pulses. Sciatic nerves were repeatedly stimulated with current pulses of increasing amplitudes until saturation. Conduction velocities were calculated for the CAP peak of A-fibers and for the first peak of the C-fiber biphasic CAP. Total areas were calculated using clampfit software (Axon Instruments). Electrophysiological recordings and analyses were performed by an experimenter blind to the provenience of the tissue.

Western blot analysis

Sciatic nerves of undisturbed Tau-null and Tau+/+ animals (6–7 animals per genotype) were isolated and homogenized in lysis buffer [10 mM HEPES pH 7.9, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10% glycerol, 1% NP-40, Complete Protease Inhibitor (Roche, Mannheim, Germany) and Phosphatase Inhibitor Cocktails II and III (Sigma, St Louis, MO)] using a Dounce glass homogenizer; extracts were sonicated and centrifuged (15.000 *g*; 10 min; 4 °C) and their protein contents were estimated (Bradford assay). After reconstitution in Laemmli buffer (250 mM Tris–HCl, pH 6.8, containing 4% sodium dodecyl sulfate, 10% glycerol, 2% b-mercaptoethanol and 0.002% bromophenol blue), 25 μg of lysates was electrophoresed on 10% acrylamide gels, and transferred onto nitrocellulose membranes (BIORAD Turbo) while transfer efficacy was checked by membrane Ponceau staining. Membranes were blocked in Tris-buffered saline containing 5% non-fat milk and 0.2% Tween-20 before incubation with myelin basic protein (MBP) antibody (Serotec; 1:500) and actin (DSHB 1:2000). Antigens were revealed by enhanced chemiluminescence (BIORAD) after incubation with appropriate horseradish peroxidase–immunoglobulin G conjugates (BIORAD). Protein ladder (Fermentas) was used to monitor molecular weight of proteins of interest. Blots were scanned and quantified using TINA 3.0 bioimaging software (Raytest, Germany). All values were normalized against actin and expressed as percentages of control.

Statistical analysis

All data sets were subjected to t-test analysis except c-fos expression data that were analyzed by 2-way ANOVA having genotype (Tau+/+ vs Tau−/−) and treatment (saline vs formalin) as factors and it was followed by Tukey post-hoc analysis (SPSS, Aspire Software, USA). Differences were considered to be significant if $p < 0.05$.

Results

Tau-null animals present decreased nociception to acute noxious but the opposite to tonic stimulation

For detailed characterization of Tau involvement in nociception, *Tau-null* and *Tau*+/+ (wild-type) animals were submitted to different pain-related behavioral tests that are used to examine relevant pain-like responses. The response latency to acute noxious heat (54 °C) on the hind paw (Hargreaves' test) was significantly increased in Tau-null when compared to wild type animals ($t = 2.865$, $p < 0.05$) indicating reduced nociception to acute noxious stimuli (Fig. 1A). Furthermore, we evaluated inflammatory pain in both genotypes using the formalin test. Pain-related behaviors such as protecting/licking and paw jerks were monitored for 1 h after subcutaneous paw injection of formalin in the right hind paw (Figs. 1B and C). In line with the above test of acute pain, the acute phase of the formalin test (0–5 min) shows that Tau-null animals spent less time engaged in pain-related behaviors as indicated by the time of protecting/licking the formalin-injected paw ($t = 2.162$, $p < 0.05$; Fig. 1B left). In addition, Tau-null animals displayed decreased number of jerks of the formalin-injected paw when compared to Tau+/+ ($t = 2.933$, $p < 0.05$; Fig. 1C left); both typical pain-related behaviors induced by formalin suggest that Tau-null animals respond less to the acute phase of formalin noxious stimulation. Interestingly, animals from the two genotypes exhibit a different response profile in the tonic phase of the formalin test (15–60 min). Specifically, an increased protecting/licking behavior was found in Tau-null animals when compared to Tau+/+ animals ($t = 2.089$, $p < 0.05$; Fig. 1B right) indicating increased pain, though no significant difference in the number of paw jerks (Fig. 1C right). Importantly, Tau-null and Tau+/+ animals presented no differences in their response threshold to innocuous light mechanical stimulus (Fig. 1D) suggesting that normal tactile sensation is preserved while the observed alterations phenotypes were restricted to pain.

Spinal cord formalin-induced c-fos expression is higher in Tau-null animals

c-fos positive cell density is used as a central correlate of pain-related neuronal activity driven by formalin stimulation of peripheral afferents (Fig. 2A). Thus, we measured c-fos-positive neurons in lumbar L4–L6 spinal cord of the formalin- and saline-injected animals. 2-Way ANOVA analysis showed that c-fos cell density in ipsilateral and contralateral I–II (superficial) and III–IV (deep) spinal cord lamina was significantly dependent on the treatment (formalin vs saline: $ipsi_{I-II}$ $F_{1,21} = 29.039$; $contra_{I-II}$ $F_{1,21} = 42.714$; $ipsi_{III-V}$ $F_{1,21} = 31.321$; $contra_{III-V}$ $F_{1,21} = 22.159$; $p < 0.001$ in all cases) and genotype (Tau-null vs Tau+/+: $ipsi_{I-II}$ $F_{1,21} = 4.560$, $p = 0.047$; $contra_{I-II}$ $F_{1,21} = 25.746$, $p < 0.001$; $ipsi_{III-V}$ $F_{1,21} = 5.372$, $p = 0.032$; $contra_{III-V}$ $F_{1,21} = 9.016$, $p = 0.008$). Also, the interaction of these factors resulted significantly in almost all comparisons – $ipsi_{I-II}$ $F_{1,21} = 4.098$, $p = 0.058$; $contra_{I-II}$ $F_{1,21} = 20.892$, $p < 0.001$; $ipsi_{III-V}$ $F_{1,21} = 5.343$, $p = 0.033$; $contra_{III-V}$ $F_{1,21} = 6.490$, $p = 0.020$. As shown in Fig. 2B, formalin injection resulted in an expected increased c-fos + density in the ipsilateral dorsal horn. However, this effect had a higher magnitude in Tau-null animals matching with their increased pain-related behavioral responses at the tonic phase of the formalin test. Interestingly, Tau-null animals demonstrated an abnormal c-fos staining at both layers I–II and III–V of the contralateral side of spinal cord as shown in Fig. 2A.

Tau-null sciatic nerves exhibit reduced myelination and decreased density of non-myelinated fibers

As Tau-null animals exhibit alteration in pain sensitivity, it was of great interest to monitor the structure and functionality of the sciatic nerve fibers that convey sensory input from the hind paw to the CNS.

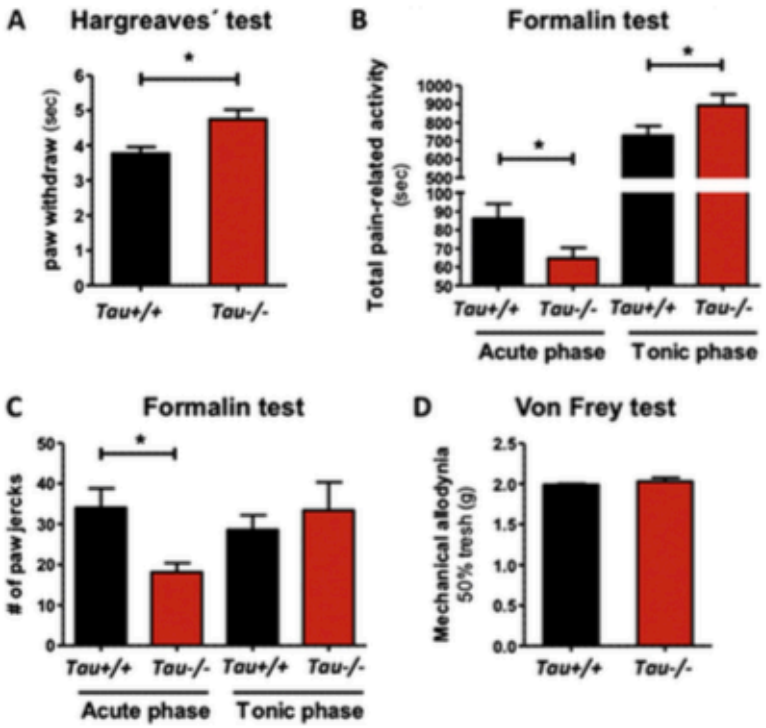

Fig. 1. Tau-null animals exhibit altered nociception. Tau-null (Tau −/−) and Tau +/+ animals were tested with noxious heat (A), inflammatory (B–C), and light mechanical stimuli (D). A) Tau −/− animals presented increased time in paw withdraw to acute noxious (heat; 54 °C) stimuli in Hargreaves' test indicating reduced pain behavior. B–C) At the acute phase of formalin test, Tau −/− animals exhibit reduced time of protecting/licking the formalin-injected paw (B; left part) as well as reduced number of paw jerks (C; left part); both pain-related behaviors reflect diminished nociception. In contrast, the licking/protecting time of Tau-null animals was higher than Tau +/+ during the tonic phase of formalin test (B, right part). D) Tau-null and Tau +/+ animals presented no differences in the response to innocuous stimulus (light mechanical). Data presented as mean ± SEM; *$p < 0.05$.

For that, we performed histological examinations of sciatic nerves using transmission electron microscopy (Fig. 3A). As shown in Fig. 3B, Tau-null animals have reduced density of non-myelinated fibers (C-fibers) ($t = 2.515$, $p < 0.05$), even though the number of non-myelinated fibers per Remak bundle was not affected (Fig. 3C). On the contrary, there was no difference in density of myelinated fibers between Tau-null and Tau +/+ animals as assessed by p-phenylene diamine (PPD) staining (Fig. 3D). Furthermore, we determined the g-ratio, an index of myelination independent of axonal diameter (Michailov et al., 2004), in sciatic nerves of animals of both genotypes (Fig. 4A). As shown in Fig. 4B, the mean g-ratio of Tau-null animals was significantly increased ($t = 10.65$, $p < 0.05$) indicating thinner myelin sheaths in their sciatic nerve. Regarding the overall axonal population, the percentage of small-diameter myelinated axons (with diameter less than 4 μm) was significantly increased in Tau-null animals compared to Tau +/+ ones (72.75% ± 0.85 and 65.40% ± 2.50, respectively; $t = 2.505$, $p < 0.05$) indicating that the biggest difference in the individual g-ratios was found in the small-diameter fibers (Fig. 4C). Moreover, we monitored the levels of one of the main proteins found in compact myelin, the myelin basic protein (MBP) in sciatic nerves of Tau-null and Tau +/+ animals using western blot analysis. As shown at Fig. 4D, we found decreased levels of MBP protein in Tau-null animals, providing further support to the hypomyelination phenotype found by g-ratio analysis.

Sciatic nerve myelinated/unmyelinated CAPs ratio is reduced in Tau-null

The above morphological and molecular findings prompted us to monitor the functionality of sciatic nerve by measuring the conduction velocity of A- and C-fibers. For that purpose, Tau +/+ and Tau −/− sciatic nerves were repeatedly stimulated with current pulses of increasing amplitudes until saturation (Figs. 5A, B). As shown in Fig. 5C, no differences were found in the conduction velocity of Tau-null and Tau +/+ animals of both large-diameter myelinated (A-) and non-myelinated (C-) fibers. However, the ratio of the total area of the compound action potentials (CAPs) of myelinated vs unmyelinated fibers was significantly increased in Tau-null animals ($t = 2.367$, $p < 0.05$; Fig. 5D) indicating an overall alteration in conduction properties of sciatic nerve of Tau-null animals.

Discussion

This study provides the first evidence that Tau depletion affects nociceptive behavior in mice and leads to morphofunctional alterations in primary afferent fibers. Our ultrastructural and biochemical analysis demonstrates a hypomyelination phenotype in sciatic nerves of Tau-null animals. Indeed, a previous cell culture-based study by Klein et al. suggests the involvement of Tau protein in CNS myelin formation through its structural interaction with Fyn protein and microtubules (Klein et al., 2002). Transgenic loss of Fyn or its function is shown to result in severe hypomyelination (Sperber and McMorris, 2001; Umemori et al., 1994) but upstream partner of Fyn can also promote myelination independently of Fyn (Biffiger et al., 2000); the latter may explain why loss of Tau does not completely abrogate the myelination process in Tau-null mice. Supporting the involvement of Tau and microtubules in myelination process, the Taiep myelin mutant rats exhibit irregular microtubules, abnormal Tau accumulation and intracellular accumulation of myelin proteins in oligodendrocytes (Song et al., 2001), while PNS myelin-deficient mice exhibited reduction of Tau as well as other microtubule-associated proteins in their sciatic nerves (Kirkpatrick and Brady, 1994). A possible mechanism suggested by Song et al. focuses on the importance of Tau and microtubules in the transport of vesicles (Dixit et al., 2008) containing myelin components to the sheath formation of myelinating cells. On the other hand, as neuron-glial cell

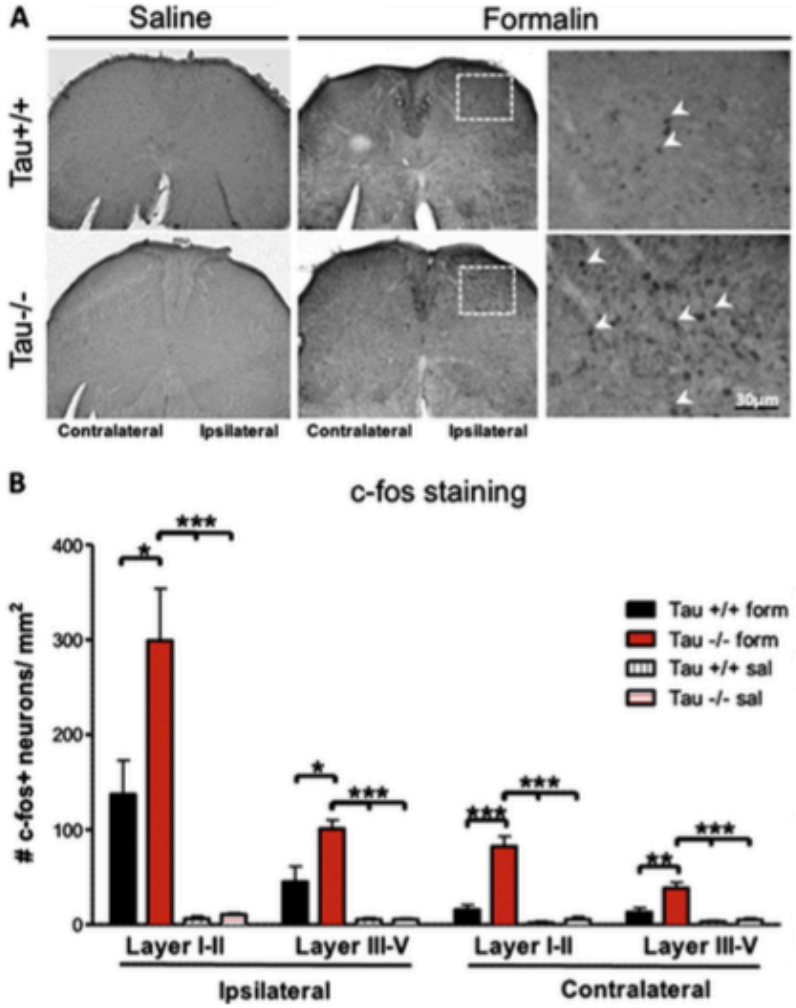

Fig. 2. Tau-null animals demonstrate higher c-fos staining in spinal cord at the end of formalin noxious stimulation. Representative immunohistochemical images (A) and quantitative analysis (B) of c-fos staining at spinal cord of Tau-null (Tau−/−) and Tau+/+ mice at the end of formalin test. Formalin injection at the right paw of the animals resulted in strong c-fos staining in the ipsilateral dorsal horn layer I/II where the majority of nociceptive signal from paw neurons arrives (superior right quadrant). Note that this formalin effect is higher in Tau-null animals than Tau+/+ ones (A, B). Right images are high-power magnifications of the rectangular areas marked in the respective low-magnification micrographs (ipsilateral dorsal horn). In addition, Tau-null animals exhibit an abnormal (contralateral) c-fos staining induced by formalin; representative images of saline-injected animals on the left. All numerical data shown represent mean ± SEM. For individual comparisons, data were analyzed by one-way ANOVA followed by Tukey HSD post-hoc. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

interaction(s) are essential for myelination, some studies showed that axonopathies are accompanied by myelin deficits (Wirths et al., 2006) raising the question whether axonal disturbances in neurons are preceded by the myelin deficits in myelinating (oligodendrocytes or Schwann) cells.

According to our observations, myelin alterations are restricted to small caliber axons (<4 μm). Indeed, Tau protein is highly expressed in small-caliber axons of both adult CNS and PNS (low and high molecular weight Tau isoforms, respectively). Large-diameter axons on the other hand express very little amount of Tau (Nothias et al., 1995; Sato-Yoshitake et al., 1989). Accordingly, Harada and colleagues found that microtubule alterations were restricted to small-size axons in Tau-KO animals (Harada et al., 1994). This may result from the fact that, in contrast to large-caliber axons, Tau is the predominant MT-associated protein in small and medium caliber axons and thus, upon its depletion, Tau function in small-caliber axons cannot easily be compensated by other MT-associated proteins such as MAP1A, which is temporally increased in Tau-KO animals (Dawson et al., 2001; Harada et al., 1994).

Furthermore, we demonstrate a reduction in unmyelinated C-fiber density in sciatic nerve of Tau-null animals; importantly, unmyelinated C-fibers and small myelinated (Aδ) fibers are the major systems conveying nociceptive inputs to the spinal cord although the participation of large myelinated Aα/β fibers has also been demonstrated (Djouhri and Lawson, 2004; Todd, 2010). Tau depletion and the resulting loss of MT–Tau interaction may have a higher impact on C-fibers than on A-fibers as the MT/neurofilaments ratio in sciatic nerve is higher in unmyelinated fibers and thus, C-fibers may be structurally more dependent on these cytoskeleton elements (Friede and Samorajski, 1970). As a result, the overall ratio between unmyelinated/myelinated fibers was affected in Tau-null animals indicating a reorganization of the overall composition of the nerve fibers; this was in line with the electrophysiological monitoring of sciatic nerve functionality that shows an overall decrease in the proportion of total area of the compound action potentials (CAPs) of myelinated vs unmyelinated fibers. However, it is important to note that there were no major conduction velocity changes in both large myelinated and the remaining unmyelinated fibers suggesting that the existing fibers are properly conducting.

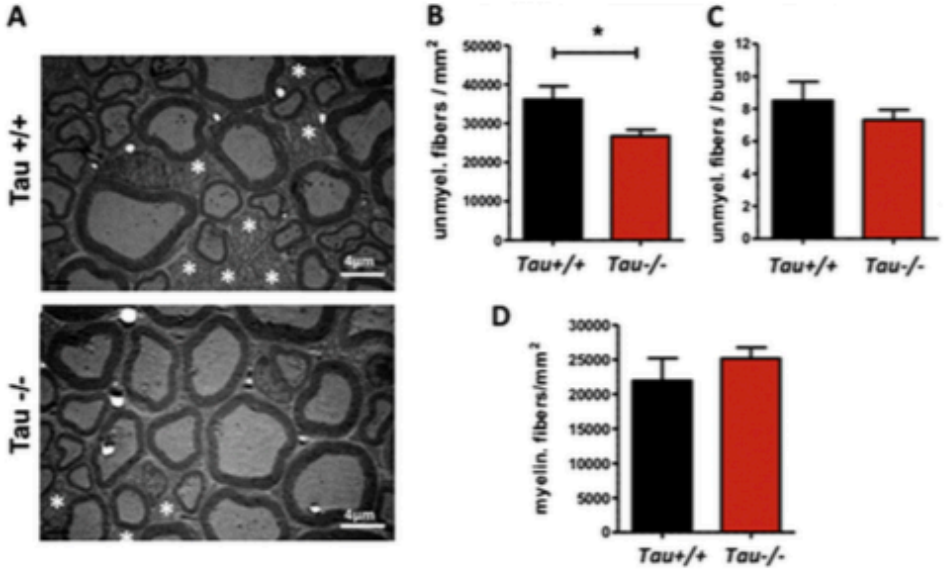

Fig. 3. Morphometric analyses of sciatic nerve ultrastructure. A) Representative images of Tau −/− and Tau +/+ sciatic nerve ultrastructure where Remak bundles are marked (white asterisk). B–C) Tau −/− animals exhibit a significant reduction of non-myelinated fiber (C-fibers) density when compared to Tau +/+ animals although no alterations in number of fibers per Remak bundle were found between the two genotypes. D) Conversely, the number of myelinated fibers was unaltered in sciatic nerves of Tau −/− animals. Data presented as mean ± SEM; *$p < 0.05$.

Tau-null animals demonstrated a decrease in pain sensitivity in the acute phase of the formalin test (first 5 min of noxious stimuli) that is in agreement with the results of the Hargreaves' test where a decrease in acute pain sensitivity in response to noxious (thermal) stimuli was also found. Although our pain-related behavioral readouts are motor-dependent, we can exclude potential confounders as no motor or

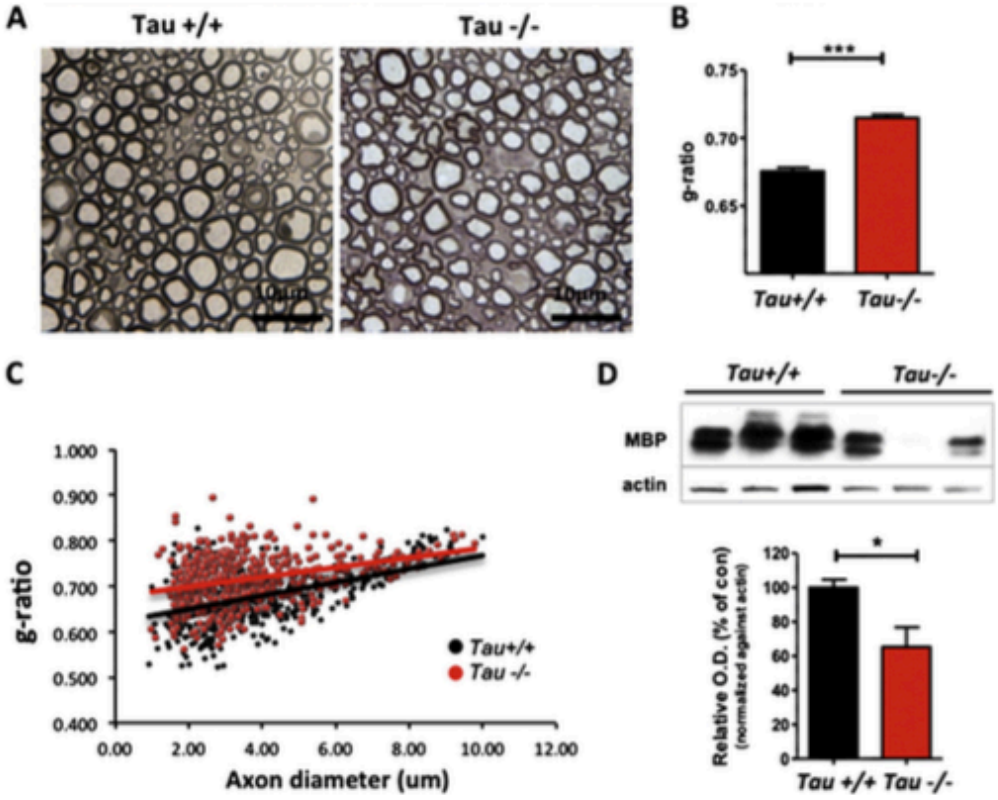

Fig. 4. Tau-null sciatic nerve presents hypomyelination phenotype. A) Representative images of PPD (p-phenylenediamine) staining of Tau −/− and Tau +/+ sciatic nerves used for g-ratio analysis. B–C) Tau −/− animals demonstrated increased g-ratio [axon diameter / (axon diameter + myelin thickness)] in comparison to Tau +/+ animals indicating hypomyelination, particularly evident in small myelinated fibers. D) Representative blots and semi-quantitative measurement of protein levels of myelin basic protein (MBP) in sciatic nerves assessed by WB analysis. Note the reduced MBP levels in sciatic nerves of Tau −/− animals. Data presented as mean ± SEM; *$p < 0.05$.

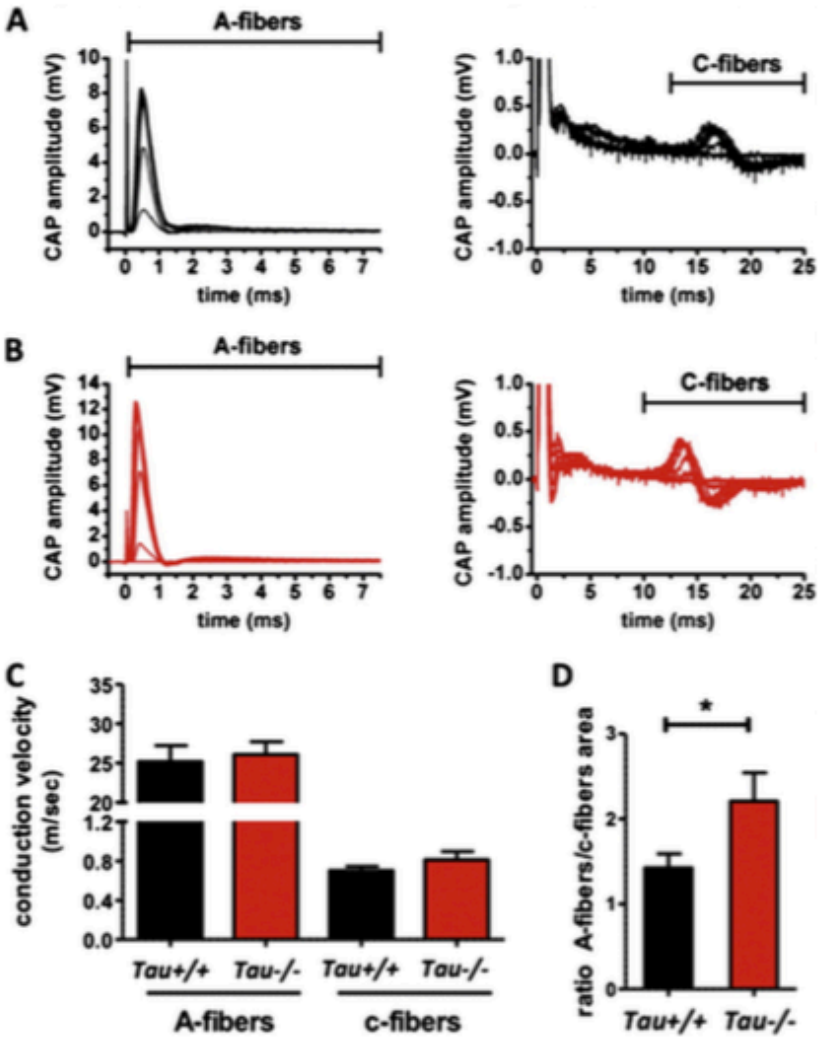

Fig. 5. Sciatic nerve conduction properties are affected in Tau absence. A, B) Representative traces of sciatic nerve CAPs are presented for A- and C-fibers of Tau +/+ (A) and Tau −/− (B). Tau +/+ and Tau −/− sciatic nerves were repeatedly stimulated with current pulses of increasing amplitudes until saturation (conduction distance: 9.3 mm and 9.6 mm for Tau +/+ and Tau −/−, respectively); traces are averages of 5 consecutive recordings. C–D) While no differences of absolute conduction velocity between Tau −/− and Tau +/+ animals were found (C), the ratio of the A-fiber/C-fiber activity (as measured by total area of the CAP responses) was increased in Tau −/− animals pointing towards altered overall conduction properties in Tau −/− sciatic nerve (D). Data presented as mean ± SEM; *$p < 0.05$.

muscle strength deficits have been found in adult Tau-KO animals used in this study (Dawson et al., 2010; Gotz et al., 2013) while Tau-KO normal response to innocuous mechanical stimuli with reflex withdrawal (Von Frey) indicates that the acute pain (thermal) deficits assessed by Hargreaves' test were not due to a disruption of flexor motor neuron function. Thus, as acute pain is primarily dependent on primary afferent transmission, with limited intervention of central sensitization mechanisms (Liu et al., 2008), this decrease in acute pain is probably dependent on the reduction in unmyelinated C-fibers and the hypomyelination of Aδ-fibers. Previous studies reported a reduced response to neuropathy-triggered mechanical allodynia in animals lacking Fyn which exhibit a clear hypomyelination phenotype (Abe et al., 2005). The close interaction between Fyn and Tau (Klein et al., 2002) could explain the similarities between our and previous observations.

Contrary to the acute phase, the tonic phase of the formalin test is centrally mediated. In agreement, c-fos expression in the dorsal horn was augmented reflecting the increased pain-related behavior observed in Tau-null animals. The increased excitability of second order spinal cord nociceptive neurons, could be explained by a mechanism where a decrease in afferent inputs, due to a restrain in synaptic transmission (D'Mello et al., 2011; Ittner and Gotz, 2011; Roberson et al., 2007), and in nociceptive conducting fibers, is compensated by an increase in somatic neuronal excitability to maintain a balanced spinal nociceptive circuit in Tau-null mice (Turrigiano, 2011). Alternatively, a decrease in spinal inhibition, caused by a decreased activity of the inhibitory circuit, could also account for such results (Zeilhofer et al., 2012). Overall, the centrally mediated hyperexcitability we described most probably also accounts for the contralateral c-fos expression observed in Tau-null mice. It is noteworthy that morphine-tolerant hyperalgesic animals exhibit a similar abnormal c-fos pattern in spinal cord (Rohde et al., 1997). Further analysis of the role of Tau on mechanisms of synaptic plasticity and neurotransmission at spinal cord is needed to clarify the above issue.

Conclusions

The present study provides the first in vivo evidence supporting the involvement of Tau protein in pain perception. This study suggests that, in the absence of Tau, two phenomena take place: i) nerve structural and functional alterations (e.g. reduced C-fiber density and Aδ-fiber

hypomyelination) leading to decreased sensation to acute noxious stimuli and ii) increased excitability of second order spinal cord nociceptive neurons resulting in heightened pain-like behaviors in the tonic phase of formalin test. In the light of recent evidence suggesting novel roles for Tau at synaptic structure and function, future studies are necessary to clarify the mechanistic role of Tau in pain sensitization as this study suggests Tau-dependent alterations in the spinal nociceptive circuitry. Altogether, these findings are likely to be of relevance to understand the mechanisms underlying the clinical observations of myelin loss and diminished pain perception in AD patients (Bartzokis, 2011; Corbett et al., 2012).

Conflict of interest statement

Authors declare no conflicts of interest associated to this work.

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