Universidade do Minho
Escola de Ciências da Saúde

Chrysoula Dioli

The role of Tau protein in the stress-induced changes in adult hippocampal neurogenesis

maio de 2016

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UMinho | 2016

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Dissertação de Mestrado
Mestrado em Ciências da Saúde

Trabalho efetuado sob a orientação do
Professor Doutor João Bessa
e do
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maio de 2016

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Título da dissertação: The role of Tau protein in the stress-induced changes in adult hippocampal neurogenesis

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Ano de conclusão: 2016

Mestrado em Ciências da Saúde

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Universidade do Minho, 18/5/2016

Assinatura:

“Good, better, best. Never let it rest.

Until your good is better and your better is best“

ACKNOWLEDGEMENTS/AGRADECIMENTOS

First, I would like to express my sincere gratitude to my supervisor, Professor João Bessa for his guidance, scientific support as well as his endless support and encouragement during my master studies, making me more mature both in science and life.

I would also like to thank my co-supervisor Dr. Ioannis Sotiropoulos for supporting and motivating me during my studies, as well as for his patient and for teaching me that research is the most fascinating obsession.

Also, an enormous thank you to Dr Luisa Pinto for her scientific guidance, for being always there for me and helping me any time.

To Professor Nuno Sousa for offering me the opportunity to be a member of NeRDs and ICVS Institute and for his guidance and support during my Master studies.

To Monica Morais that she was the best company, the best advisor and always willing to help me in every step of my thesis. Thank you for your friendship, guidance, for making me lauph and the most important for teaching me that life is not only black and white.

To Carlos Portugal- Nunes, Joana Silva, Sofia Lopes, Sara Rodrigues and Rita Trindade for being my team, my lab mates during these studies. For helping me, supporting me and making me feel a part of team that has a one goal: help each other with not asking anything back.

To Goreti L. Pinto for accompanying me all these endless hours in the confocal microscope, for all the funny moments we shared and being available all the time for every new request.

To Sara Silva, Patricia Patricio, Dinis Alves, Antonio Pinheiro, Catarina Ferreira for their patient for all these hours they spent with me helping me learn all the techniques.

To all the NeRDs for their endless help in any scientific or technical problem I faced during my Master and for their kindness and friendship.

Last, I would like to thank my family, my parents Maria and Tryfon, my brother Angelos and my sister Sofia as well as all my friends in Greece who always support my choices and passion for science.

RESUMO

A função da proteína Tau nas alterações induzidas pelo estresse na neurogénese adulta do hipocampo

A exposição ao stress cronico aumenta a susceptibilidade para patologias ligadas ao cérebro e esta associada com alterações de neuroplasticidade e défices cognitivos. O stress-induz alterações estruturais/funcionais no hipocampo contribuindo para o aparecimento de doenças como a depressão, tendo particular foco no giro denteado do hipocampo onde o stress é conhecido como um agente que reduz a neurogénese no cérebro adulto. No entanto, os mecanismos celulares subjacentes ao stress-induz alterações na neurogénese são pouco compreendidos. Os nossos estudos anteriores mostraram que o stress crónico leva a uma hiperfosforilação e a um incorrecto funcionamento da proteína Tau conduzindo a atrofia neuronal e défices de memória. Além disso, a hiperfosforilação da Tau foi relacionada como causa para o mau funcionamento neuronal e diminuição da neurogénese na doença de Alzheimer. Baseado nisto, neste trabalho pretendemos clarificar o papel da Tau no stress-induz redução da neurogénese num nicho neurogénico adulto- o hipocampo. Com esse objectivo, submeteram-se ratinhos macho Tau knockout (Tau-KO) e os suas respectivas ninhadas controle (WT) durante nove semanas ao paradigma de stress crónico imprevisível e avaliou-se a proliferação, diferenciação e sobrevivência das novas células formadas no giro denteado adulto assim como o seu papel em tarefas dependentes do hipocampo usando técnicas de análise molecular, celular e comportamental. Os nossos resultados indicaram que enquanto o stress cronico reduz a proliferação das células no giro denteado dos animais WT, não sendo estes efeitos observados nesta mesma população de células nos animais Tau-KO. Além disso, os neuroblastos e os novos neurónios estão também reduzidos nos animais WT, mas não nos animais Tau-KO, sugerindo um papel mediador da Tau nas alterações da proliferação celular e na diferenciação neuronal induzida pelo stress crónico. Pelo contrário, os novos astrócitos estavam diminuídos em ambos os grupos WT e Tau-KO após exposição a stress, indicando que a proteína Tau não é necessária para o stress-induz redução de astrócitos. Mais ainda, o stress cronico reduz cascatas de sinalização conhecidas por regular a sobrevivência e proliferação celular como PI3K/GSK3β/β-catenin pathway seguido pela concomitante redução na sinalização mTOR nos animais WT, mas não nos Tau-KO, no giro denteado. Estas conclusões destacam o papel crucial da Tau como mediadora do stress-induz défices na neurogénese no hipocampo adulto adicionando conhecimento ao mecanismo das cascatas celulares que podem estar associadas ao papel patológico do stress cronico no cérebro e na sua plasticidade.

Palavras-Chave: Tau * Stress * Neurogénese adulta * Giro Denteado * mTOR

ABSTRACT

The role of Tau protein in the stress-induced changes in adult hippocampal neurogenesis

Exposure to chronic stressful conditions is suggested to increase susceptibility to brain pathology as it is associated with neuroplastic deficits as well as impaired cognition and mood. Specifically, structural/functional changes of hippocampal formation are shown to contribute to the pathophysiology of different stress-related disorders, e.g. depression, with particular focus on the dentate gyrus (DG), a region of the hippocampus where stress is shown to suppress neurogenesis in the adult brain. Yet, the underlying cellular mechanisms of the stress-driven neurogenic deficits are poorly understood. Our previous studies show that chronic stress triggers hyperphosphorylation and malfunction of the cytoskeletal protein Tau that leads to neuronal atrophy and memory deficits. In addition, Tau hyperphosphorylation has been causally related to neuronal malfunction and diminished neurogenesis in Alzheimer's disease. Based on the above findings, we hereby aim to clarify the role of Tau on stress-driven suppression of neurogenesis in adult hippocampal neurogenic niche. For that purpose, we have exposed male Tau knockout animals (Tau-KO) and their wildtype littermates (WT) to nine weeks of a chronic unpredictable stress paradigm and evaluated proliferation, differentiation and survival of newly-born cells in the adult DG as well as their significance in hippocampus-dependent function using molecular, cellular and behavioral analysis. We found that, while chronic stress decreased proliferating cells in the DG of WT animals, this effect on the above population was not found in Tau-KO animals. Moreover, neuroblasts and newly-born neurons were also found to be reduced in stressed WT, but not in Tau-KO animals, suggesting an essential mediation of Tau in the damage of cell proliferation and neuronal differentiation induced by chronic stress. In contrast, newly-born astrocytes were decreased in both WTs and Tau-KOs after stress exposure, indicating that Tau is not necessary for stress-induced reduction in the DG astrocytic pool. Furthermore, chronic stress reduced cascades known to regulate cell survival and proliferation in the DG such as PI3K/GSK3β/β-catenin pathway followed by concomitant reduction in mTOR signaling in WT, but not Tau-KO. The above findings highlight Tau as a crucial mediator of stress-driven neurogenic deficits in adult hippocampus adding to our mechanistic understanding of the cellular cascades that may convey the pathogenic role of chronic stress in the brain and its plasticity.

Keywords: Tau * Stress * Adult neurogenesis * Dentate Gyrus * mTOR

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ABBREVIATIONS

3R-Tau – 3- repeat Tau

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Aβ – Amyloid β

Ab – Antibody

ACTH – Adrenocorticotropic hormone

AD – Alzheimer's disease

APP – Amyloid Precursor Protein

AVP – Arginine Vasopressin

BDNF – Brain-derived Neurotrophic Factor

BrdU– 5-bromo-2'-deoxyuridine

cdk5 – Cyclin-dependent kinase 5

CFC – Contextual Fear Conditioning

CNS – Central Nervous System

CON – Control

CORT – Corticosterone

CRH – Corticotropin releasing hormone

CUS – Chronic Unpredictable Stress

DAPI – 4',6-diamidino-2-phenylindole

DCX – Doublecortin

DG – Dentate Gyrus

DGCs – Dentate Granule Cells

EC – Entorhinal Cortex

FBS – Fetal Bovine Serum

FST – Forced Swim Test

GABA – γ-Aminobutyric acid

GCs – Glucocorticoids

GR – Glucocorticoid receptors

GSK3β – Glycogen Synthase Kinase -3β

GFAP – Glial fibrillary acidic protein

HPA axis – Hypothalamic-Pituitary-Adrenal axis

IF – Immunofluorescence

LTD – Long-term depression

MAP – Microtubule-associated Protein

MAPT – Microtubule-associated Protein Tau

Mol – Molecular Layer

MR – Mineralocorticoid receptor

MRI – Magnetic Resonance Imaging

mTOR – mammalian target of rapamycin

MTs – Microtubules

NFTs – Neurofibrillary Tangles

NMDA – N-methyl-D-aspartate receptor

NOR – Novel Object Recognition

NPCs – Neural Progenitor Cells

NSCs – Neural Stem Cells

OB – Olfactory Bulb

OCT – Optimal cutting temperature compound

PBS – Phosphate-buffered saline

PFA – Paraformaldehyde

PI3K – Phosphoinositide 3-kinase

PP1 – Protein Phospatase 1

PP2A – Protein Phospatase 2A

PP – Perforant pathway

PSD-95 – Postsynaptic density - 95

PVN – Paraventricular Nucleus

RT – Room Temperature

SGZ – Subgranular Zone

SVZ – Subventricular Zone

STR – Stress

TA – Temporoammonic pathway

Tau-KO – Tau knockout

Tg – Transgenic

t-Tau – Total Tau

USVs – Ultrasonic Vocalizations

VEGF – Vascular endothelial growth factor

WB – Western blot

WT – Wild type

ZT – Zone Time

1

Introduction

1. INTRODUCTION

Epidemiological studies around the world show that increasing life expectancy has been unfortunately accompanied with increased risk for the development of brain disorders while our mechanistic understanding of both normal and pathological brain aging remains poor. One of the unique characteristics of adult brain is its plasticity to different external and internal stimuli while brain plasticity is shown to be diminished or damaged by aging or under different pathological conditions e.g. depression, Alzheimer's disease[1]. One of the main mechanisms of brain plasticity is neuronal remodeling related to alterations in dendritic length and arborization as well as the number and structure of synapses. These alterations affect the neuronal connectivity with other cells (neurons or glial cells) and subsequently, the formation/function of neuronal circuits and brain networks with obvious impact in the behavioral outputs modulated by these networks. A different mechanism of brain plasticity is related to the genesis of newly-born neurons (neurogenesis) in adult brain while different brain disorders e.g. Alzheimer's disease, depression, schizophrenia are accompanied by impaired neurogenesis[2–4].

1.1. NEUROGENESIS IN THE ADULT BRAIN

Neurogenesis has been defined as a constant process of generating functional neurons from neural stem cells (NSCs) and progenitor cells (NPCs)[5]. In 1965, it was shown for the first time that neurogenesis occurs not only in embryonic and perinatal stages, but also in adult mammals[5,6]. Altman and Das described the presence of proliferating cells in the dentate gyrus of young rats, after they injected thymidine- H^3, that was capable to incorporate in the DNA of dividing cells[6]. Using the same technique in 1984, Paton and Nottebohm described that new neurons in the adult central nervous system (CNS) could integrate to different brain circuits[7]. Some years later, the first NSCs were isolated from the mouse brain and cell cultures were obtained from these cells[8,9]. From the early 90's till today, the use of bromodeoxyuridine (BrdU), a synthetic analog of Thimidine, is the main tool in understanding the neurogenesis process[5].

Adult neurogenesis is well described in two specific brain regions, the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone (SVZ) of the lateral ventricles, but a special interest has also been given to the striatum and hypothalamus (Figure 1)[5,10,11]. The hippocampus

is involved in the control of affective and cognitive (memory and learning) functions, thus adult SGZ has been considered as a niche that provides new neurons to sustain these processes, while neurogenesis in SVZ leads to the migration of newly-born cells to the olfactory bulb where they differentiate and are implicated in learning and memory processes[5,12].

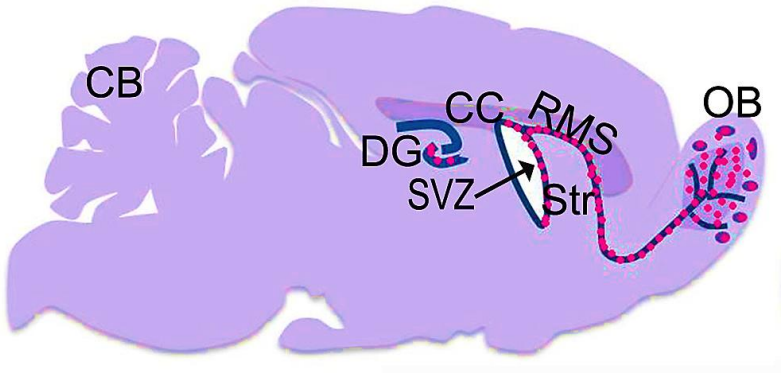


Figure 1 – Main neurogenic niches in adult mouse brain. *Adult neurogenesis occurs mainly in two brain areas: hippocampal dentate gyrus (DG) and subventricular zone (SVZ) of lateral ventricles, where newborn cells migrate through the Rostral Migratory Stream (RMS) to the olfactory bulb (OB) (adapted from Kandasamy et al., 2015)[13].*

The hippocampus consists of different interconnected areas which includes CA1, CA3 and the dentate gyrus (DG) (Figure 2), where both neurogenesis and gliogenesis occur in adult brain; the latter results in the generation of astrocytes and oligodendrocytes. During the last years, a plethora of studies have contribute to the characterization of the different steps of hippocampal neurogenesis (Figure 2-insert). In the first step, adult type 1 NSCs (slowly proliferating cells exhibiting a radial process crossing the entire granule cell layer of DG) that are located in the SGZ of DG, generate type 2 NSCs (fast proliferating cells).[12,14] Type 2 NSCs give rise to neuroblasts that differentiate into glutamatergic dentate granule cells (DGCs) and migrate to the granule cell layer[15]. In the last step, these cells maturate forming dendrites that reach the DG molecular layer and receive inputs (e.g. glutamatergic) from the entorhinal cortex (EC). Newly-born and pre-existing neurons send projections (mossy fibers) to the pyramidal cells in CA3 hippocampal area. In turn, CA3 pyramidal neurons relay signals to CA1 pyramidal neurons which, then, send projections to the subiculum, and the deep-layer of the EC[16–18]. Newborn neurons require three to four weeks to become partly functional while their full maturation lasts for several months with continuous formation of spines and synaptic connections with target cells in CA3 (Figure 2)[19]. However, recent evidence demonstrates that synaptic inputs from CA3 neurons project to newly-born neurons in DG that could be involved in the formation of memories[20,21]. While still

under intensive investigation, this synaptic formation is suggested to inhibit the activity of some newborn neurons impacting on DG- and hippocampus-dependent cognitive performance such as memory acquisition and pattern separation [19,21].

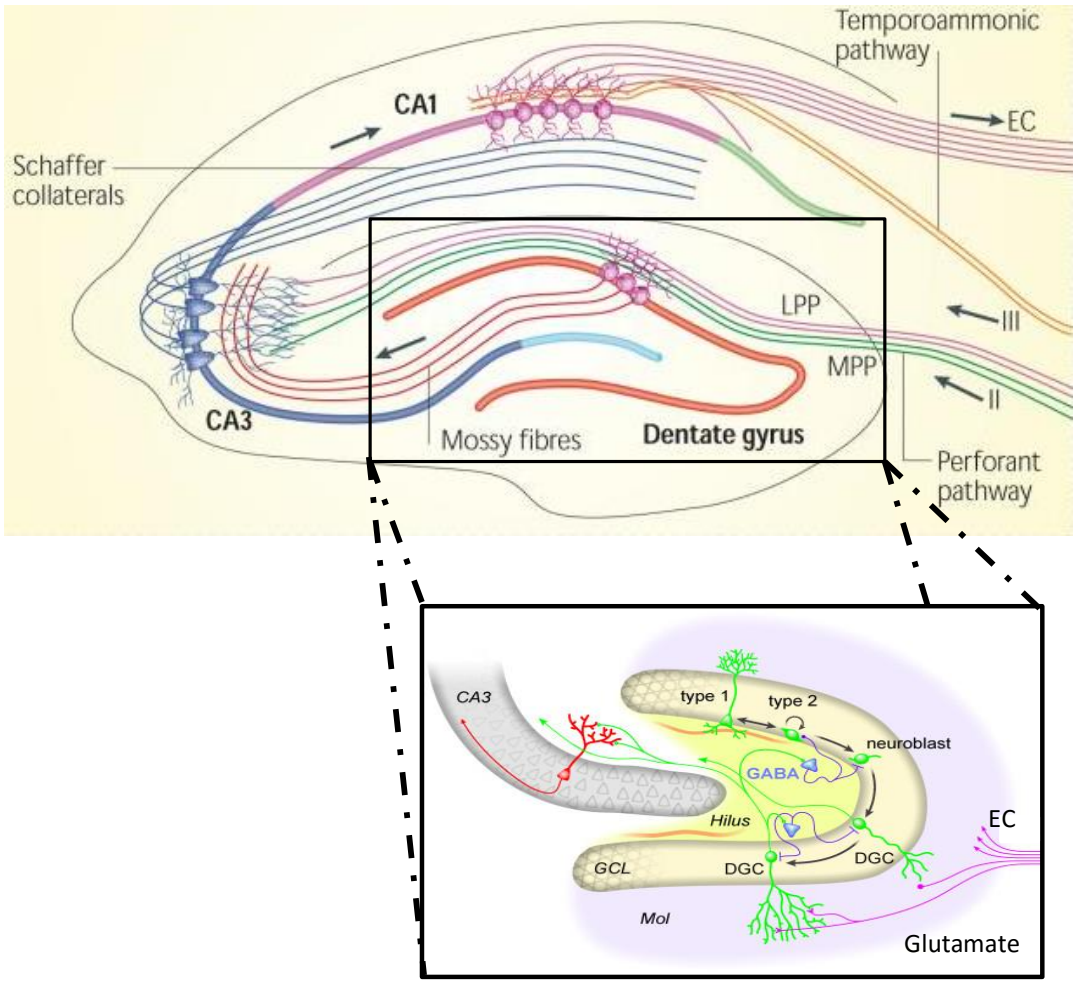


Figure 2 – Schematic representation of hippocampal circuitry and adult hippocampal neurogenesis. *The axons of layer II neurons in the entorhinal cortex (EC) project to the dentate gyrus (DG) through the perforant pathway (PP), while the DG sends projections to the pyramidal cells in CA3 through mossy fibres. Next, pyramidal neurons of CA3 send axonal projections to CA1 hippocampal area and CA1 pyramidal neurons send back-projections into the EC. Note that CA3 also receives direct projections from EC layer II neurons through the PP and CA1 receives direct input from EC layer III neurons through the temporoammonic pathway (TA). Details of DG neurogenesis are presented in the insert where adult neurogenesis occurs in five steps: 1) activation of quiescent radial glia-like cell (type 1), 2) proliferation of non-radial precursor and intermediate progenitors (type 2), 3) generation of neuroblasts, 4) integration of neuroblasts to the dentate granule cell layer (DGC) and 5) maturation of granule cells to the molecular layer (Mol) that receive inputs from the Entorhinal Cortex (EC) and project to CA3 (red neuron) (adapted from Mu and Cage, 2011 and Deng et al., 2010)*[12,22].

1.1.1. ROLE OF HIPPOCAMPAL NEUROGENESIS IN ADULT BRAIN FUNCTION

Different brain outputs and behavioral domains related with cognition and mood require hippocampal function while damage of hippocampus has been implicated in different brain disorders related to memory and other cognitive deficits as well as depressive and anxious behaviors. Specifically, hippocampus has been involved in different aspects of memory such as the formation of short-term memories and their conversion to long-term memories that will be stored in the cortex[19,23]. Furthermore, animal models have identified a role for adult hippocampal neurogenesis in the acquisition and consolidation of spatial memory, the formation of associative memory as well as in pattern separation; the later refers to the DG-dependent formation of distinct representations of similar inputs[24–26]. For example, the depletion of hippocampal neurogenesis by low dose X- irradiation in adult mice impaired pattern separation in an 8-arm maze test as well as in a contextual fear discrimination learning test[26,27] Furthermore, many studies have also shown that mice with genetically- or pharmacologically-reduced levels of neurogenesis reveal poorer performance in a set of hippocampus-dependent tests including contextual fear conditioning (CFC), Novel Object Recognition (NOR), Y-maze and eye-blink conditioning [28,29,30]. Moreover, using Morris Water Maze test to assess spatial memory, Kee et al., showed that mainly newborn neurons, but not mature neurons, are preferentially activated by spatial learning, suggesting that new neurons make a unique contribution to memory processing in DG[19,31]. On the other hand, the enhancement of hippocampal neurogenesis is shown to increase pattern separation thus strengthening the notion that levels of adult DG neurogenesis critically contribute to hippocampal-related cognitive functions[26].

A plethora of studies suggest that cognition and emotion are interconnected behavioral domains (Figure 3)[32–34] with the hippocampus participating in brain networks associated with emotional behaviors for example, interacting with the amygdala[35], which is considered the integrative center for emotional behavior and emotional memories. Thus, different parameters that affect neurogenesis have been also shown to affect mood and emotional status. Becker et al., reported that a four-week social defeat paradigm in rats led to reduced proliferation levels in the DG followed by depressive-like pathology, measured by two behavioral tests, the Forced Swim Test (FST) and the sweet water consumption[36]. On the other hand, physical exercise (running wheel) in rats increased proliferating cells in the DG ameliorating their cognitive performance measured in the NOR test[37]. Environmental enrichment conditions, that included physical exercise, increase both proliferation and cell survival in rodents improving their emotional status, measured by their emission of ultrasonic vocalizations (USVs)[38].

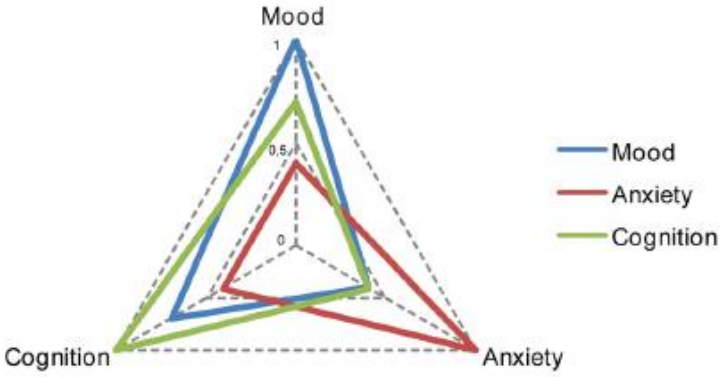


Figure 3 – Graphical representation of correlations between the different behavioral domains *where cognition (memory deficits), mood (depressive-like behavior) and anxiety are shown to be interconnected as measured by previous studies of our team using chronic stress paradigms (Bessa et al., 2009)*[34]*.*

1.1.2. DIFFERENT PARAMETERS THAT INFLUENCE ADULT DG NEUROGENESIS

Different environmental, physiological and pathological stimuli and conditions are known to influence adult hippocampal neurogenesis impacting on various steps of neurogenesis such as proliferation and maturation as well as integration and survival of newborn neurons[39]. Specifically, previous studies have shown that neurogenesis in the adult brain DG is positively or negatively influenced by sex hormones, physical exercise, environmental enrichment, sleep disruption, inflammation and exposure to prolonged stressful conditions[40–42]. Based on the increased loads of distress in modern lifestyle and societies and its association with different pathologies e.g. depression and Alzheimer's disease, special focus has been given over the last decades to the role of chronic stress on brain plasticity and specifically on neurogenesis.

1.2. STRESS AND ITS PHYSIOLOGICAL ROLE IN ADAPTATION

Living organisms need to continuously maintain the integrity of their 'internal environment' using homeostatic mechanisms[43]. Stress has been defined as a disruption of homeostasis following the arrival of internal or external challenges ("stressors")[43,44]. The stress response represents a process of "maintaining stability through change", also referred to as allostasis, a process involving the activation of the autonomic, neuroendocrine and immune systems. There are different types of stressful stimuli depending on the nature, severity, chronicity, as well as the predictability and controllability of the stressor by the animal that can influence the physiological response of the organism to stress[45,46]. The most easily and frequently measureable and critical neuroendocrine response to stress involves the release of glucocorticoids (GCs) through the activation of hypothalamic-pituitary-adrenal axis (HPA)[47] (Figure 4). Upon its arrival, stressful stimuli trigger the release and secretion of corticotropin releasing hormone (CRH) and vasopressin (AVP) from the hypothalamus (hypothalamic paraventricular nucleus; PVN) into the pituitary portal vessel system and activate the synthesis of adrenocorticotropin hormone (ACTH) in the pituitary gland[48,49]. ACTH secretion into the bloodstream stimulates GCs synthesis and secretion from the adrenal cortex. GCs serve as a feedback inhibitory signals back to the hypothalamus and pituitary[47,48,49]. GCs, cortisol in humans and corticosterone (CORT) in rodents, bind to two GC-activated receptors: the type I mineralocorticoid receptors (MRs) with high affinity and type II GC receptors (GRs) with low affinity[49]. GRs and MRs are expressed in different patterns in the brain: GRs are widely distributed throughout the brain in most neurons and glial cells, while MRs are restricted to neurons and mainly to the hippocampus[49,50]. GCs receptors are more abundant in the hippocampus, medial prefrontal cortex and amygdala, making it reasonable to associate their location to their functional properties[51,52]. Each of these brain areas are modulated by GCs, exhibiting a negative feedback effect on HPA axis activity (Figure 4)[48,52,53]. Additionally, based on the role of each of these brain areas, it can be conducted that GCs are able to modulate memories associated with spatial cognition and emotional learning[52].

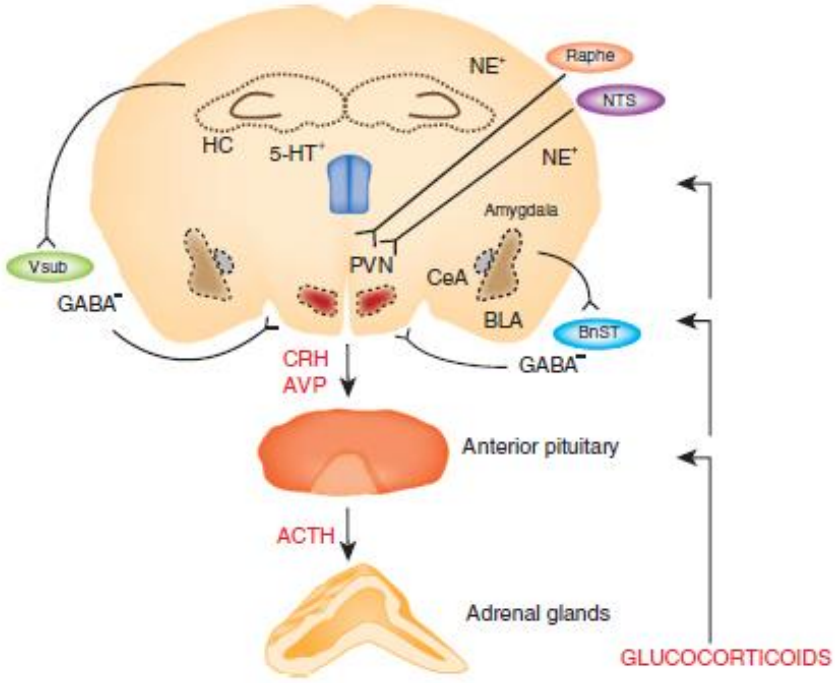


Figure 4 – Schematic representation of the HPA axis and its regulation. *A stressful stimulus triggers the release of corticotropin releasing hormone (CRH) and vasopressin (AVP) from the paraventricular nucleus (hypothalamic PVN), which are secreted into the pituitary portal vessel system and activate the synthesis of adrenocorticotropin hormone (ACTH). ACTH secretion into the bloodstream stimulates glucocorticoid (GCs) synthesis and secretion from the adrenal glands where GCs drive a negative feedback on pituitary and hypothalamus. Note that different brain regions (e.g. hippocampus and Raphe), also exhibit a regulatory role of HPA axis (adapted from Arnett et al., 2016)*[49].

1.2.1. CHRONIC STRESS, NEUROGENESIS AND BRAIN PATHOLOGY

Whereas acute stress is generally beneficial and adaptive, a series of pathophysiological changes can occur, if the stress response is prolonged in time, including alterations in the brain structure and function as well as different peripheral targets e.g. immune and reproductive systems, cardiovascular function and others[54,55]. It is suggested that, under chronic stressful conditions, HPA axis activity may become dysregulated and result in impaired GC signaling with a potential threat for health[56,57,58]. Indeed, severe or prolonged stress is well known to increase the risk of developing psycho- and neuro-pathologies such as depression, anxiety and Alzheimer's disease in susceptible individuals. Many studies have shown that vulnerability and resilience to chronic stress effects is different among individuals and this difference is suggested to be determined by genetic, environmental and epigenetic factors[44].

Among the most studied effects of chronic stress in the brain is related to hippocampal plasticity as numerous studies in humans and animals have shown that prolong exposure to stressful conditions causes neuronal atrophy, synaptic loss and reduced neurogenesis in hippocampus (Figure 5)[59]. Using a chronic unpredictable mild stress paradigm, previous studies in rats showed that the survival of newly-born cells in the DG is reduced after exposure to stress[60] followed by a reduction in the number of neuroblasts in stressed animals[61]. Furthermore, Lagace et al. showed that a ten-day protocol of social defeat can decrease the number of proliferating cells in mice[62]. These studies also report that these animals present anxious and depressive-like behavior[60,61,63,64]. Additionally, besides neurons, glial cells are also affected by chronic stress with previous studies showing that chronic unpredictable stress paradigm in rats leads to a reduction in the number of astrocytes in DG[65].

Human studies focusing on different stress-related pathologies, e.g. anxiety and depression, also demonstrate altered neurogenesis in hippocampus; however, despite the fact that these studies are very limited, their findings are controversial[63]. Lucassen et al. measured the total number of DG progenitor cells in postmortem tissue of patients suffering from depression or anxiety, reporting a significant decrease in progenitor cells in depressed patients[66]. In line with this, another human study reported a non significant tendency for reduction in proliferating cells[67]. However, a third study using human postmortem DG tissue found no differences in the proliferating cell subpopulation in DG[68]. While the studies that monitor cell proliferation in the human adult brain are limited, many postmortem and high-resolution magnetic resonance imaging (MRI) volumetric studies have shown smaller DG size and reduced DG granule cell numbers in patients suffering from depression or anxiety disorders[63,69,70]. On the other hand, the use of antidepressants restores the levels of adult neurogenesis and the depressive-like phenotype in animal models and increases the total dentate granule cell number and dentate gyrus size in humans[69,71–73].

Moreover, it has been shown that hippocampal neurogenesis is also altered in transgenic animal models and in the human brain in Alzheimer's disease (AD), the most common form of dementia[74], that is recently related to chronic stress[75,76] as it will be discussed later. Specifically, animal studies using different AD transgenic models showed either a reduction or increase in neurogenesis levels [74,77,78]. The same inconsistency has been observed in human studies showing either a decrease or a reduction in the levels of neurogenesis[74,79,80]. A possible explanation for these controversial findings in AD brain could be based on the different stages of AD pathology where reduced neurogenesis occurs in the initial, early

stages of the disease whereas the increase of neurogenesis could serve as a compensation mechanism against the severe neuronal loss that is found in later stages of AD.

1.2.2. MECHANISMS OF STRESS-DRIVEN SUPPRESSION OF NEUROGENESIS

Different molecular cascades and signaling mechanisms have been suggested to underlie the detrimental effect of chronic stress on adult brain neurogenesis. Indeed, the physiological underpinnings of stress-related neurogenic deficits focus on GCs that are shown to inhibit proliferation, differentiation and survival of DG cells in rodents through GCs binding to their receptors and their translocation from the cytoplasm to the nucleus where they activate or repress the transcription of specific genes[81–83]. *In vitro* studies on human cells showed that exposure to low cortisol levels induce a MR-dependent increase of proliferating cells, while exposure to high cortisol levels induce a GR-dependent decrease[84]. In addition, exposure to chronic stress also influences the levels of neurotrophic factors in the brain, such as brain- derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF)[85]. Specifically, BDNF levels have been shown to be decreased after stress, and indirectly modulate other factors such as VEGF [83,86]. The neurogenesis-related actions of BDNF focus on its ability to promote differentiation and maturation of newly-born neurons by enhancing the release of gamma-aminobutyric acid (GABA) from interneurons[83,87]. Another cellular cascade shown to be involved in stress-triggered suppression of neurogenesis is the wingless (WNT) signaling through the canonical WNT/β-catenin pathway that affects the proliferation and differentiation of DG cells[83,88]. The stress-driven effect on proliferation involves the transcriptional control of genes that regulate the cell cycle while the impact of stress on neuronal differentiation involves the regulation of the promoter of Neurogenin-1 gene[89,90]. Other studies report that stress diminish cell proliferation in the DG by changing the activation level of NMDA glutamatergic receptors[91]. Specifically, rat studies, showed that antagonism of NMDA receptors increases the production of granule cells in the DG, while the activation of NMDA receptors results in the opposite effect[92]. Furthermore, as different chronic stress paradigms are often used as animal models of depression, different antidepressant treatments reverse the effect of stress in the hippocampal neurogenic niche[59,61,93]. It is suggested that antidepressants can increase the levels of neurotrophic factors, like BDNF and VEGF which are decreased due to stress (Figure 5)[59,93]. Additionally, the WNT proteins are upregulated following antidepressant treatment[93]. While the above mechanisms appear after a significant period of antidepressant treatment, a rapid antidepressant effect is also

described with the use of the NMDA receptor antagonist Ketamine, but its effect on neurogenesis is yet to be clarified[94].

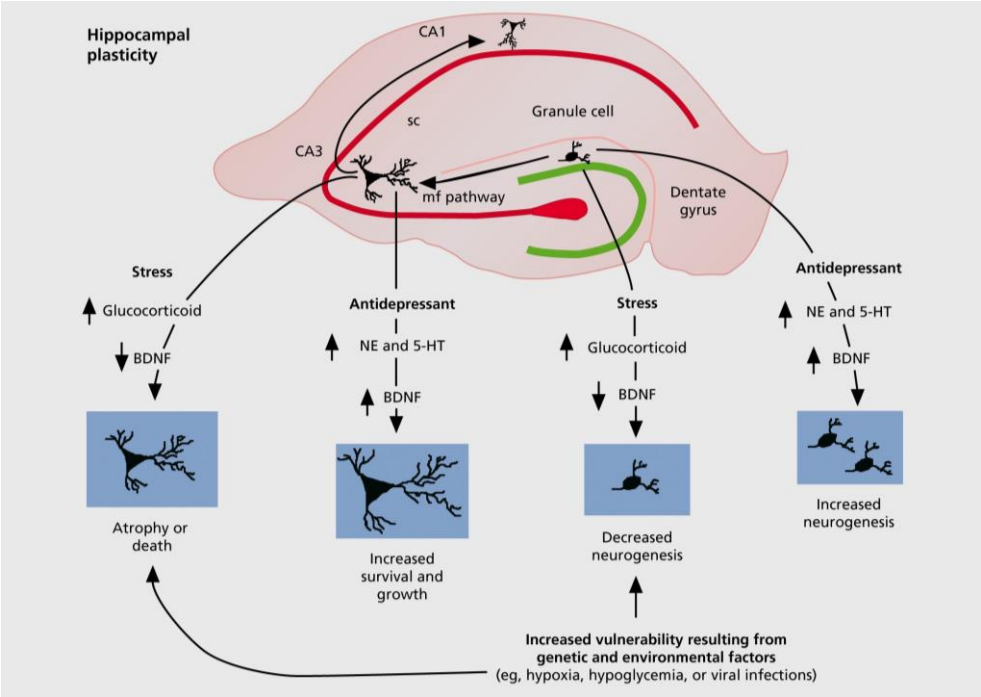


Figure 5 – Chronic stress and antidepressant opposite effects on hippocampal plasticity. *Exposure to stressful conditions results in elevated GCs levels triggering decrease neurogenesis in parallel with neuronal atrophy while antidepressants reverts both stress-driven deficits on hippocampus (adapted from Duman, 2004)[89].*

1.3. CYTOSKELETAL CHANGES IN NEUROGENESIS; A POSSIBLE ROLE FOR TAU PROTEIN

Despite the fact that the cell proliferation and axonal elongation processes requires cytoskeletal support, little attention has been given to the importance of cytoskeletal dynamics and the required high degree of plasticity in newborn cells which allows them to divide, migrate, differentiate and integrate into the existent hippocampal network. One of the main cytoskeletal proteins is Tau, which is a microtubule-associated protein (MAP), mainly expressed in the central and peripheral nervous system[95,96]. Tau is mainly found in neurons but it is also expressed in lower levels by astrocytes and oligodendrocytes[97–100]. Neuronal localization of Tau is also different among intracellular compartments as it is more abundant in neuronal axons and less abundant in neuronal somata and dendrites, as recently shown (Figure 6A)[101].

The most well-known function of Tau is its ability to bind to microtubules (MTs) modulating MTs stability and assembly (Figure 6B)[96]. However, Tau protein has various cellular targets, with many of them having cytoskeletal and/or signaling properties, thus highlighting Tau protein as a converging molecule in the different ways that cytoskeletal structure interacts with cellular cascades (Figure 6B)[100,102]. Specifically, Tau binding to MTs has been considered to regulate axonal transport, by interfering with the binding of motor proteins to the MTs[100,103,104]. Tau is also involved in the transport of axonal and dendritic mitochondria[105], thus participating in many cellular processes that required mitochondria support (e.g. synaptic function, energy supply and Ca+2 absorbance). In addition, Tau can also bind to actin (F- actin) filaments and bundle them[105], representing a potential structural link between actin and MTs[100,106–108].

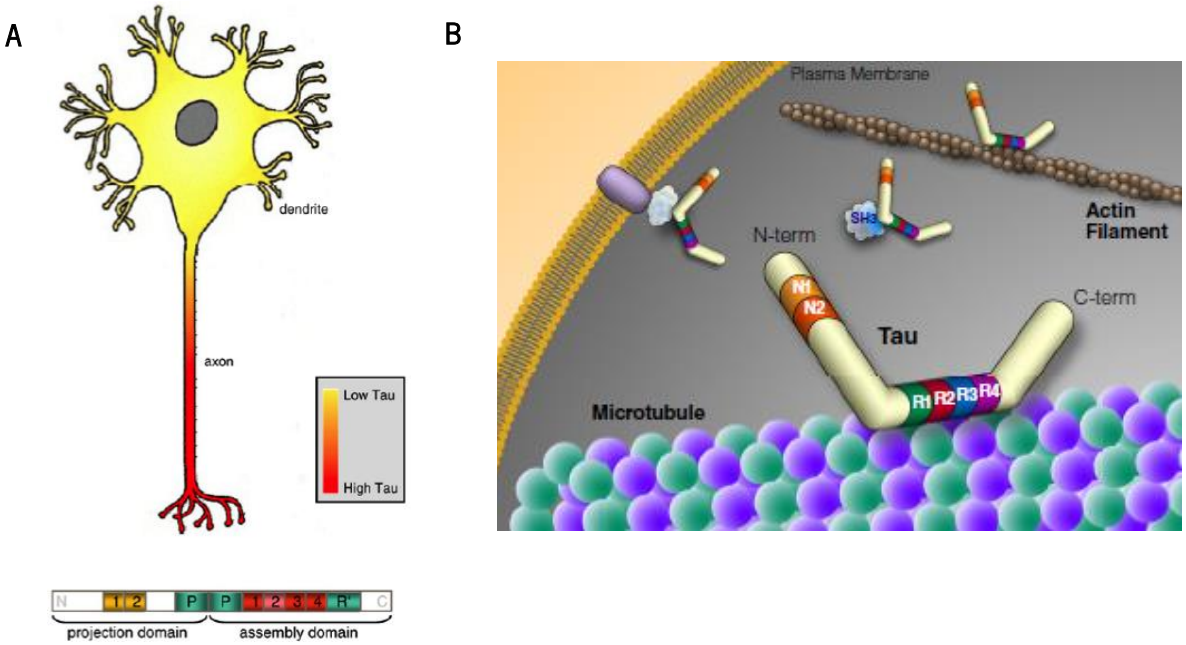


Figure 6 – Tau localization in neurons and its different intracellular targets . *A) Schematic representation of Tau in neurons showing that higher levels of Tau are present in axonal compartment (red) while neuronal soma and dendrites express lower Tau levels (yellow). B) Different cellular targets of Tau that includes microtubules, actin filaments and Fyn protein (binding at SH3 domain of Fyn) (adapted from Morris et al., 2011)*[100].

Furthermore, regarding the role of Tau on neurite outgrowth and neuronal differentiation, in vitro studies of hippocampal neuronal cultures from Tau knockout (Tau-KO) mice showed impairment in the differentiation phase of these cells, with significant delay in the axonal and dendritic extension[109] while other studies suggest a role of Tau in the distal axon where it contributes to the organization of the

MTs[110]. Moreover, expression of human Tau protein in Tau-KO mice restored normal axonal growth[109]. Recently, Tau has been suggested to participate in synaptic structure and function interacting with different scaffold proteins and receptors such as NMDA receptors, PSD-95 and Fyn affecting both synaptic plasticity and signaling[111,112]. Namely, Frandemiche et al. showed a neuronal activity-triggered movement of Tau in the synapse when it interacted with actin, while a previous study from our team have also shown that the long-term depression (LTD) mechanism of synaptic plasticity is diminished in Tau-KO animals and cells[111]. Furthermore, other studies relate neuronal malfunction with mislocalization of Tau at synapses showing that Tau binds to Fyn and transports it to the dendritic spines interacting with NMDA receptors and PSD-95 scaffold protein[112].

1.3.1. TAU PROTEIN, ITS ISOFORMS AND THEIR DEVELOPMENTAL PROFILE

Tau protein is the product of alternative mRNA splicing of exons 2, 3 and 10 of MAPT gene that is located in chromosome 17, which yields in six main isoforms[113] (Figure 7A). 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 microtubule binding domains at the C-terminal (Figure 7A)[100,114]. The repeat region is responsible for the binding to MTs and promotion of their assembly and Tau isoforms with four repeats (4R-Tau) present greater affinity to MTs than isoforms with three repeats (3R-Tau)[114–116]. The expression of Tau isoforms is developmentally regulated (Figure 7B). Indeed, 3R-Tau isoforms are found at early developmental stages, while 4R-Tau isoforms are mainly found in later developmental stages[117,118]. However, 0N/3R tau isoform is the only embryonic isoform that is present in adulthood, but in lower levels[119,120]. 3R-Tau isoform has been found to colocalize with most of the cells that express the neurogenic markers of neuroblasts, doublecortin (DCX) and PSA-NCAM in the SGZ of hippocampus and with few cells that express calbindin, a marker of mature neurons[120,121]. These findings suggest that 3R-Tau isoform is present mainly in the early stages of adult hippocampal neurogenesis (neuroblasts) and is almost absent in mature neurons. It has been proposed that the reason of this preference of Tau isoform during adult neurogenesis is due to the fact that early stages of neurogenesis require a more dynamic cell shape (which 3R-Tau isoform could offer) that could promote cell division and differentiation, while the stronger ability of 4R-Tau isoforms to promote microtubule assembly is necessary for stabilization of mature cells[118,122].

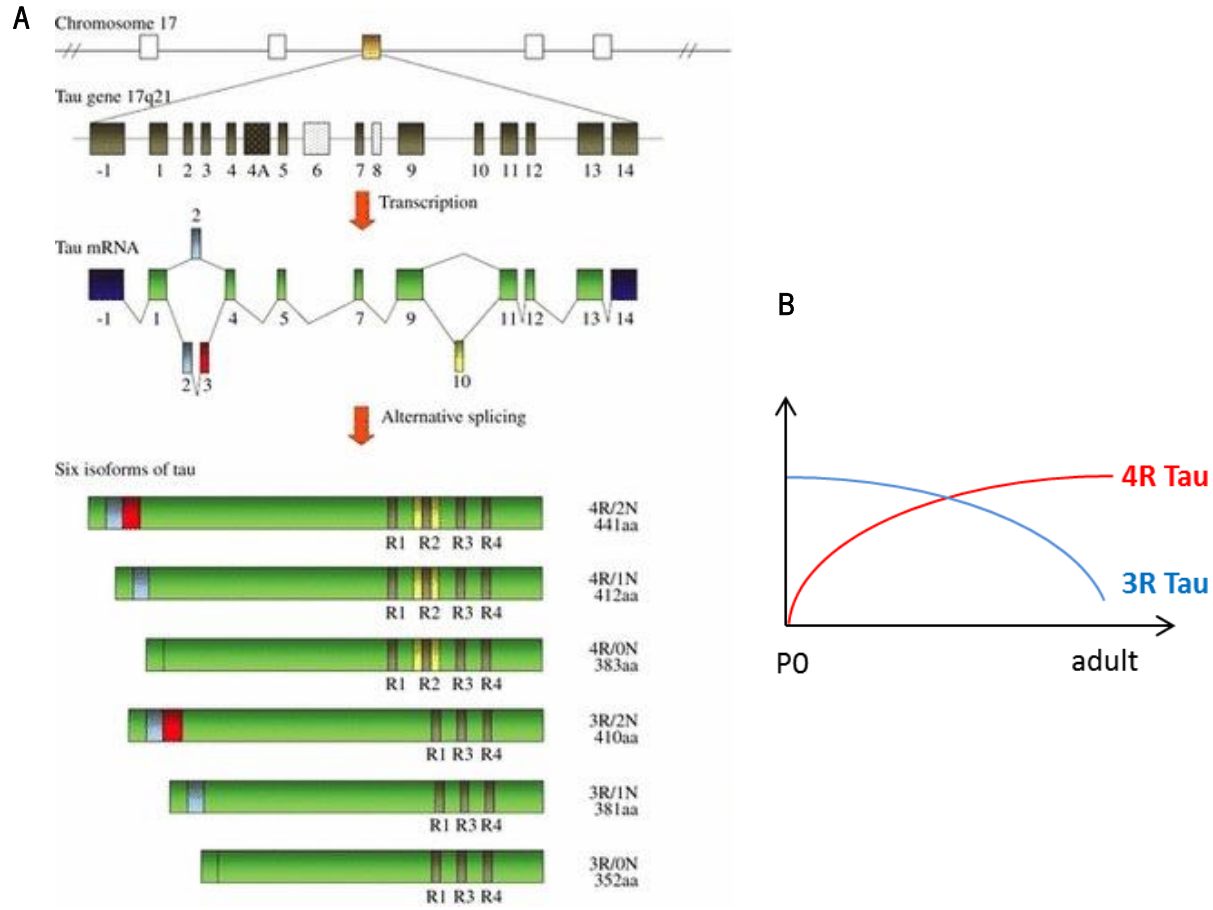


Figure 7 – Different Tau isoforms and their developmental profile of expression. *A) Tau gene is located to chromosome 17q21 in humans (upper panel of A) and its transcription to mRNA is followed by alternative splicing in exons 2, 3, and 10. This results to the generation of six Tau isoforms subdivided in 4R- and 3R-Tau groups; 4R- Tau present four repeated binding domains (R1 to R4), while 3R-Tau content three domains. Each isoform also differs in the number of N-terminal exons (in blue and red) that generate 2N, 1N or 0N isoforms (adapted from Conrad et al., 2007)*[113]*. B) The expression of Tau isoforms is developmentally regulated with 3R-Tau levels progressively decrease after the postnatal period (P0) while 4R-Tau expression exhibit the opposite profile (drawn by C. Dioli).*

1.3.2. TAU PHOSPHORYLATION AND IT'S ROLE ON TAU FUNCTION AND MALFUNCTION

Many studies have shown that the function(s) of Tau protein can be influenced by different posttranslational modifications[123] and amongst them, phosphorylation has received a lot of attention due to its role on regulating the binding capacity of Tau to MTs and to the membrane[118,123]. While Tau phosphorylation at certain sites results in conformational changes of Tau and decreased MTs binding assembly, dephosphorylation promotes MTs polymerisation and stability[124]. A nice example of the role of

phosphorylation-dependent involvement of Tau in cytoskeletal changes focuses on the expansion of branches in the growing axons (Figure 8)[125]. The growth and guidance of axon branches requires regulation of actin and MTs dynamics in different levels and steps[125], with the initial steps requiring F-actin branching and elongation. As short fragments of MTs enter the newly forming branches, Tau detaches from MTs and other MAPs, like MAP1B, which also interact with actin filaments to stabilize the MTs, regulating branch formation (Figure 8b)[125]. At a later step, dephosphorylated Tau binds to MTs offering the required stability that supports elongation of axon branch (Figure 8d).

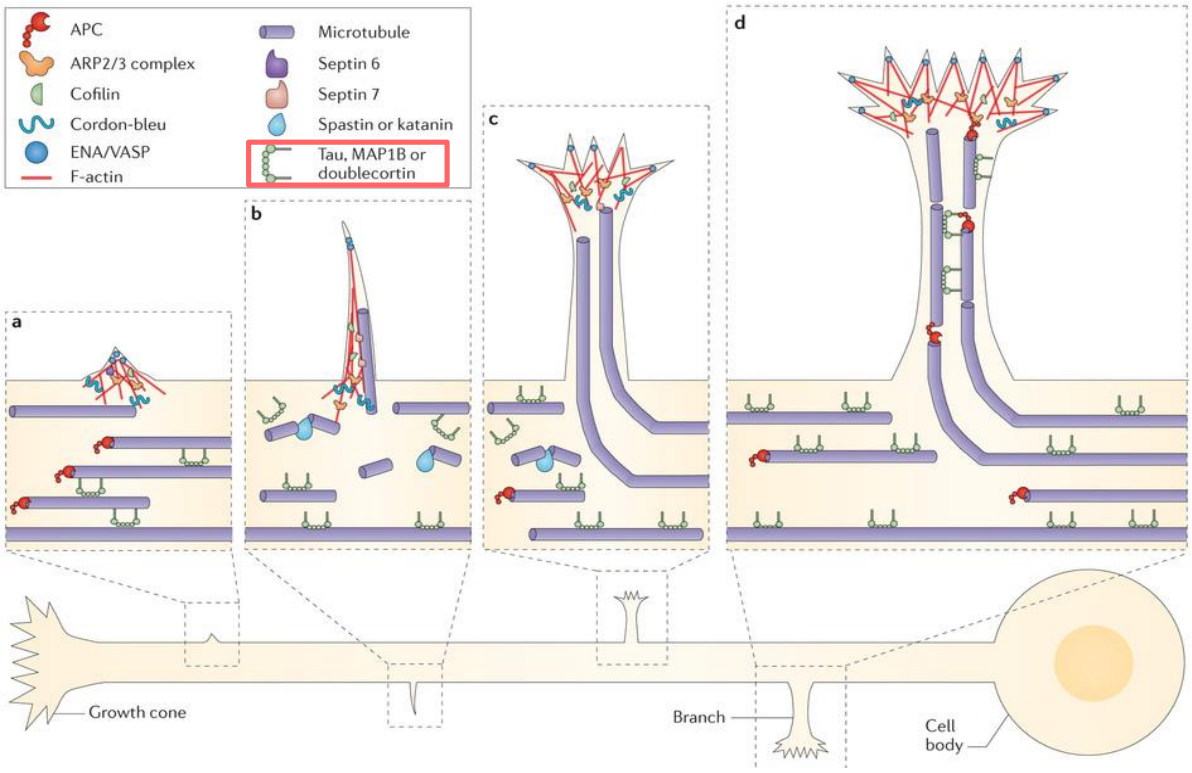


Figure 8 – Presence of Tau protein in the growing branches of neuronal axon. *Tau is present in the growing axons in two forms: the phosphorylated forms of Tau that detaches from the MTs allowing spastin and katanin to severe MTs promoting a MT dynamic status (b) and, in a second step, dephosphorylated forms of Tau binds to MTs leading to the formation and stabilization of the branches that supports elongation of axon branches (d) (adapted from Kalil and Dent, 2014)*[125]

There are at least 80 known phosphorylation sites of Tau that are localized at different Ser or Thr aminoacids along the protein including the microtubule-binding domains (shown as R1, R2, R3, R4 in Figure 9A)[126]. Under normal conditions, Tau phosphorylation and dephosphorylation is a dynamic process regulated by different kinases and phosphatases (Figure 9C). Most of these phosphorylation sites are on Ser–Pro and Thr–Pro motives[127]. In pathological conditions, like AD, Tau is

hyperphosphorylated in many and different epitopes e.g. Ser202, Thr205, Thr231, Ser262 or 396/404 while some of them are mainly found to be phosphorylated in AD brain (aberrant phosphorylation; e.g. Thr212 and Ser214) (Figure 9A)[127]. Phosphorylation of those epitopes can diminish or abolish MT-binding capacity of Tau leading in cytoskeletal pathology and malfunction (Figure 9D)[101]. For example, hyperphosphorylation in Thr231 and Ser262 epitopes are considered to induce destabilization of the neuronal cytoskeleton, causing hippocampal atrophy[128,129].

Different kinases regulate the phosphorylation of Tau (Figures 9C,D)[117,118,130], with the major Tau kinases including glycogen synthase kinase 3β (GSK-3β) and cyclin-dependent kinase 5 (cdk5)[131,132]. GSK3 phosphorylates Tau at Thr231 affecting microtubule binding, while phosphorylation at Ser396 or 404 does not affect microtubule binding[133]. Additionally, cdk5 also phosphorylates different Tau epitopes such as Ser202, Thr205, Thr231, Ser396 and Ser404[134]. Interestingly, some epitope phosphorylation (e.g. Thr231 and Ser404) demands the combined action of cdk5 and GSK-3β where GSK-3β requires a priming phosphorylation of this residue by cdk5[135]. On the other hand, different phosphatases dephosphorylate Tau protein, such as protein phosphatase 1 and 2 (PP1 and PP2A, respectively) [135]. In AD brains, GSK3β is highly activated, cdk5 is upregulated whereas PP2A is downregulated contributing to Tau hyperphosphorylation[117,131,136].

Similarly to Tau isoform expression, the phosphorylation of Tau is developmentally regulated[118]. Specifically, Tau is highly phosphorylated in embryonic neurons and is increasingly dephosphorylated by aging[117,130]. However, mitotic cells in SGZ of DG also exhibit an AD-like Tau phosphorylation profile by expressing high phosphorylated Tau in Ser396/404 mostly in the stage of differentiation to neuroblasts[118,137]. The presence of phosphorylation sites during the early stage of adult neurogenesis is not surprising, since phosphorylation decreases the affinity of Tau protein to the microtubules, confering neuroblasts a less stable and more dynamic MT network[118].

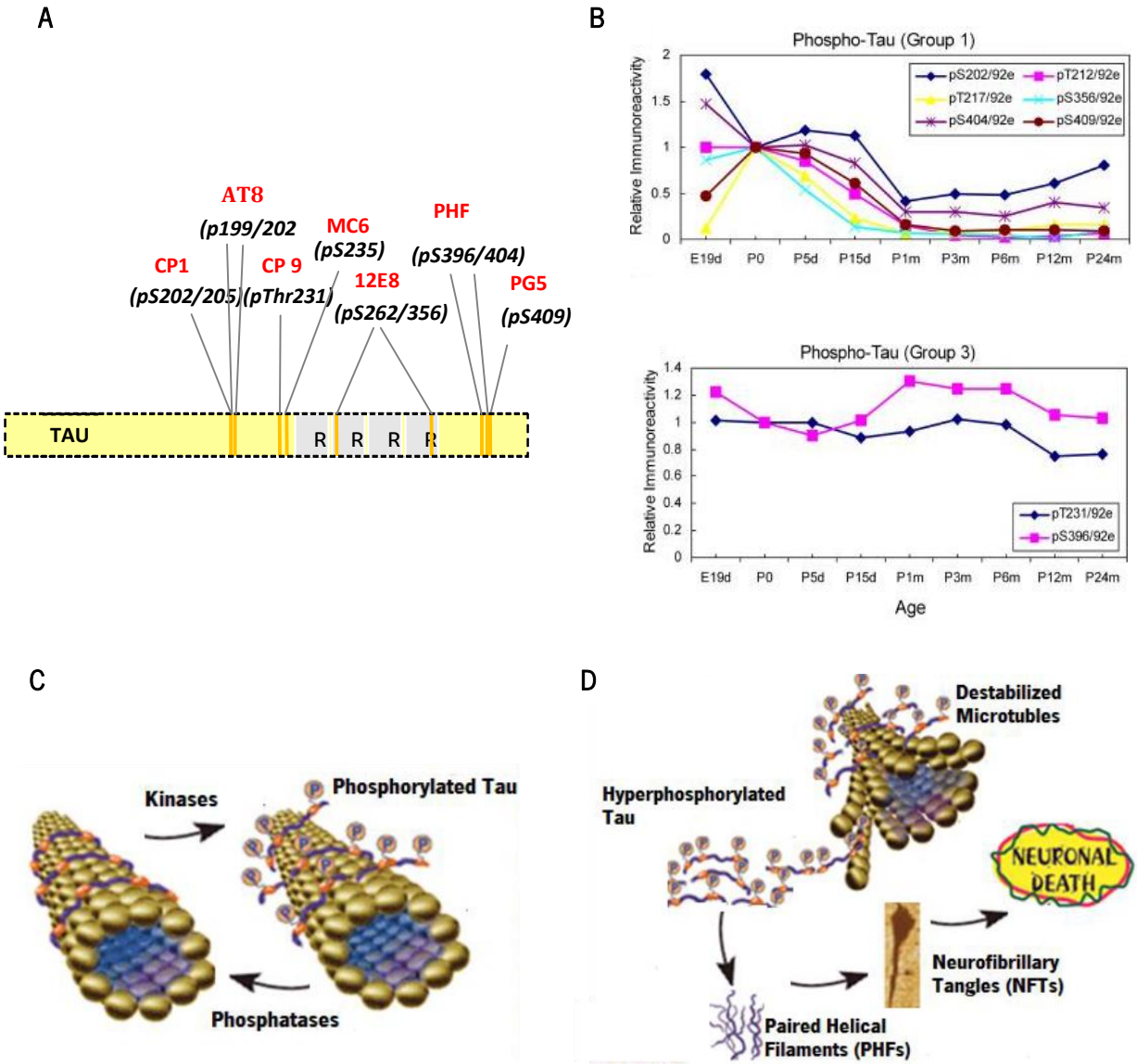


Figure 9 – Tau phosphorylation in brain development and pathology. *A) Schematic representation of Tau phosphorylation sites that are hyperphosphorylated in AD (drawn by I. Sotiropoulos). B) Temporal profile of different Tau phospho-epitopes from embryonic day 19 (E19d) until 24 months old (P24m). Note that some phosphorylation sites are highly phosphorylated in embryonic stages and are decreased during the postnatal period (upper part of panel B), while others remain unchanged during aging (lower part of panel B) (adapted from Yu et al., 2009)*[130]*. C) Tau phosphorylation/dephosphorylation is a normal and dynamic procedure occurring through the balanced action of kinases and phosphatases regulating cytoskeletal dynamics. D) In pathological conditions, e.g. AD, Tau hyperphosphorylation leads to its detachment from MTs, resulting in cytoskeletal damage and neuronal death while the free-unbound Tau is aggregated in the neuronal somata forming the typical Neurofibrillary Tangles (NFTs) (adapted from http://slideplayer.org/slide/791618/).*

Interestingly, the developmentally regulated changes in the phosphorylation of Tau is not similar for all phospho-epitopes (Figure 9B). Some epitopes, like Ser202, Thr212, Thr217, Ser356, Ser404, and Ser409, are highly phosphorylated during development until the postnatal day 15 (P15d) and then decline, while others like Thr231 and Ser396, are stable during development and adulthood (Figure 9B)[130].

1.3.3. CHRONIC STRESS AND TAU HYPERPHOSPHORYLATION IN AND BEYOND AD

Previous studies have shown that different stressful conditions, such as acute stress, cold water, hypothermia and starvation are able to modulate Tau phosphorylation[138–140]. As clinical studies have suggested that chronic stress can be a risk for AD, particular attention has been given to the detrimental role of chronic stress and elevated GCs levels on the initiation and progression of AD.

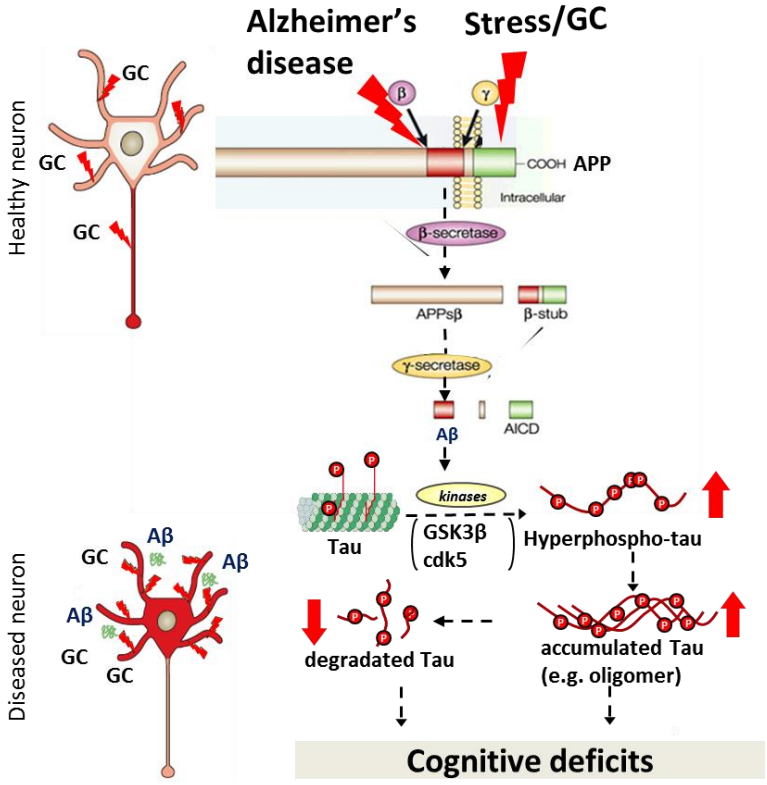


Figure 10 – Chronic stress and GCs impact on AD. *Chronic stress triggers APP misprocessing towards the Aβ generation which in its turn, evoke Tau hyperphosphorylation, its reduced degradation and increased accumulation followed by Tau missorting at the somatodendritic compartment and spines (in red in diseased neuron) . Note that Tau is mainly found at the neuronal axons (red in healthy neurons) (drawn by Ioannis Sotiropoulos).*

Using different transgenic and non-transgenic models of AD, our previous studies have demonstrated that exposure to chronic stress or prolong treatment with the synthetic GC, dexamethasone, trigger hyperphosphorylation of Tau affecting different phosphorylation epitopes such as Thr231, Ser262, Ser396/404 that leads to Tau accumulation in neuronal somata (Figure 10)[4,138,141,142]. Later studies from our team showed that, similarly to AD Tg mice models, chronic stress also evokes Tau hyperphosphorylation in wild-type animals that is related to the detrimental effects of stress on both cognition (e.g. memory loss) and mood (depressive-like pathology). Specifically, recent studies from our team demonstrated that chronic stress and/or GCs treatment cause Tau hyperphosphorylation in different phospho-epitopes while in parallel, triggers intracellular translocation of different Tau isoforms to the dendrites and dendritic synapses, a phenomenon called synaptic missorting of Tau that leads to dendritic and synaptic atrophy[101]. Indeed, previous studies from AD field also suggest that Tau hyperphosphorylation is implicated in cytoskeletal imbalances and cytoskeletal pathology exhibiting synaptic loss and dendritic atrophy and, thus leading to memory impairment (Figure 10)[3,101,138]. In line with animal studies, brain post-mortem analysis of AD patients revealed a correlation between the extent of Tau hyperphosphorylation, synaptic loss and impairments in memory and executive functions[138,143]. Altogether, these findings suggest that chronic stress and AD share some neurobiological mechanisms, highlighting Tau and its hyperphosphorylation as a key regulator of neuroplastic changes related to neuronal and synaptic atrophy[138,144,145].

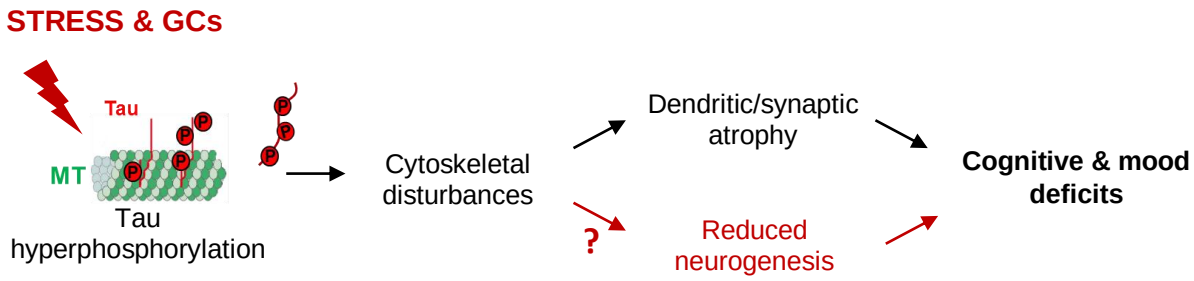


Figure 11 – Hypothetical model of this Master study. *Previous studies from our lab have shown that stress and GCs trigger Tau hyperphosphorylation which in turn, evoke cytoskeletal disturbances related to dendritic atrophy and synaptic loss leading to cognitive and mood deficits[101,138]. Thus, based on the role of Tau on cytoskeletal dynamics and plasticity, these Master studies will monitor the potential involvement of Tau in the stress-induced suppression of DG neurogenesis in adult brain (drawn by C. Dioli & I. Sotiropoulos).*

Based on the essential role of Tau protein on cytoskeletal stability and dynamics and the mentioned role in neuroremodelling induced by stress, this Master thesis aims to monitor the potential involvement of Tau on the mechanisms through which chronic stress suppresses hippocampal neurogenesis in the adult brain (Figure 11).

2

Objectives

2. OBJECTIVES

Accumulating evidence suggests that chronic stress and Alzheimer's disease pathology share common neurobiological mechanisms highlighting Tau and its malfunction as a converging protein between these pathologies[4]. Previous studies show that chronic stress triggers Tau hyperphosphorylation relating it to cognitive and mood deficits induced by chronic stress[142,138], while recent work suggests that absence of Tau protein could block detrimental impact of stress in neuronal structure and function[3].

As Tau is a cytoskeletal protein involved in many cellular processes, e.g. cell proliferation and neuronal differentiation, this Master study aims to explore the potential role of Tau on the mechanism through which chronic stress damages neurogenesis and gliogenesis in adult hippocampus answering to the below objectives:

I. Monitor the impact of Tau loss in newborn cells as well as their differentiation in neurons and glia in adult hippocampus.

II. Investigate the potential role of Tau in deficits of cell proliferation and neuronal and glial differentiation induced by exposure to chronic stress

III. Clarify the cellular cascades underlying the stress-driven neurogenic damage and verify whether similar stress-driven pathways operate in the reduction of newborn neuronal and glia populations.

3

Material and Methods

3. MATERIAL AND METHODS

3.1. ANIMALS

6-7 months-old male Tau knockout animals (Tau-KO)[109] and their wildtype littermates (WT) (C57BL/6J background) were used in this study. 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 ◦C; relative humidity of 55%, ad libitum access to food and water). 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. All experiments were conducted in accordance with the Portuguese national authority for animal experimentation, Direcção Geral de Veterinária (ID: DGV9457).

3.1.1. EXPERIMENTAL DESIGN

Mice of both genotypes were divided in the following experimental groups (Figure 12): i) control, non-stressed animals (CON; N=10 per genotype per experimental replicate) and, ii) stressed groups (STR; N=10 per genotype per experimental replicate); the whole experiment has replicated twice. Stressed mice of both genotypes were submitted to a nine-week protocol of chronic unpredictable stress while control animals remained undisturbed in their cages. During the last week of this protocol, CON and STR animals performed a battery of behavioral tests and one day after the last behavioral testing, they were sacrificed (Figure 12). For assessment of cell proliferation, six animals of each experimental group were injected with 5-Bromo-2′-deoxyuridine (BrdU; 50mg/kg/day for three consecutive days) before sacrifice (Cohort A). For monitoring cell survival and differentiation, six animals were injected with BrdU (50mg/kg/day for 3 consecutive days) four weeks before sacrifice (Cohort B) (Figure 12). Seven- eight animals from each experimental group were used for molecular analysis.

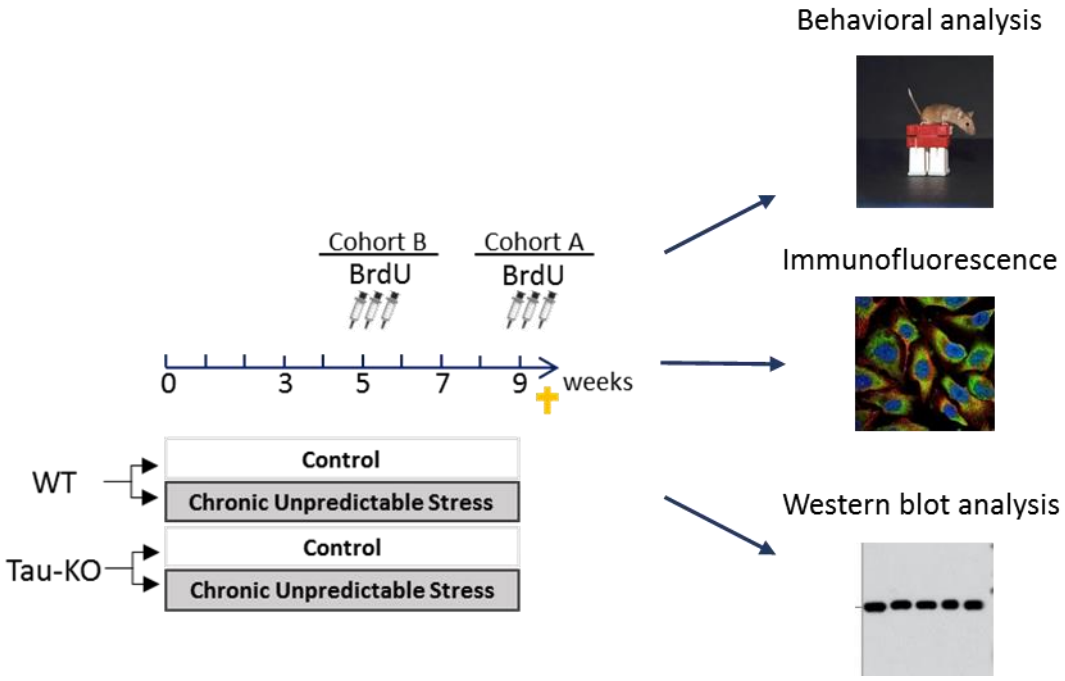


Figure 12 - Experimental groups and timeline. *Illustration of the experimental timeline showing the nine weeks-long stress protocol as well as the two time points of injections with 5-Bromo-2'-deoxyuridine (BrdU; 50mg/kg/day for three consecutive days). Specifically, some animals of each group were injected with BrdU at the end of nine weeks period (just before sacrifice; Cohort A) while other animals were injected with BrdU five weeks after the initiation of stress (four weeks before sacrifice; Cohort B). At the end of week 8 all the animals performed behavioral tests. After sacrifice, brains were used for immunofluorescence and Western Blot analysis.*

3.1.2. STRESS PROTOCOL

Chronic Unpredictable Stress (CUS) protocol included four different stressors (overcrowding (3h); restraint (3h); vibrating plate (3h) and hair dryer (30 min)) and was applied during nine sequential weeks. To prevent habituation, a single, randomly chosen, stressor was applied in a daily basis at a different time of the day[138,146]. Animal body weight and serum corticosterone (CORT) levels were measured as indicators of stress efficacy as exposure to stressful conditions is known to reduce body weight in rodents as well as to increase circulating levels of CORT[138,146]. At the end of the stress protocol, blood was collected from all animals at 8:00 p.m. and 8:00 a.m., centrifuged for 10 min at 13000 rpm and serum was isolated and stored at -80 °C. Serum CORT levels were measured using a radioimmunoassay kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

3.1.3. BEHAVIORAL ASSESSMENT

Before performing the behavioral tests, CON animals were daily handled for eight days to reduce potent, unwanted testing stress (e.g. transportation and handling). Three different behavioral tests were used to assess cognitive and emotional performance: i) Novel Object Recognition test monitoring long-term recognition memory ii) Y-maze test for assessment of short-term spatial memory iii) Ultrasonic Vocalizations for measuring overall emotional status.

Novel Object Recognition test

Novel Object recognition (NOR) test lasted five days and was performed in an arena consisted of a white rectangular box (33cm x 33cm x 33cm). Animals were placed inside the empty arena (with no objects to explore) for 20 minutes for three consecutive days for habituation. In the following day, animals were placed in the test arena, which contained two identical objects (equally distant) and were allowed to explore the objects for 10 min; then, the animals were returned to their home cage. In the fifth day, the animals were again placed in the arena and were presented to one new (novel) object and one old (familiar) object for 10 min; both objects were generally consistent with height and volume but they were different in shape and appearance. Each animal was placed equidistant from the two objects and was recorded by a camera on the top of the experimental room. Videos were analyzed with the Kinoscope software (http://sourceforge.net/projects/kinoscope/) and the time spent exploring each object was measured. As rodents exhibit prefer to interact with the novel object, the discrimination index between novel and familiar object was used as an index of recognition memory. The index was calculated based on the formula DI = (N – F) / (N + F).

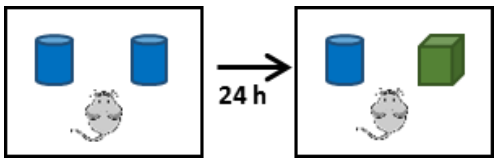


Figure 13 – Novel Object Recognition (NOR) test*. After habituation in empty area for three sequential days (20 min per day), each animal was placed in the testing arena (10 min), which contained two identical objects. At the following day, the animal was placed to the same area containing an old (familiar) and one new (novel) object for 10 min. Animal behavior was recorded by a camera and the time that the animals explored each object was analyzed (drawn by C. Dioli).*

Y-maze Test

For monitoring short-term memory, a Y-maze test was used. The Y-maze apparatus consists of a start, familial and a novel arm while there are spatial references marks on the wall of the experimental room. During the first trial (10 minutes), each animal was allowed to explore the start and familial arm of the apparatus and then return to its home cage. After sixty minutes, the animal was placed back in the Y-maze apparatus with access to all, three, arms and was allowed to freely explore for 5 minutes. Animal behavior was recorded and analyzed by video tracking system and software (Viewpoint, Champagne-au-Mont-d'or, France). The time and frequency of animal visits in the novel arm were used as indexes of short-term spatial memory.

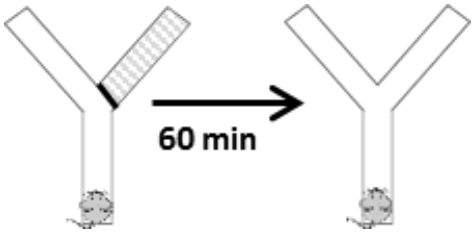


Figure 14 - Y-maze test. *Each animal was placed in the start arm of the Y maze apparatus and allowed to freely explore for 10 min the two arms (start and familiar; down and left arm in the scheme). Then, animal returned in its home cage. Sixty minutes later, the animal was presented to the start arm and left to explore the three arms for 5 min (drawn by C. Dioli).*

Ultrasonic vocalizations

For monitoring overall emotional status, measurement of ultrasonic vocalizations (USVs) was performed as previously described[147,148,149]. Briefly, a female mouse was placed in a mouse's (male) cage that was previously isolated for 24 hours. The female-induced USVs were recorded using the Avisoft-Recorder (version 5.1.04) and manually analyzed with AvisoftSASLab Pro (version 5.1.22, Avisoft Bioacoustics).

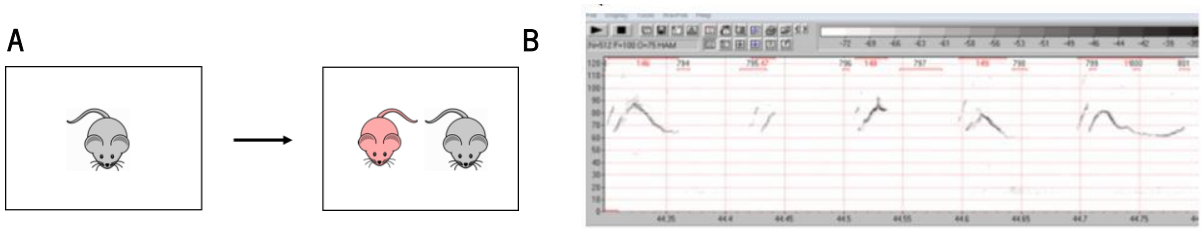


Figure 15 – USV test and analysis. *A) In a cage of a previously single-house male animal (left part of scheme), we placed a young female animal (right part of scheme) and ultrasound vocalizations (USVs) were recorded for 15 minutes B) The icon show examples of USV's recordings and the corresponding spectrograms with frequency resolution ~1.2 kHz and temporal resolution ~0.4ms. The total amount of USVs was used as an index of overall emotional status.*

3.2. BRAIN TISSUE COLLECTION

After behavioral testing, animals that were injected with BrdU were deeply anaesthetized [ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg)] and transcardially perfused with 0, 9% saline solution followed by 4% paraformaldehyde (PFA). After careful removal from the skull, brains were post-fixed with 4% PFA for 2h (RT) and transferred to 30% sucrose solution for two overnights (4 ◦ C) (Figure 16). Later, the brains were divided in two hemispheres and the left part was kept at 1x PBS (with sodium azide) at 4 ◦C, until being cut in the vibratome. The right part was frozen (using Optimal Cutting Temperature compound (OCT)) and kept in -20 ◦C, until being cut in the cryostat. The remaining animals were killed by decapitation for molecular analysis. Brains were collected immediately and dentate gyrus was macrodissected and stored at -80°C until further analysis.

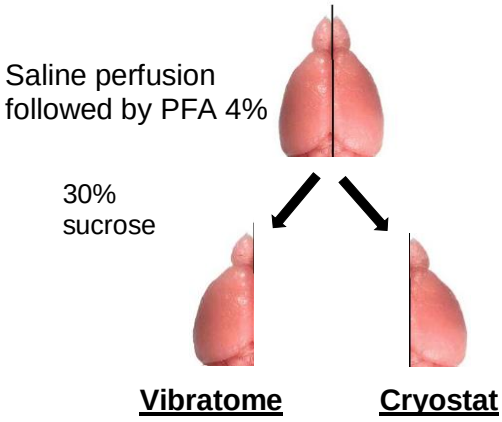


Figure 16 – Schematic representation of brain tissue collection for immunofluorescence analysis. *Anesthetized animals were sacrificed with saline perfusion followed by PFA 4% perfusion. The whole brain was placed in 30% sucrose for two overnights (4◦C) and then, was cut in two part (hemispheres). Left hemisphere was placed in PBS and was cut in vibratome, while the right hemisphere was placed in OCT and cut in cryostat (drawn by C. Dioli).*

3.2.1. IMMUNOSTAINING

Coronal cryosections (20 μm) and/or vibratome sections (50 μm) of brain were used for immunofluorescent (IF) analysis. For monitoring proliferation, we performed BrdU and Ki67 double staining in mice of Cohort A. Briefly, free floating sections were placed in hydrochloric acid (HCl) for 30 min and then in fetal bovine serum (FBS) 10 % for 30 min for blocking. Next, were incubated for one overnight with BrdU (1:200; Abcam, Cambridge, UK) / Ki67 (for proliferating cells; 1:500; Merck Millipore, Darmstadt, Germany) Ab's diluted in PBS-Triton 0, 5% buffer. At the following day, the sections were incubated with secondary Ab's: Alexa Fluor anti-rat 488 (for BrdU; Invitrogen) and Alexa Fluoer anti- rabbit 594 (for Ki67; Invitrogen) in room temperature (RT) for 2 h (all secondaries were used in 1:1000). Then, sections were stained with 4',6-diamidino-2-phenylindole (DAPI) (1:1000) diluted in PBS-Triton 0, 5% for 10 min. During the whole protocol, PBS 1x was used in all washing steps. For measuring the number of neuroblasts in mice of Cohort A, we performed BrdU and DCX double staining. For that staining, cryosections were placed in citrate buffer (pH=6) for 15 min in 95 ◦C for antigen retrieval, then in HCl for 30 min, followed by incubation in FBS 10 % for 30 min. Then, sections were incubated for two overnights with DCX (for neuroblasts; 1:250; Santa Cruz Biotechnology, Dallas, Texas, U.S.A) Ab diluted in PBS-Triton 0, 5% buffer. At the next step, sections were incubated with the secondary Ab: Alexa Fluor anti-goat 568 in RT for 2 h. Next, after incubation with FBS 10 % for 30 min, sections were presented to BrdU (1:200, overnight). At the next day, Alexa Fluor anti- rat 488 was used as secondary Ab (RT, 2 h). At the end, sections were stained with DAPI (1:1000) diluted in PBS-Triton 0, 5% buffer. PBS-Triton 0, 5% buffer was used in all washing steps. For measuring the number of newborn astrocytes, we used cryosections from mice of Cohort A and stained them with BrdU and GFAP. Section were placed in citrate buffer (pH=6) for 15 min in 95 ◦C, then in HCl for 30 min, followed by incubation in FBS 10 % for 30 min. Then, sections were incubated for one overnight with BrdU (1:200)/ GFAP (for glia; 1:200; Dako, Glostrup, Denmark) Abs diluted in PBS-Triton 0, 5% buffer. At the following day, sections were incubated with the secondary Ab: Alexa Fluor anti-rat 488 (for BrdU) and Alexa Fluor anti- rabbit 594 (for GFAP) in RT for 2 h. At the last step, sections were stained with DAPI (1:1000) diluted in PBS-Triton 0, 5% buffer. PBS-Triton 0, 5% buffer was used in all washing steps. For PHF1 staining, we used sections of Cohort A mice that were placed in citrate buffer (pH=6) for 15 min in 95 ◦C, then blocked in V- block for 5 min followed by incubation for two overnights with BrdU (1:200)/ PHF1 (1:200; detecting phosphorylated forms of Tau at Ser396/404 epitopes; kind gift from Dr Peter Davies, Albert Einstein College, NY, U.S.A.) diluted in PBS-Triton 0, 5% buffer. Then,

sections were incubated with the secondary Ab Alexa Fluor anti-rat 647 (for BrdU) and Alexa Fluor anti-mouse 594 (for PHF1) in RT for 2 h. At the last step, sections were stained with DAPI (1:1000) diluted in PBS-Triton 0, 5% buffer.

For detection of newly-born neurons, we used vibratome sections (50 μm) from Cohort B mice. Sections were incubated overnight with NeuN (for mature neurons; 1:100; Merck Millipore, Darmstadt, Germany) after their blocking with FBS 10% (30 min). At the following day, sections were incubated with Alexa Fluor anti-rabbit 647 and then, were placed in HCl for 30 min followed by incubation with BrdU (1:200, overnight). At the next day, we performed Alexa Fluor anti-rat 594 secondary incubation (1:1000) followed by DAPI (1:1000). For astrocytic detection, we followed the same protocol for GFAP as described above.

For Cohort A animals, BrdU-positive cells and their double labelling with the above antibodies were counted manually within the subgranular zone of the DG. For Cohort B animals, BrdU-positive cells and their double staining with the above antibodies were also manually counted in the granular zone of the DG using confocal microscope (Olympus FluoViewTM FV1000, Hamburg, Germany). In order to assess cell densities, the corresponding DG areas were determined using an Olympus BX61 optical microscope and the Stereo Investigator v. 5.65 Newcast software (Microbrightfield, VT).

3.2.2. WESTERN BLOT ANALYSIS

After the animals' sacrifice by decapitation, brains were fast removed from the skull and dentate gyri were macrodissected and immediately frozen. Frozen 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)); extracts were cleared by centrifugation (14.000 g) and their protein contents were estimated by Bradford assay. After the addition of Laemmli buffer (250mM Tris-HCl, pH 6.8, containing 4% sodium dodecyl sulfate, 10% glycerol, 2% b-mercaptoethanol and 0.002% bromophenol blue), lysates were electrophoresed on 10-12% acrylamide gels using Biorad tetrapack gel electrophoresis apparatus. Then, proteins were transferred onto nitrocellulose membranes using the Trans-Blot® TurboTM Blotting System (BioRad). Transfer efficacy was monitored by ponseau staining of the membrane immediately after the end of the semi-dry transfer. Then, the membranes were blocked in 5% nonfat milk in TBS-Tween 0,1% buffer

before incubation with the following antibodies: PI3K p85 (1:1000, Cell signaling), mTOR (1:1000, Abcam), phospho-GSK3β pTyr216 + GSK3α pTyr279 (1:500, Invitrogen), GSK3α ,β (1:1000, Invitrogen), β- catenin (1:1000, Santa Cruz), Tau-5 (1:5000, Abcam), pThr231-Tau (1:1000, Abcam), PHF1 (1:1000, Abcam), 3R-Tau (1:1000, Millipore), 4R-Tau (1:1000, Millipore) and actin (1:5000, Abcam). After incubation with the appropriate secondary antibody, antigens were revealed by ECL (Clarity, Bio-Rad). Signal monitoring and quantification was achieved using a BIORAD ChemiDoc and ImageLab BIORAD software. All values were normalized and expressed as a percentage of control values.

3.3. STATISTICAL ANALYSIS

Numerical data are presented as group means ± SEM. All data sets were subjected to two-way ANOVA analysis (Stress and Genotype were considered as two independent factors) before application of appropriate post hoc comparisons (GraphPad v.6.01 La Jolla, CA). For molecular analysis of Tau and its phosphorylation levels, t-test analysis was performed, as only WTs animals (control and stressed ones) express Tau. Values of $p<0.05$ were considered as significant differences.

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4

Results

4. RESULTS

This study aims to clarify the role of protein Tau and its involvement in mechanisms through which chronic stressful conditions suppress cell proliferation as well as both neurogenesis and gliogenesis in adult hippocampus. For that purpose, the following studies have used mice lacking Tau protein (Tau knockout; Tau-KO) and their wildtype (WT) littermates. Animals of both genotypes were subjected to chronic unpredictable stress (CUS) for 9 weeks (see also Figure 12).

4.1. THE PHYSIOLOGICAL RESPONSE OF WT AND TAU-KO MICE TO CHRONIC STRESS IS SIMILAR

Hypothalamus-pituitary-adrenal (HPA) axis and the secreted corticosteroids are central part of stress response mechanisms while HPA axis hyperactivity, increased corticosteroid levels and subsequently altered body weight are characteristic symptoms of chronic stress. Thus, we monitored body weight and serum corticosterone levels in WT and Tau-KO animals at the end of the stress protocol. Chronically stressed animals of both genotypes exhibited decreased weight gain compared to their control (non-stressed) littermates [p_{WT} = 0.006 and p_{KO} =0.01; 2-way ANOVA Stress overall effect ($F_{1,76}$ = 21.7, $p < 0.001$; Figure 17A). Furthermore, we found an overall effect of Stress on basal circulating corticosterone (CORT) levels at the onset of the dark period (ZT12 / 8:00 p.m.; 2-way ANOVA, $F_{1,34}$ = 15.34, p = 0.0004) (Figure 17B); post-hoc analysis revealed a significant increase in CORT levels in both stressed WT (p = 0.038) and Tau-KO (p = 0.046) animals when compared to the corresponding control (non-stressed) animals. Similarly, we found a Stress effect on CORT levels monitored at the beginning of the light phase (ZT00 / 8:00 a.m.; $F_{1,34}$= 6.797, p = 0.013)

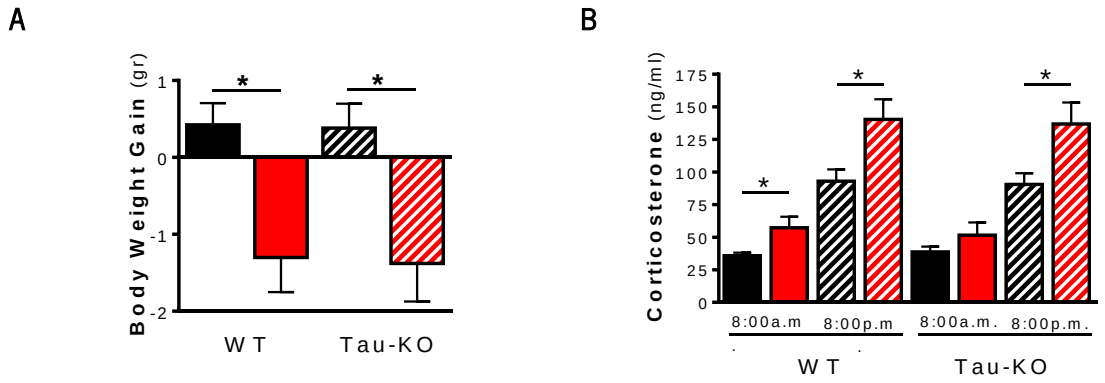


Figure 17 – Chronic stress impact on body weight and corticosterone levels of both WT and Tau-KO stressed mice*. A) Stressed (STR) animals of both genotypes exhibited reduced body weight (negative gain) as compared to their corresponding (CON) counterparts. B) Chronic stress resulted in elevated corticosterone levels in both WT and Tau-KO animals measured at 08:00 p.m. (peak of corticosterone secretion) with similar findings at 08:00a.m. (nadir time-point of corticosterone secretion) . All numerical data are shown as mean +/- SEM; * p < 0.05*

4.2. Depletion of Tau blocks the stress-evoked reduction of proliferating cells in the adult DG

For evaluating alterations in the fast proliferating cells and the number of newly-born cells in the subgranular zone (SGZ) of the dentate gyrus (DG), animals of cohort A were injected with BrdU (50mg/kg/day for three consecutive days before the sacrifice) due to its property of being incorporated into the newly synthesized DNA and labeling dividing cells (Figure 18A). Our BrdU immunofluorescence analysis (Figure 18B) in DG of WT and Tau-KO showed a clear interaction between Stress and Genotype (2-way ANOVA, $F_{1,32} = 6.538$, $p = 0.015$) of BrdU-positive cells density, with post-hoc analysis revealing a significant reduction in stressed WT ($p = 0.007$) but not Tau-KO ($p = 0.917$) animals when compared to their corresponding controls (Figure 18B). As proliferating cells in the DG remained in clusters of two to four cells in the neurogenic niches, we also monitored the number of DG clusters. Similarly to the cell density, 2-way ANOVA statistical analysis showed a Stress x Genotype interaction in the number of clusters ($F_{1,108} = 7.628$, $p = 0.060$) with stressed WT exhibiting less clusters than stressed Tau-KO ($p = 0.02$) (Figure 19). To provide an additional piece of evidence of the impact of Tau-KO on hippocampal proliferation we performed staining with the endogenous cell cycle marker Ki67 (Figure 18A). Analysis of Ki67 and BrdU double-labeled cells showed a Stress x Genotype interaction in cell density at the DG ($F_{1,26} = 9.498$, $p = 0.004$) with stressed WT animals showing a reduction in Ki67/BrdU cell density when

compared to their corresponding controls (p = 0.021). In contrast, stressed Tau-KO were not different from their control (p = 0.492) (Figure 18C). No differences were found between control animals of both genotypes (p=0.336). These findings suggest that Tau absence, while not affecting the DG proliferation per se, blocks the reducing effect of chronic stress on cell proliferation in the adult hippocampal DG.

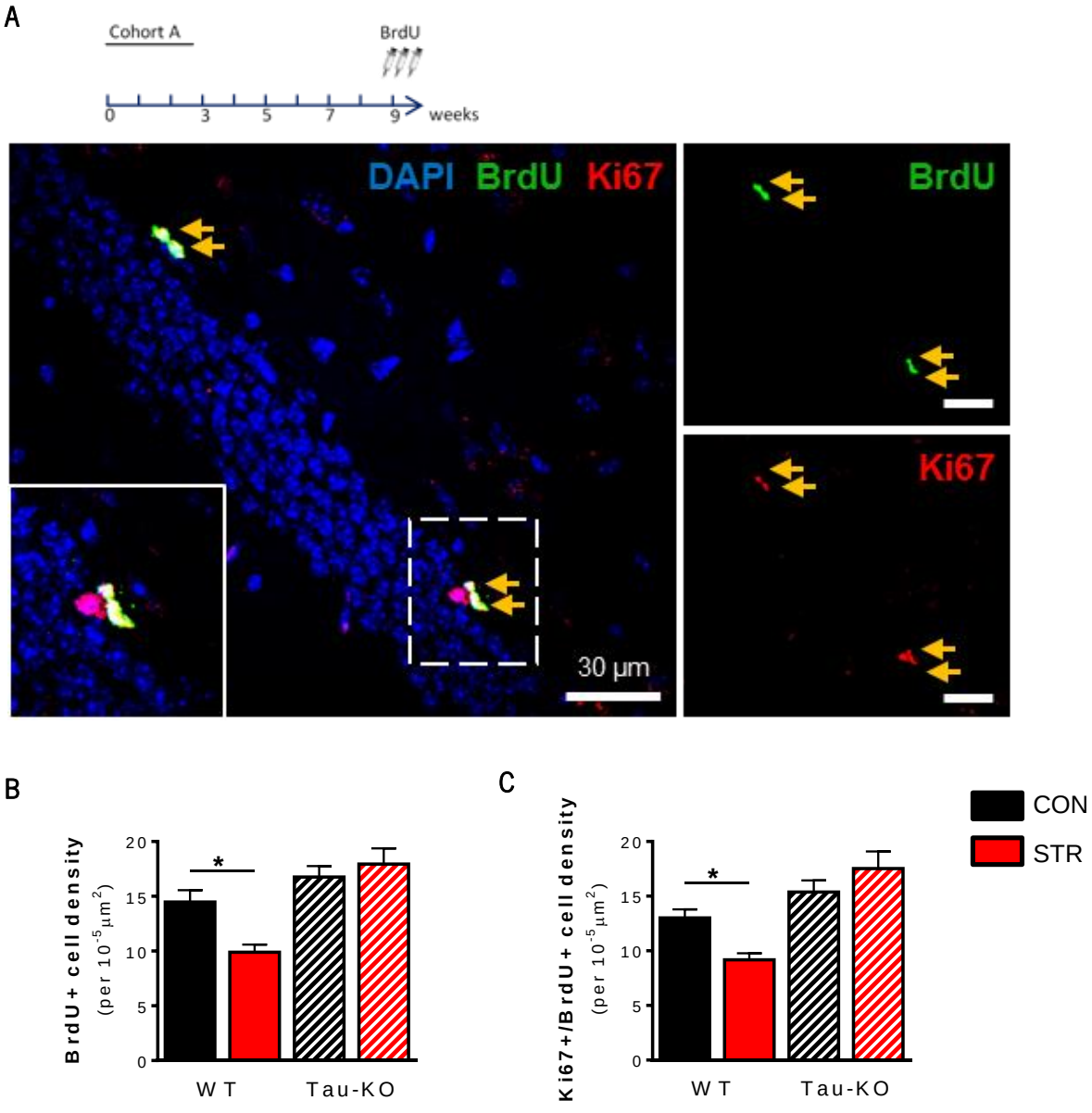


Figure 18 – Exposure to chronic stress results in reduction of proliferating cells of WT, but not Tau-KO, mice. *A) Representative microphotos of BrdU (green) /Ki67 (red) labelled cells in the hippocampal DG of animals from Cohort A (BrdU three days before sacrifice – see experimental design above the microphotos); a higher magnification of an example of BrdU/Ki67 double-labelled cells is shown in the left-down insert of panel A while single staining microphotos for BrdU (upper right) and Ki67 (down right) are also shown. B) Quantification graph of BrdU+ cells showing that exposure to chronic stress reduced the BrdU+ cell density in DG of WT animals; this stress effect was not found in Tau-KO animals. C) Similarly, density of Ki67/BrdU-positive cells was reduced in WT, but not in Tau-KO, animals by stress. All numerical data are shown as mean +/- SEM; * p < 0.05.*

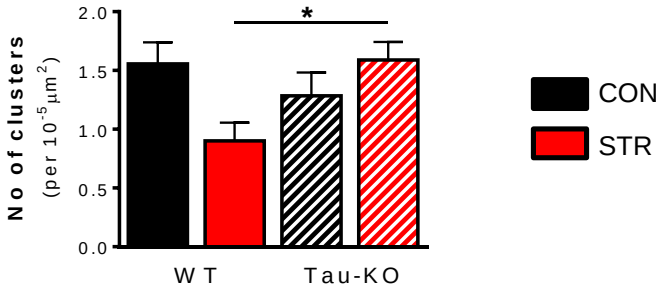


Figure 19 – Differential impact of chronic stress on clustering of BrdU-positive cells in WT and Tau-KO DGs. *Stressed (STR) WT animals exhibited reduced number of BrdU clusters in DG when compared to stress Tau-KO animals. All numerical data are shown as mean +/- SEM; * $p < 0.05$*

4.3. TAU ABLATION ATTENUATES THE STRESS-EVOKED SUPPRESSION OF NEUROGENIC, BUT NOT ASTROCYTIC, POOL IN THE ADULT HIPPOCAMPAL DG

Next, we monitored the impact of stress on the DG neuroblasts measuring BrdU-labelled cells that are co-stained with Doublecortin (DCX), a cytoskeletal protein expressed in neuroblasts, in the subgranular zone of DG (Figure 20A). 2-way ANOVA revealed an interaction of Stress x Genotype in density of DCX/BrdU double-labelled cells at the DG ($F_{1,42}$ = 5.441, p = 0.024; Figure 20B). Further analysis showed that exposure to chronic stress reduced the DG density of DCX/BrdU neuroblasts in WT animals (p = 0.04); this was not found in the DG of Tau-KO animals where stressed and control mice exhibited similar levels of this cell subpopulation (p = 0.986, Figure 20B).

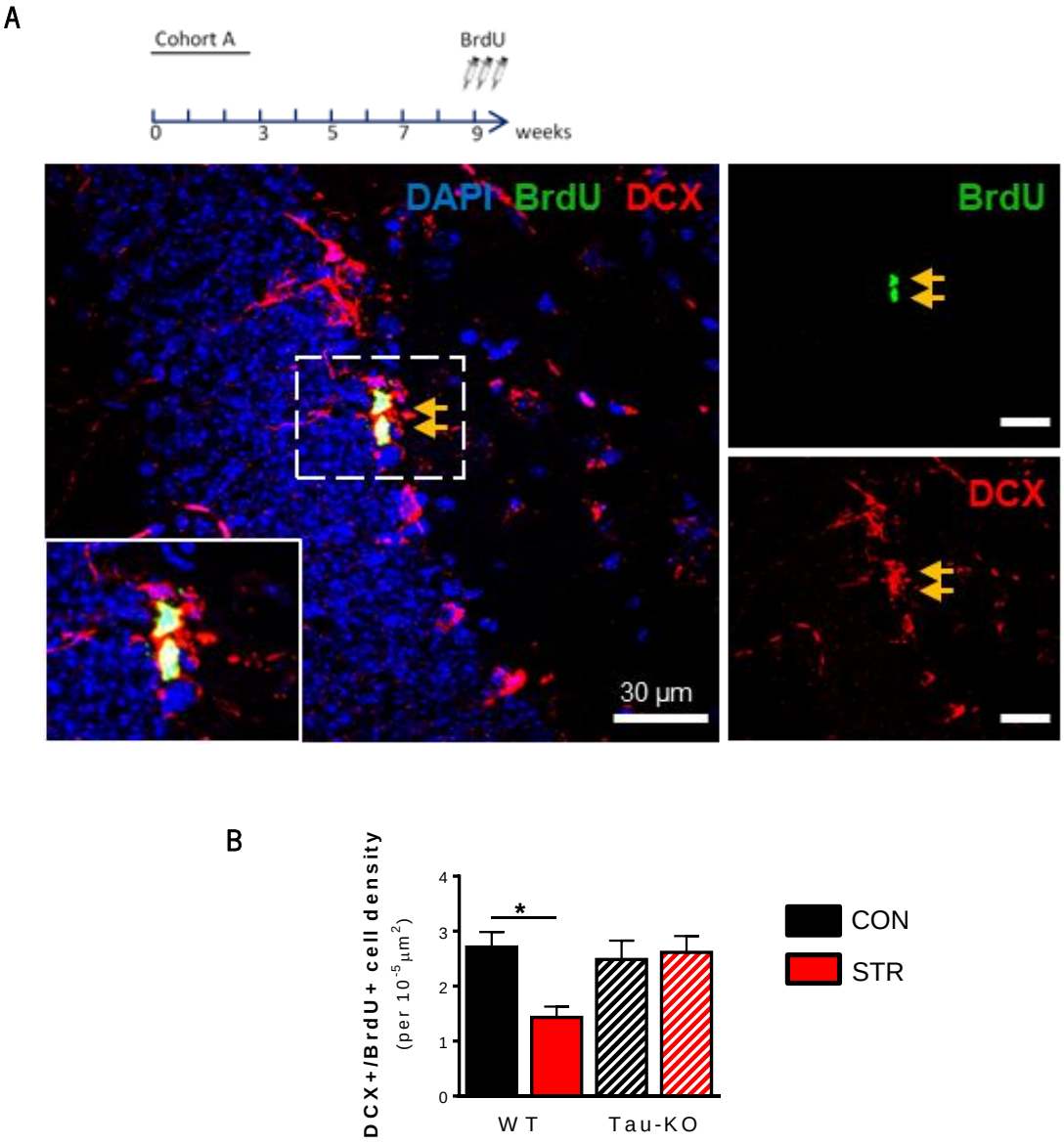


Figure 20 – Stress-induced reduction of neuroblasts in WT and not Tau-KO mice. *A) Microphotos of immunofluorescent staining of DCX (red)/BrdU (green) -positive cells in the subgranular zone of DG of animals of Cohort A. A higher magnification of an example of DCX/BrdU double-labelled cells is shown in the left-down insert of panel A while single staining microphotos for BrdU (upper right) and DCX (down right) are also presented. B) Quantification graph showing that, in contrast to Tau-KOs, WT stressed animals exhibited reduced DCX/BrdU density when compared to their control littermates. All numerical data are shown as mean +/- SEM; * p < 0.05.*

We additionally monitored the newborn astrocytic pool by counting the GFAP/BrdU double-labelled cells (Figure 21A). Interestingly, chronic stress had a similar effect on both WT and Tau-KO DG ($p = 0.035$ and $p = 0.026$, respectively), reducing the number of BrdU/GFAP cells ($F_{1,56} = 16.21$, $p < 0.001$; Figure

21B). These findings support a specific role for Tau protein in the stress-triggered reduction of neuroblasts/immature neurons in the DG.

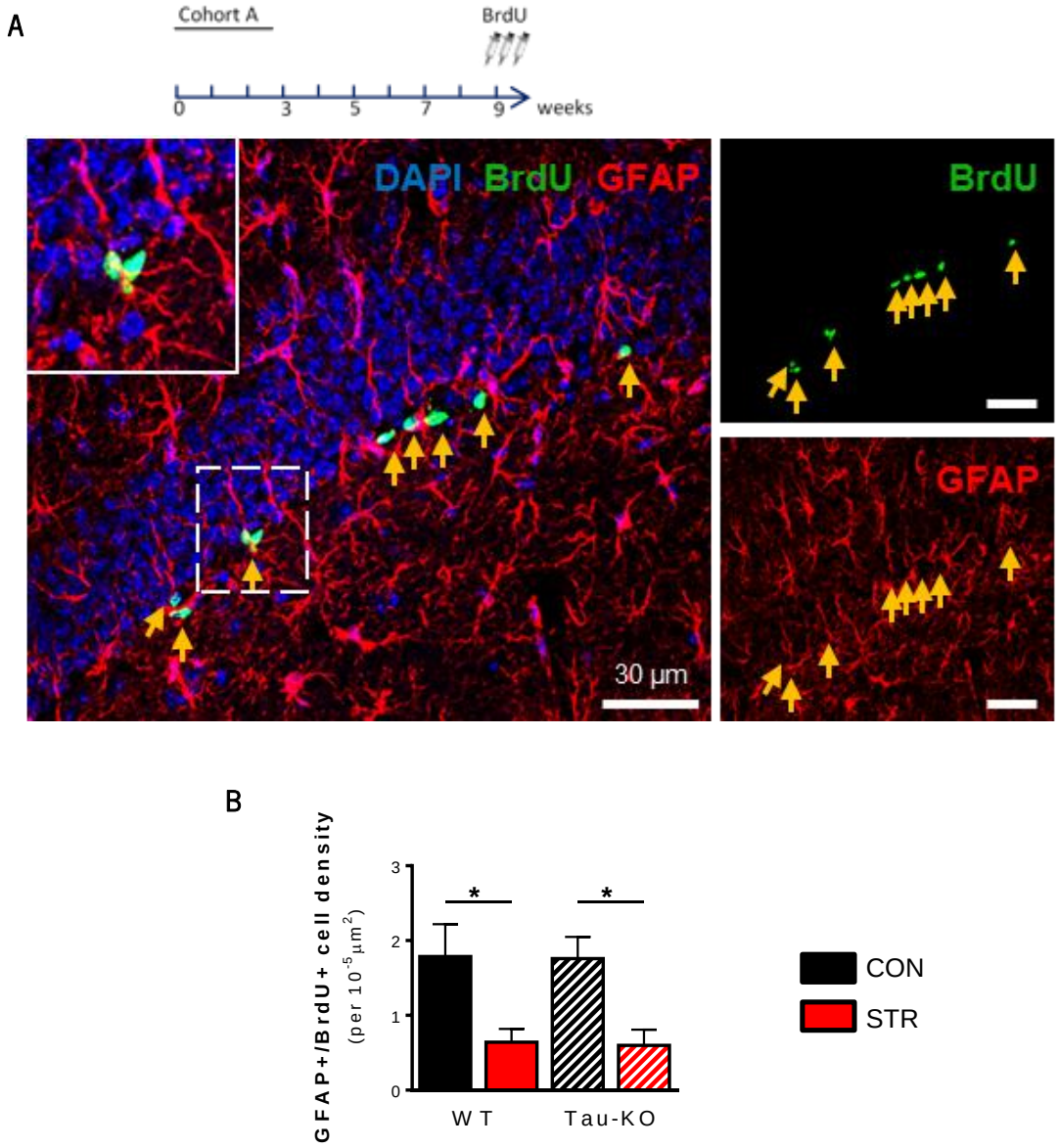
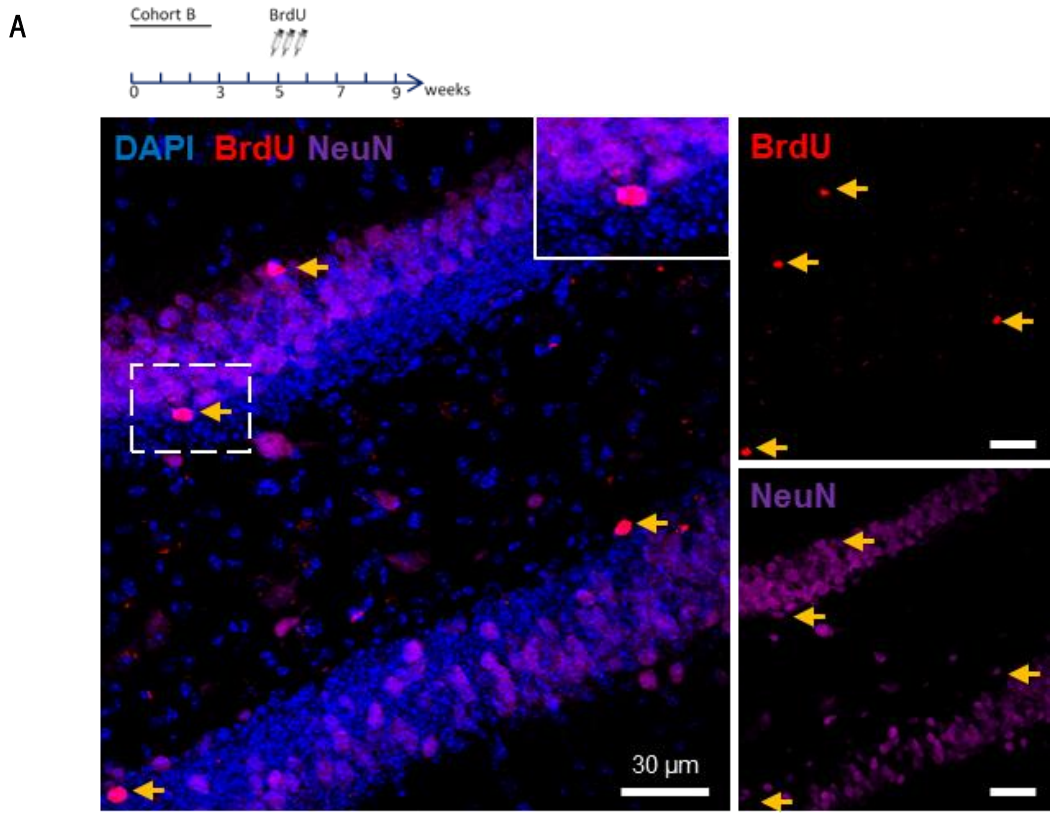


Figure 21 – Newborn astrocytes are reduced in both WT and Tau-KO stressed mice. *A) Microphotos of immunofluorescent staining of GFAP(red)/BrdU(green)-positive cellular subpopulations in the subgranular zone of DG of Cohort A animals. A higher magnification of an example of GFAP/BrdU double-labelled cells is shown in the left-up insert of panel A; single staining microphotos for BrdU (upper right) and GFAP (down right) are also shown. B) GFAP/BrdU-positive cell density was similarly decreased by stress in animals of both genotypes. All numerical data are shown as mean +/- SEM; * p < 0.05*

Next, we monitored the effect of chronic stress on the maturation and survival of newly-born neurons using a different cohort of animals that were injected with BrdU four weeks before the sacrifice (Figure 22A). In this cohort, BrdU was injected five weeks after the initiation of the chronic stress protocol and cells were analyzed four weeks later. In that way, BrdU staining allowed us to monitor the number of surviving newly-generated cells. 2-way ANOVA analysis of BrdU-stained cells in this animal cohort showed a clear interaction between Stress and Genotype in BrdU+ cell density ($F_{1,26} = 4.366$, $p = 0.046$) with stressed WT exhibiting a reduction compared to their controls ($p = 0.046$). Note that stress had no effect on Tau-KO animals ($p = 0.812$) (Figure 22B). These findings suggest that chronic stress reduces the number of newly-generated cells in the DG of WT animals whereas Tau ablation blocked this stress effect. To assess the phenotype of these BrdU-labelled cells, we performed double labeling of NeuN and BrdU or GFAP and BrdU (Figure 22A, 23A). Our statistical analysis showed a Stress x Genotype interaction on NeuN/BrdU-positive cell density (2-way ANOVA; $F_{1,26} = 4.321$, $p = 0.047$) demonstrating that chronic stress reduced the number of new neurons in WT, but not Tau-KO, DG ($p = 0.030$ and $p = 0.909$, respectively) (Figure 22C). Furthermore, GFAP/BrdU analysis showed that stress had the same impact on the number of astrocytes in both stressed groups, with 2-way ANOVA showing overall Stress effect ($F_{1,27} = 25.24$, $p < 0.0001$) and significant reduced number of GFAP/BrdU- positive cell density of WT and Tau-KO stressed animals ($p=0.009$ and $p=0.006$ respectively) (Figure 23B).



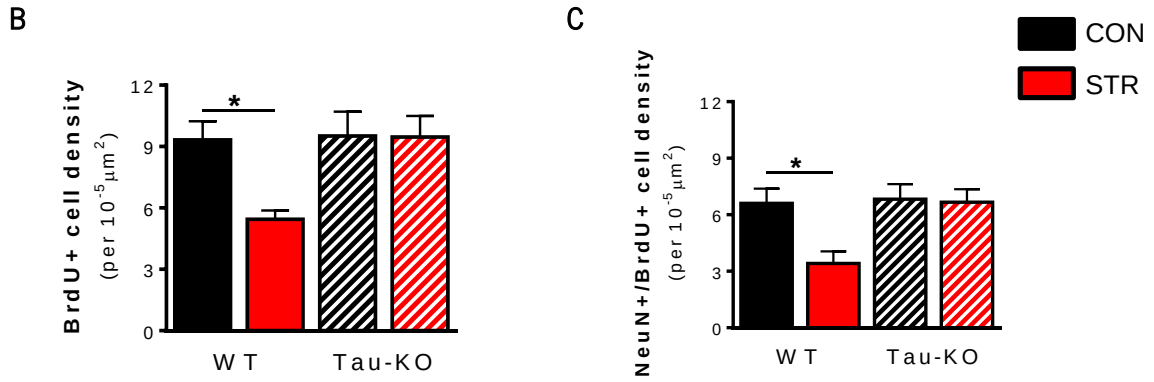
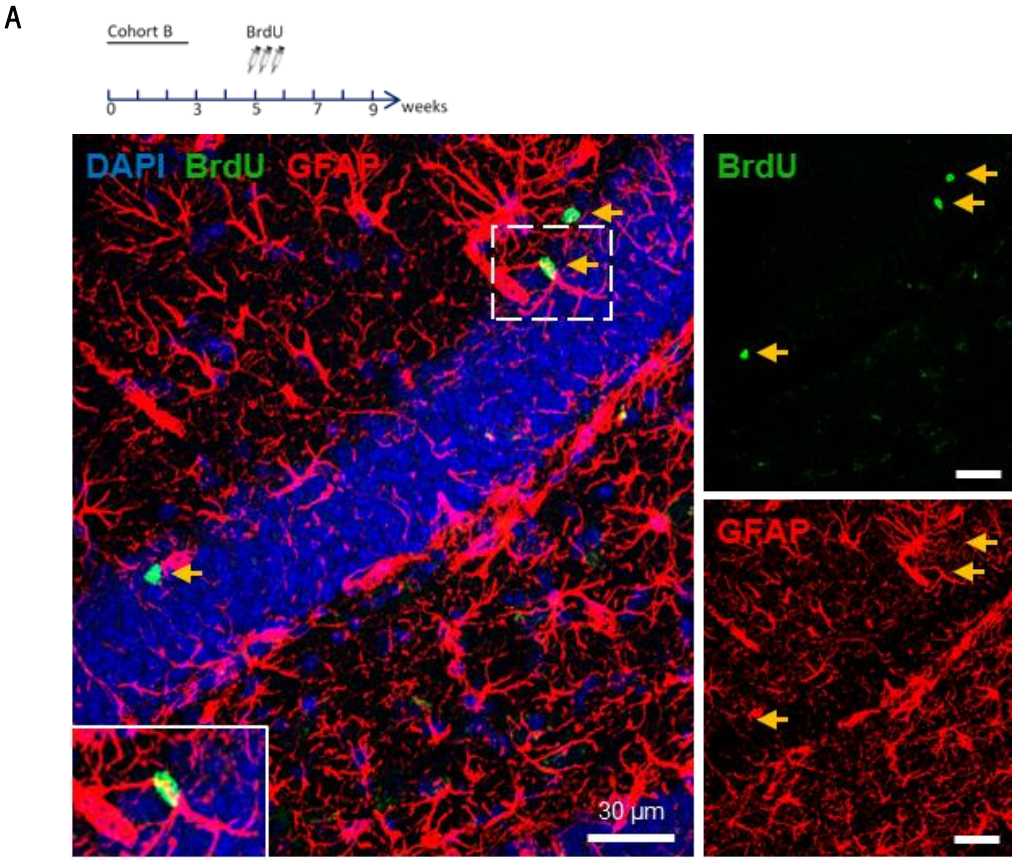


Figure 22 – Absence of Tau attenuates the stress-driven neurogenic deficits in adult DG. *A) Analysis of newly-born neurons in animals injected with BrdU four weeks before sacrifice (cohort B- see experimental design above the microphotos) based on double staining for NeuN (purple) /BrdU (red). A higher magnification of an example of NeuN/BrdU double-labelled cell is shown in the upper insert of panel A while single staining microphotos for BrdU (upper right) and NeuN (down right) are also presented. B) Stress evoked a decrease in BrdU+ cell density in WT, but not Tau-KO DG. C) Similarly, stress reduced the cell density of double labelled NeuN/BrdU subpopulation only in WT animals whereas no effect of stress was found in Tau-KO animals. All numerical data are shown as mean +/- SEM; * $p < 0.05$.*



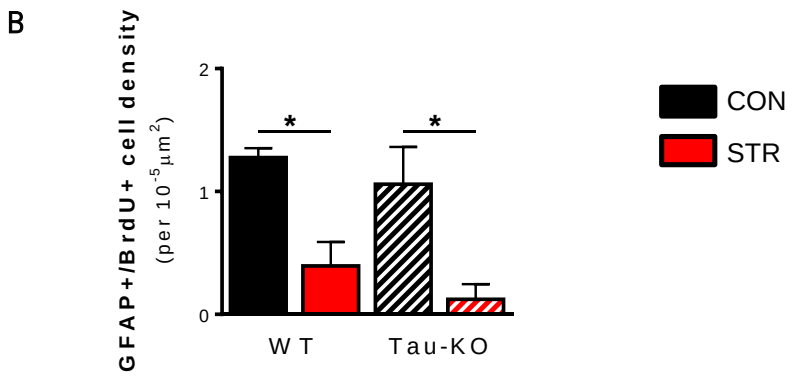


Figure 23 – Stress-induced reduction of newborn astrocytes in adult DG does not depend on Tau protein. *A) Microphotos of immunofluorescent staining of GFAP (red) /BrdU (green) -positive cellular subpopulations in the granular zone of DG of Cohort B animals. A higher magnification of an example of GFAP/BrdU* double-labelled cell *is shown in the left-down insert of panel A; single staining microphotos for BrdU (upper right) and GFAP (down right) are also shown. B) Chronic stress reduced the density of GFAP/BrdU double labelled cells in all animals independently of Tau presence. All numerical data are shown as mean +/- SEM; * p < 0.05.*

4.4. CHRONIC STRESS AFFECTS TAU PHOSPHORYLATION AND ISOFORMS IN ADULT DG

Previous studies, including some from our team, have shown that chronic stress triggers Tau hyperphosphorylation and accumulation in CA1 and CA3 hippocampal areas associating Tau malfunction with neuronal atrophy of neurons in these areas. Based on above findings, we also monitored whether chronic stress can also affect Tau phosphorylation status in DG and specifically, in newly-born cells. Thus, we perform a double IF staining for BrdU and PHF1 antibody (Figure 24A); the later recognized phosphorylated isoforms of Tau at Ser396 and Ser404 epitopes. We found that chronic stress increased the density of PHF1/BrdU positive cells in DG of WT animals ($p = 0.03$) (Figure 24B), that was also reflected in a significant increase in the percentage of PHF1 positive cells among the BrdU DG subpopulation in stressed animals ($p = 0.04$).

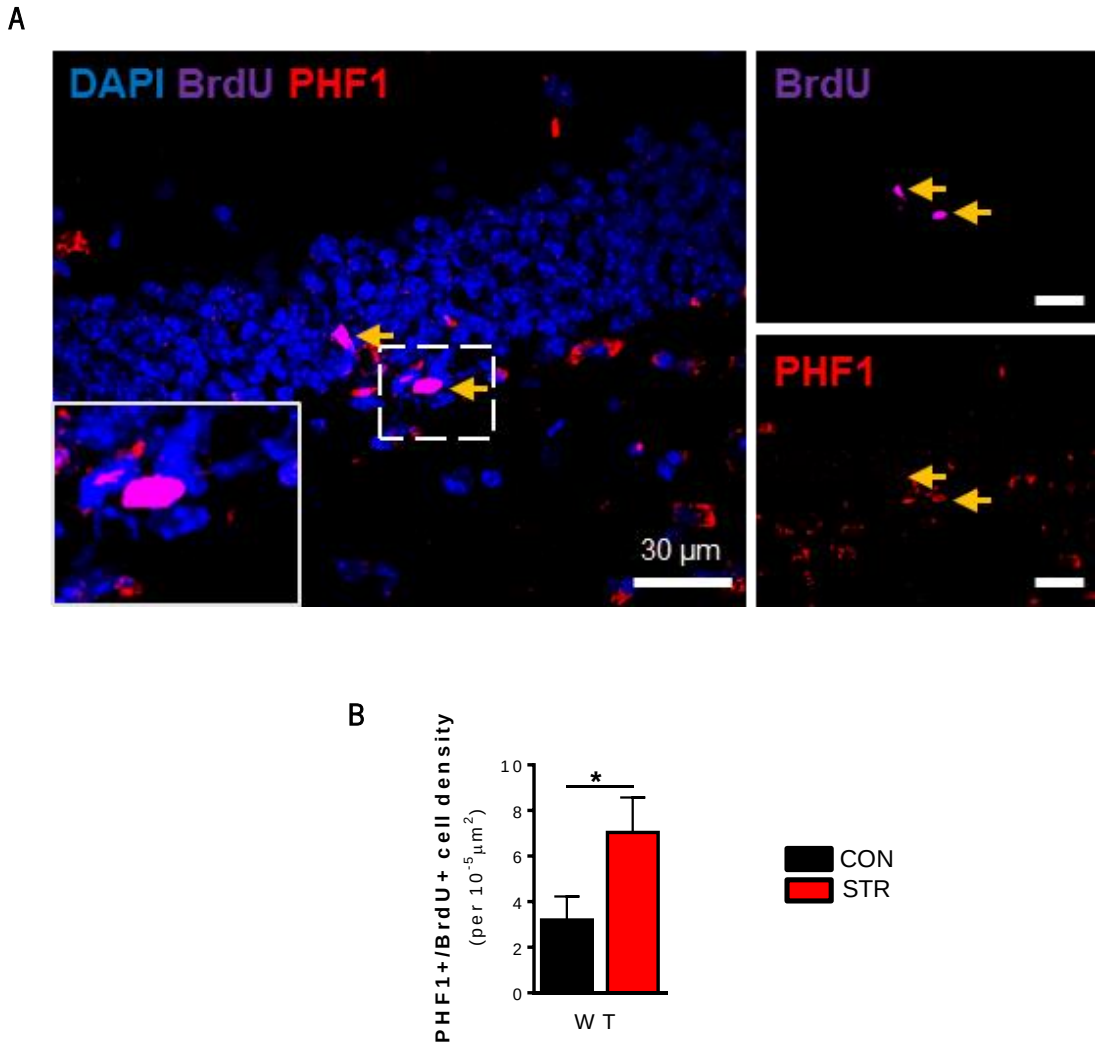


Figure 24 – Stress increases Tau phosphorylation in newly-born cells of DG. *A) Representative microphoto of PHF1 (red) /BrdU (purple) double stained cells in the subgranular zone of DG of WTs. A higher magnification of an example of PHF1/BrdU double-labelled cell is shown in the left-down insert of panel A while single staining microphotos for BrdU (upper right) and* PHF1 *(down right) are also shown. B) Quantification graph showing that exposure to chronic stressful conditions increases the density of PHF1/BrdU+ cells. All numerical data are shown as mean +/- SEM; * p < 0.05.*

We further analyzed the impact of stress on Tau phosphorylation and its isoforms by using Western Blot analysis of DG protein extracts (Figure 25). As shown at Figure 25B, chronic stress increased PHF1-detected levels of phosphorylated Tau ($p = 0.02$) confirming our IF-based findings (see also Figure 24B) while, as expected, Tau-KO animals have no signal (right part of Figure 25A). In addition, we also monitored another antibody detecting Tau phosphorylated at pThr231 epitopes, known to be increased in AD (Figure 25A)[138]. Chronic stress also increased Tau phosphorylation in this epitope ($p = 0.03$) (Figure 25B) while total Tau levels were not significantly altered by stress (Figure 25C). We next

monitored the two different isoforms of Tau which are known to be developmentally regulated[150]. Measurement of 3R-Tau, characteristically expressed in neuroblasts and immature neurons, showed that chronic stress significantly reduced its expression (p =0.02) while levels of 4R-Tau, mainly expressed in mature neurons, were increased in WT DG (p = 0.03) (Figure 25C).

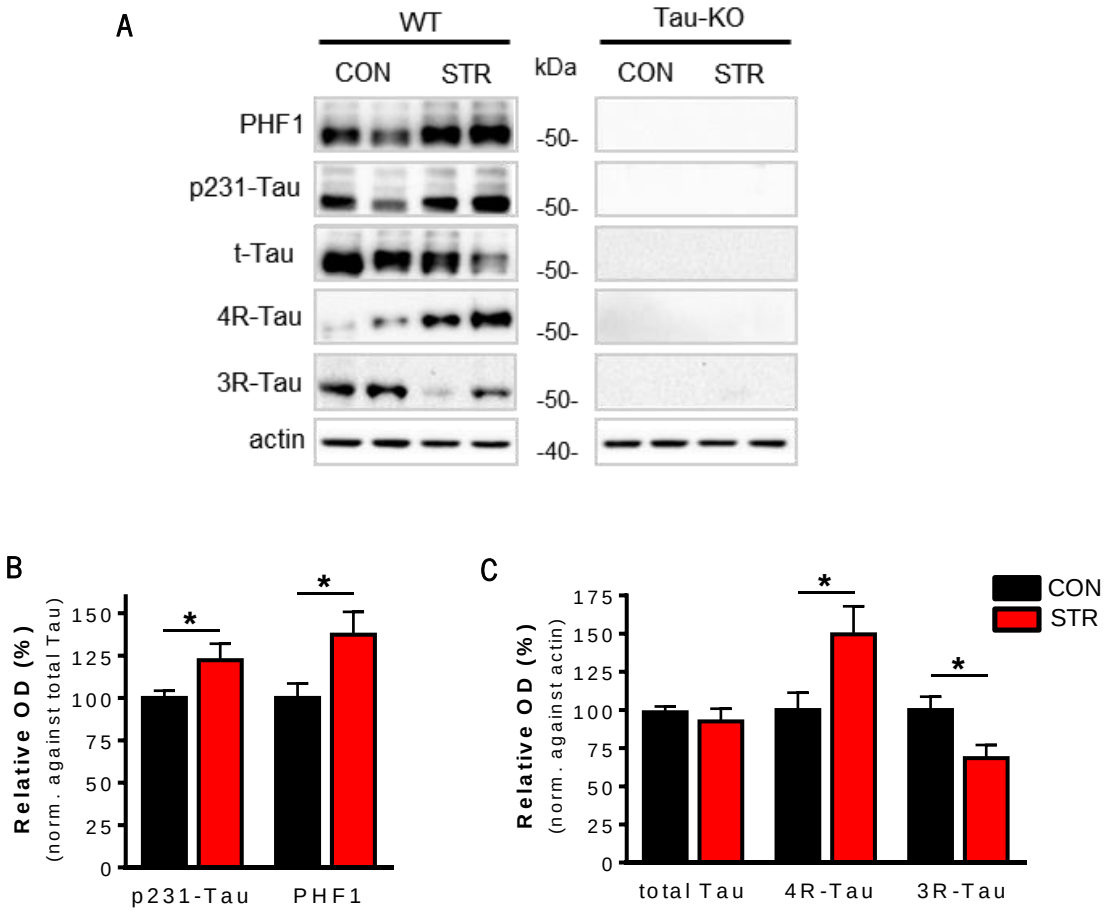
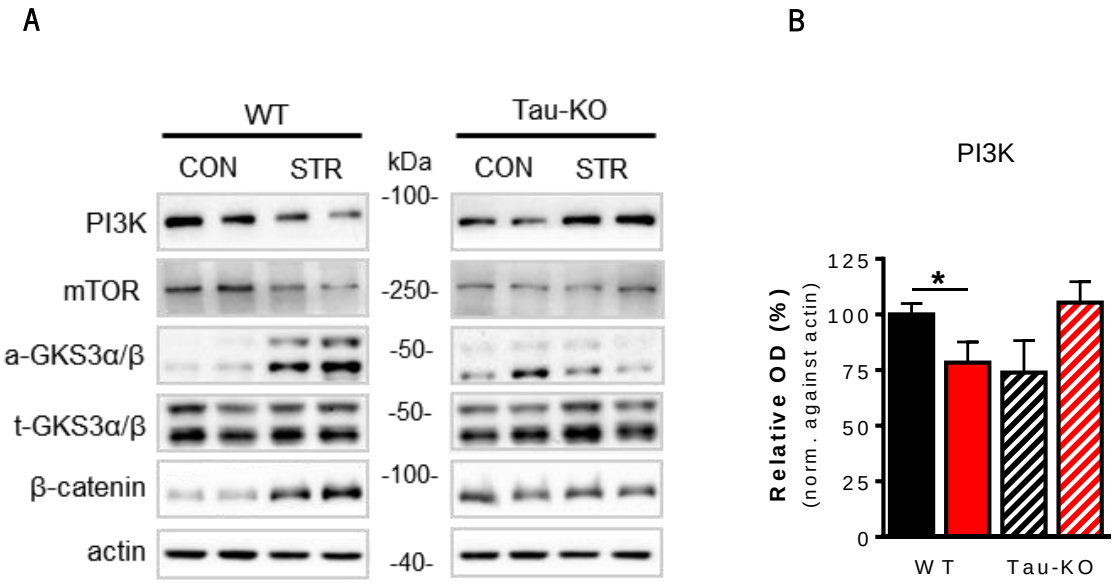


Figure 25 – Chronic stress triggers increased Tau phosphorylation and affects Tau isoforms in DG of WT animals
*A) Representative blots of different phosphorylated forms of Tau as well as 4R- and 3R-tau isoforms. B) Quantification analysis showed that chronic stress increased Tau phosphorylation at Thr231 and Ser396/404 (PHF1) epitopes in WT DG. C) While total Tau (t-Tau) levels are not altered by stress, levels of 4R-Tau and 3R-Tau isoforms were increased and decreased, respectively in stressed DG of WT animals. All numerical data are shown as mean +/- SEM; * p < 0.05.*

4.5. TAU- DEPENDENT SUPPRESSION OF PI3K /MTOR /GSK3Β/Β- CATENIN NEUROPLASTIC SIGNALING BY STRESS

We next monitored the PI3K/mTOR/GSK3β/β-catenin molecular pathway, known to regulate cell survival and proliferation in the adult DG[135,151,152]. Molecular analysis of the DG revealed that chronic stress decreased PI3K protein levels in WTs ($p = 0.04$) while there was a tendency to increase it in Tau ($p = 0.07$) [2-way ANOVA Stress x Genotype interaction ($F_{1,42} = 8.373$, $p = 0.006$)] (Figure 26B). Furthermore, chronic stress decreased the levels of mTOR in WT ($p = 0.0146$) but had no effect on Tau-KO DG [Stress x Genotype interaction ($F_{1,42} = 4.206$, $p = 0.046$] (Figure 26C). On the other hand, exposure to chronic stress increased levels of pTyr216-GSK3β in WTs ($p = 0.026$) followed by corresponding increased cytoplasmic levels of β- catenin in WTs only ($p = 0.026$) (Stress x Genotype interaction ($F_{1,42} = 7.016$ $p = 0.011$)] (Figure 26D, E). Note that these stress-evoked changes were not found in Tau-KO animals suggesting that absence of Tau is involved in activation of GSK3β (Figure 26D). Altogether, the above findings indicate that chronic stress suppresses the PI3K-driven cellular cascade that leads to reduced mTOR levels and simultaneously increased levels of active GSK3β; both molecules regulation is shown to diminish cell proliferation and survival[135,151,152]. In contrast, the absence of Tau seems to confer resilience to the above stress-induced cellular cascades that may preserve cell proliferation and survival levels in the DG.



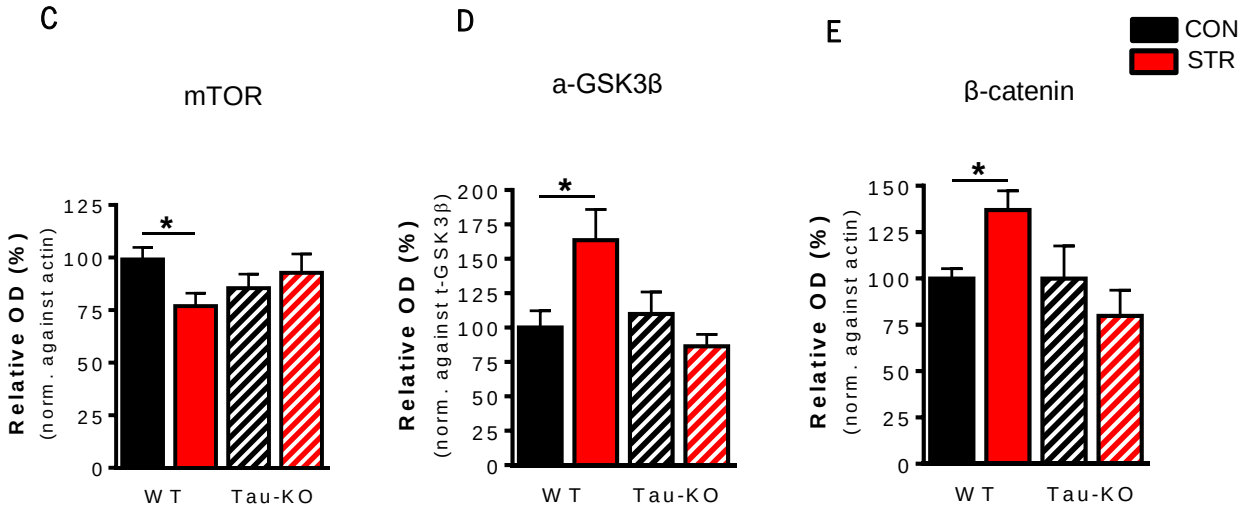


Figure 26 – Tau-dependent suppression on PI3K/mTOR/GSK3ß/β- catenin pathway in stressed DG. *A) Representative blots and B-E) quantification of the cellular cascade(s) analyzed in DG of WT and Tau-KO animals. Chronic stress reduces the expression of PI3K (B) and mTOR (C) protein levels followed by increased levels of (active) pTyr216-GSK3β (a- GSK3β) (D) and β-catenin (cytoplasm) (E) in DG of WT, but not Tau-KO, animals. All numerical data are shown as mean +/- SEM; * p < 0.05.*

4.6. CHRONIC STRESS IMPAIRS COGNITIVE AND EMOTIONAL STATUS IN WTS BUT NOT TAU-KOS

As deficits in hippocampal neurogenesis are previously shown to have a critical role in alterations of cognitive as well as mood behavior, we next monitored cognitive performance and emotional status in control and stressed animals of both genotypes. For monitoring short-term memory, we performed Y-maze test. 2-way ANOVA analysis revealed an interaction between Stress and Genotype on duration and frequency that animals visited the novel arm of the Y-maze apparatus [dur $F_{1,40}$ = 4.879, p = 0.033; frequ $F_{1,40}$ = 4.985, p = 0.031] (Figure 27Ai,ii). Further analysis showed that chronic stress reduced the duration and frequency of novel arm visits in WT animals (p_{dur} = 0,019; p_{frequ} = 0,010) but not in Tau-KO animals (p_{dur} = 0.999; p_{frequ} = 0,888) (Figure 27Ai, ii). The above findings were in agreement with our findings at Novel Object Recognition (NOR) test where we monitored long-term recognition and discrimination memory. We found that stressed WT animals exhibited reduced discrimination index compared to their corresponding controls (p = 0.035) whereas stressed and control Tau-KOs exhibit no differences (p = 0.861) [2-way ANOVA Stress x Genotype interaction ($F_{1,70}$ = 6.122, p = 0.015)] (Figure 27B). Next, we also monitor overall emotionally of our animals monitoring the Ultrasonic vocalizations

(USVs) that animals emit as an index of overall emotional status. We found that exposure to chronic stress differentially affected overall emotional status in WTs and Tau-KOs. Specifically, 2-way ANOVA showed a Stress x Genotype interaction of the number of female-induced USVs ($F_{1,34}$ = 5.010, p = 0.031) while further analysis revealed that stressed WTs showed less USVs compared to their controls (p = 0.042) (Figure 27C). Importantly, Tau-KO USVs were not altered by exposure to chronic stress (p = 0.294) and no differences were found between WT and Tau-KOs of both genotypes in all the above behavioral parameters tested (Figure 27).

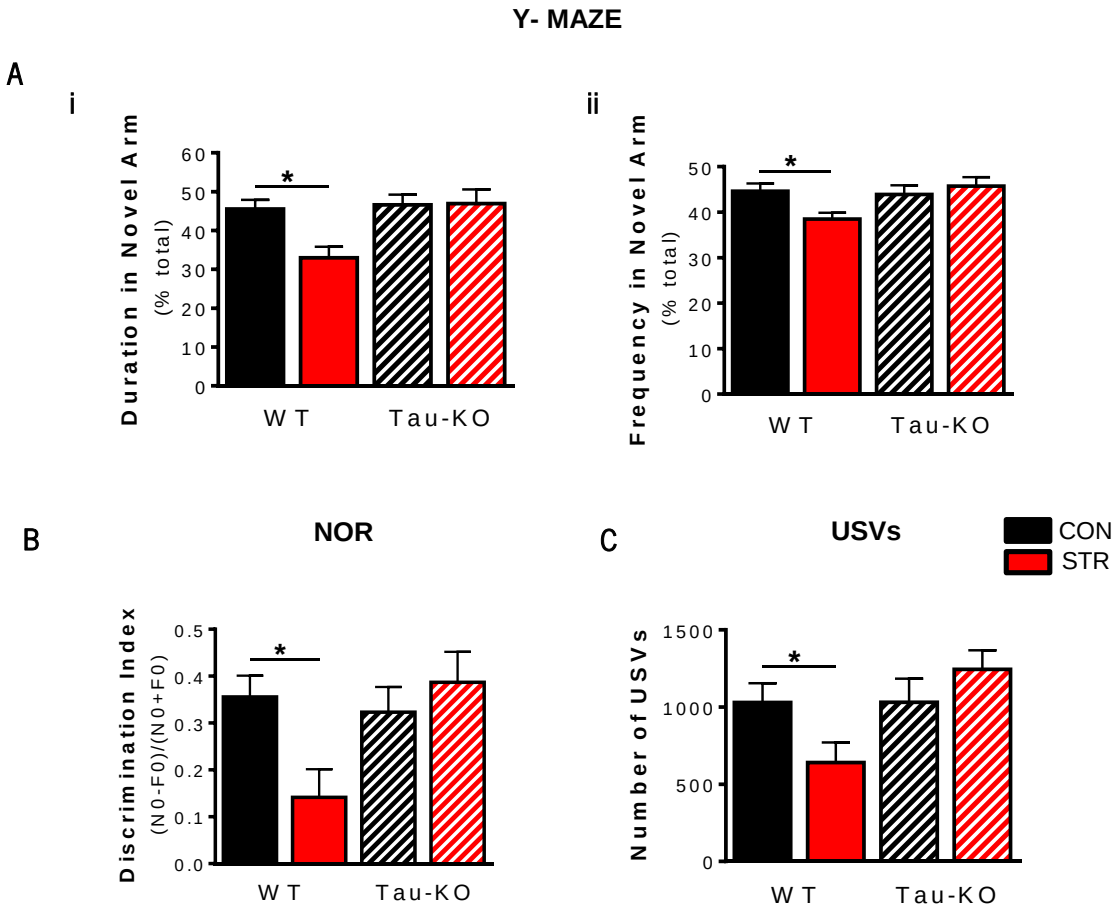


Figure 27 – Stress results in deficits of cognitive performance and emotional status of WT, but not Tau-KO animals. *A) In Y-maze test, stressed WT animals exhibited reduced duration (i) and frequency (ii) of visits in novel arm of apparatus as compared to control WTs; this reduction was not observed in Tau-KO animals. B) Similarly, discrimination index in Novel object recognition (NOR) test was reduced by stress in WT, but not Tau-KO, animals suggesting a significant resilience of Tau-KO animals to chronic stress. C) Ultrasonic vocalizations (USVs) were also reduced in stressed WT when compared to control animals suggesting a stress-driven deficit on emotional status; Tau-KO USVs was not affected by stress. All numerical data are shown as mean + SEM. (* p < 0.05).*

Altogether, the above findings support a clear involvement of Tau protein in the suppression of adult neurogenesis in the DG as well as deficits of cognitive function and emotional status that are evoked by chronic stress, suggesting the potential implication of disturbances in isoform and phosphorylation profile of Tau.

5

Discussion

5. DISCUSSION

Despite the considerable progress in the understanding of the pathophysiology and neurobiology of stress-related disorders over the past three decades, our knowledge about the cellular targets and cascades related to the detrimental actions of stress and GCs on brain plasticity remains limited. One of the main brain areas exhibiting remarkable and continuous structural remodeling in adulthood under both control and stressful conditions is the hippocampal DG due to its ability to produce new cells throughout life[41,83,153]. Many studies have demonstrated that prolonged exposure to stress and excessive GCs levels suppress DG neurogenesis but our mechanistic understanding of these phenomena remain limited. While the suggested mechanisms involved in stress-reduced neurogenesis include GC-driven glutamate release and NMDA receptors signaling and/or reduced levels of several growth/neurotrophic factors, little attention has been given to the importance of cytoskeletal dynamics and the required high degree of plasticity of newborn cells which allows them to divide, migrate, differentiate and synaptically integrate into the existent hippocampal network[41,91]. Based on the phosphorylation-dependent role of Tau on microtubules (MTs) stabilization and its recently suggested involvement in NMDA-mediated excitotoxicity, these Master studies monitor the involvement of Tau in the suppression of neurogenesis by chronic stress[112,154]. This Master thesis demonstrated for the first time that Tau ablation blocks the stress-driven suppression of neurogenesis in the adult DG indicating a critical role for Tau in the neurogenic damage by stress.

Although discovered in 1975, Tau´s precise biological function(s) and cellular role(s) are still debated with their in vivo significance remaining uncertain. Tau is a cytoskeletal protein involved in different cellular processes such as cell proliferation and neuronal differentiation, as many in vitro studies of hippocampal neurons from Tau-KO mice showed an impaired in the differentiation phase, with significant delay in their axonal and dendritic extension[109]. Moreover, expression of human Tau proteins in Tau-KO mice restored normal axonal growth[109]. However, adult Tau-KO mice do not exhibit any disturbances in brain and neuronal structure as the cytoskeletal actions of the remaining microtubule-associated proteins are suggested to compensate for the loss of Tau function (in Tau-KO) during development[100,155]. This could explain the findings reported in the current and previous studies that overall levels of proliferation and neurogenesis of Tau-KO are not different from WT under control conditions[118]. It's also important to mention that some studies show that Tau-KO animals exhibit

differences in the migration of neuroblasts in the hippocampal DG, but also in the subventricular zone (SVZ), the other main adult neurogenic niche[137]. However, these authors did not detect any numerical changes on any stages of neurogenesis between WT and Tau-KO mice (e.g. number of proliferating cells and neuroblasts), highlighting the importance of Tau protein in migration, but not generation, of neuroblasts during the early stages of neurogenesis[137]. Further studies should confirm these findings in other Tau-KO models and clarify the mechanisms through which Tau affects the neuroblasts migration. In addition, the use of a conditional Tau-KO model would help us clarify the role of Tau on neurogenesis, avoiding potential competition mechanisms that occur during development which may mask the real function of Tau by other microtubule-associated proteins (e.g. MAP1A, MAP2)[100,155]. Furthermore, in order to understand whether the potential impact of Tau on adult hippocampal neurogenesis has a cell-autonomous profile, further studies should also perform conditional deletion of Tau in specific subpopulation of DG e.g. neuroblasts.

Previous studies have reported that the expression of Tau is developmentally regulated[119,150]. Similarly, the expression of Tau isoforms that include three or four MT-binding domains (3R-Tau and 4R-Tau, respectively) is shifted during neurogenesis in the adult brain[118]. Under normal physiological conditions, 3R-Tau isoforms, that bind with less affinity to MTs, are present in neuroblasts (neuronal precursors) co-localized with the cytoskeletal protein doublecortin (DCX), providing the necessary cytoskeleton plasticity[118,137]; note that DCX is suggested to complement the low 3R-Tau ability for microtubule stabilization[156]. On the other hand, 4R-Tau isoforms, which exhibit higher affinity to MTs, are expressed in mature neurons offering the necessary stability for the establishment and maintenance of dendritic/axonal structures of the newly integrated neuron. Interestingly, this Master thesis findings show that chronic stress triggered a decrease in the protein levels of 3R-Tau followed by a simultaneous elevation of 4R-Tau isoforms in the adult DG (Figure 28). Genetically induced-expression of 4R-Tau is shown to suppress proliferation in cells and mouse hippocampus leading to downregulation of transcripts involved in cellular growth and proliferation and in tandem upregulation of transcripts implicated in cell death[157,158]. Furthermore, imbalance of 4R/3R-Tau and the consequent cytoskeletal disturbances are causally related to neuronal pathology in Alzheimer's disease brain where impaired adult neurogenesis maybe an early event in the pathology[12,159]. While the biochemical findings (based on WB analysis) of this Master thesis demonstrate that chronic stress increased the 4R:3R Tau ratio without overall differences in total Tau levels, these results were obtained in whole DG lysates, making it

difficult to understand if Tau isoform alterations occur mainly in newly-born cells or in all the DG neurons (including the pre-existing, old neurons). As previous studies have shown that 3R-Tau is expressed in all neuroblasts (monitored with DCX)[121], the reduced levels of 3R-Tau monitored by WB in stressed WT DG, could also reflect the reduced number of neuroblasts found by IF in these mice. Future studies should monitor in detail the potential impact of stress on the mechanisms that regulate alternative splicing of Tau and the expression of different Tau isoforms (see Figure 7 of Introduction). Interestingly, the involvement of Tau in stress-driven suppression of DG cell genesis was only detected in neuronal, and not astrocytic, population. The above findings of this Master thesis support the view that different cellular cascades operate in the stress-triggered reduction of newborn neuronal and glial populations[95]. As Tau is mainly expressed in neurons while astrocytes express low levels of Tau, it is possible that astrocytic cytoskeleton and its changes underlying cell genesis are not dependent of Tau protein.

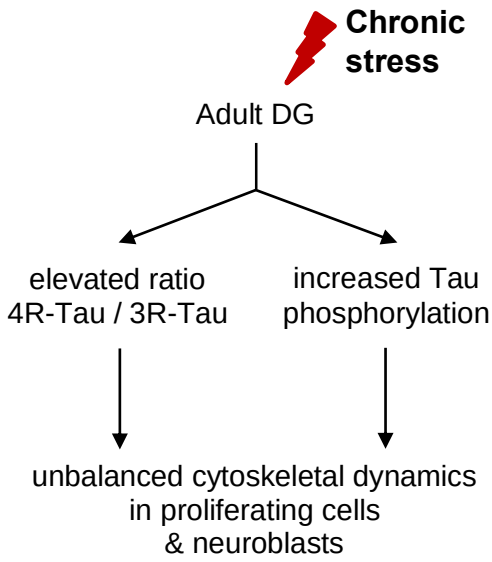


Figure 28 – Chronic stress impact on Tau expression and phosphorylation profile in DG. *Schematic representation of the effects of chronic stress on Tau protein in DG showing a stress-evoked elevated ratio of 4R-Tau:3R-Tau accompanied by increased Tau phosphorylation; both alterations may damage the necessary control on cytoskeletal dynamics required for cell proliferation and neuronal differentiation (drawn by C. Dioli).*

The phosphorylation profile of Tau is also developmentally regulated, with fetal Tau and Tau in mitotic cells exhibiting high phosphorylation levels which are reduced later in adulthood[130]. Many Tau phospho-epitopes, e.g. Ser404, are highly phosphorylated during development and then, decline; in contrast,

others Tau epitopes, e.g. Thr231 and Ser396, do not change during development[130]. As previously suggested, alterations in the control of Tau phosphorylation and isoforms that are necessary for actively dividing cells like neuronal precursors, can result in aberrations of proliferation[160,161]. Accordingly, this Master thesis shows by WB analysis that chronic stress increases Tau phosphorylation in DG of WT animals in epitopes Thr231 and Ser396/404 (detected by PHF1 antibody), but the total Tau levels are not affected[101,138]. In line with the WB analysis, IF analysis revealed increased phosphorylation of Tau at Ser396/404 epitopes by chronic stress in the proliferating cells. Previous studies suggest that Tau phosphorylation in neuroblasts and newly differentiating neurons of pre-pathological age of AD Tg mice (2 months old) may underlie, at least in part, the impaired proliferation and neuronal maturation in DG of these animals[162]. Thus, stress-driven increase in the phosphorylation of these epitopes, shown to decrease MT-affinity of Tau, could damage the complex and dynamic regulation of Tau phosphorylation diminishing the cellular control over the cytoskeletal and network essential for neuroblasts[163,164]. Many studies from our and other research teams have reported that different types of acute or chronic stressors trigger Tau hyperphosphorylation at different phospho-epitopes, such as Ser202, Thr231, Ser396/404, leading to accumulation of total Tau levels in whole hippocampus lysates and/or neuronal somata and dendrites of CA1 and CA3 subareas of hippocampus[138,141]. In line with these studies, the findings of this Master thesis show for the first time that chronic stress increases Tau phosphorylation in the DG detected by pThr231- and pSer396/404-Tau antibodies. However, we did not detect a stress-evoked increase in total Tau levels in the DG but we found that stress increased the levels of 4R-Tau and decreased the 3R-Tau isoforms, opening a novel window of research related to the impact of chronic stress on overall Tau dynamics.

While Tau hyperphosphorylation is a well recognized phenomenon in AD neuropathology, other studies also support a view of the neuroplastic role of Tau hyperphosphorylation in neuronal atrophy beyond AD, as both Tau hyperphosphorylation and neuronal/synaptic atrophy could be reversible during hibernation and hypothermia as well as in acute stress[139,140,165]. Indeed, phosphorylation and dephosphorylation of Tau is a continuous and dynamic cellular process, controlled by different kinases and phosphatases, with essential contribution to cytoskeletal plasticity. When this phosphorylation/dephosphorylation balance is disturbed, Tau becomes hyperphosphorylated resulting in cytoskeletal damage that may underlie the suppression of DG neurogenesis under stress. Future

studies should monitor whether stress-driven effects on Tau phosphorylation and isoforms can be reverted after a stress-free period [165].

Importantly, the above Tau phospho-epitopes are targets of the kinase GSK3β, whose active form is also increased in hippocampal DG of stressed WT, but not Tau-KO animals[164,166]. Note that GSK3β is a component of the Wnt signaling pathway (Figure 29) where activation of PI3K inhibits GSK3β and in turn, triggers the translocation of β-catenin from cytoplasm to nucleus promoting proliferation and differentiation of progenitor cell during both brain development and neuronal survival[135,151,152]. This Master thesis findings suggest that chronic stress suppresses the above pathway in WT, but not Tau-KO, which may underlie the stress-driven reduction of hippocampal neurogenesis found only in WT animals. In line with our findings, both in vivo and in vitro evidence suggest that Tau ablation attenuates the neuronal malfunction in different pathologies including Alzheimer's disease and glutamate-driven excitotoxicity while it has recently been shown that deletion of Tau blocks the activation of GSK3β induced by amyloid beta (Aβ); however, the exact mechanisms are under intensive investigation[167–169]. On the contrary, previous studies demonstrated that increased levels of Tau increase vulnerability to different insults including GCs and Aβ [12,170,171]. Our findings suggest that exposure to chronic stress revealed a Tau-dependent activation of GSK3β and reduction of the levels of mTOR that have previously been shown to suppress neurogenesis in the DG[172]. These findings are in line with previous experimental studies showing that chronic stress and/or GCs activate GSK3β as well as suppress mTOR signaling in hippocampus, while a recent work directly connects GSK3 and mTOR, showing that GSK3 suppress mTOR signaling in hippocampus[138,141,173–175]. Importantly, human studies report clear deficits in mTOR signaling in patients with depression, a disease state causally related to chronic stress while experimental evidence suggest a repressing role for GCs on mTOR pathway in the hippocampal DG[43,47,173,176,177]. Moreover, activation of mTOR is shown to regulate the spatial and local control of Tau mRNA translation (via 5-TOP sequence of Tau-mRNA) towards axonal formation and neuronal polarization at the developing neuron without affecting levels of other cytoskeletal proteins e.t. actin, β-tubulin[178]. Thus, inhibition of mTOR reduces the expression levels of Tau protein and blocks neuronal polarity in the developing neuron[178]. However, further investigation of downstream targets of mTOR protein, like p70s6k that regulates cell growth and differentiation remain to be explored[179]. Together, the stress-driven suppression of mTOR, activation of GSK3β and Tau alterations presented in this study

(Figure 28, 29) suggest a possible Tau-dependent mechanism through which chronic stress initiates a signaling cascade that impacts on cell survival, proliferation and neuronal cell fate in the adult DG.

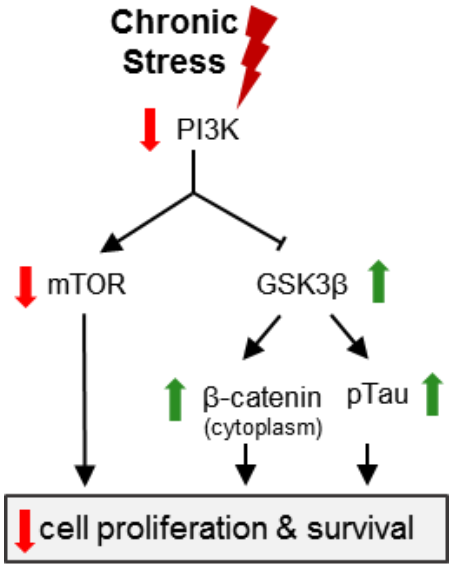


Figure 29 – Proposed cellular mechanisms underlying the stress-induced suppression of cell proliferation and neuronal survival in adult hippocampal. *Exposure to chronic stress decreased mTOR levels while in parallel increased GSK3β activity resulting in accumulation of β-catenin in cytoplasm as well as increased Tau phosphorylation. Interestingly, this stress effects seems to be Tau-dependent suggesting a novel role of Tau in mechanisms underlying stress-driven brain plasticity (drawn by C. Dioli).*

While other cellular pathways involving e.g. MAP kinases cannot be excluded[83], this study provides the first evidence that Tau protein is a selective mediator of stress-driven neurogenic deficits in adult hippocampus adding to our mechanistic understanding of the cellular cascades that may convey the pathogenic role of chronic stress in the brain and its relation with the development of stress-driven pathologies, e.g. depression, where neurogenesis has been implicated in the etiopathogenesis and antidepressant treatment.

6

Conclusions

6. CONCLUSIONS

Modern lifestyle appears to increasingly present a high load of daily distress in our societies while a plethora of clinical and experimental studies have associated the prolonged exposure to stressful conditions with the development of different brain disorders related to cognitive and mood deficits, e.g. Alzheimer's disease and depression. Thus, better understanding of the mechanisms through which chronic stress affects the brain and its circuits will help us to develop specific therapeutic targets and strategies against these brain pathologies.

These Master studies provide novel insights regarding the molecular underpinnings of the detrimental impact of chronic stress on one mechanism of adult brain plasticity, related to the genesis of newly-born neurons (neurogenesis) in hippocampus. Our in vivo studies demonstrated for the first time that Tau is essential for the suppression of neurogenesis, but not astrogliogenesis that chronic stress evokes in the adult hippocampus since animals lacking Tau (Tau knockout) were protected against the above effects of stress. These results are in line with previous experimental studies suggesting that Tau deletion or reduction could be protective against different neuropathologies such as Alzheimer's disease, epilepsy and excitoxocity[167–169]. Moreover, our findings suggest that chronic stress suppresses a known mechanism that regulates cell proliferation and survival involving PI3K/mTOR/GSK3β/β-catenin cascade while this stress-triggered suppression depends on the presence of Tau protein.

This Master thesis presents the first set of novel experimental evidence about the role of protein Tau in the hippocampal neurogenic deficits induced by exposure to chronic stress. These findings add to our limited understanding of stress-triggered cellular cascades and molecular pathways that may underlie the neuro-remodeling and neuroplastic action of chronic stress and GCs. While further studies are necessary to clarify how Tau absence affects the cellular response(s) to stress, this Master thesis opens new roads of research investigation that could monitor:

a) the neuroprotective role of Tau reduction in brain pathologies beyond AD e.g. stress-driven depression; note that Tau reduction has been suggested as a therapeutic approach against AD neurodegeneration.

b) the potential involvement of Tau on the beneficial effects of antidepressants against stress-driven neuronal atrophy and suppressed neurogenesis.

7

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