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Original Article

ORIGINAL ARTICLE

Tau Deletion Prevents Stress-Induced Dendritic Atrophy in Prefrontal Cortex: Role of Synaptic Mitochondria

Sofia Lopes[1,2], Larysa Teplytska[3], Joao Vaz-Silva[1,2], Chrysoula Dioli[1,2], Rita Trindade[1,2], Monica Morais[1,2], Christian Webhofer[3,4], Giuseppina Maccarrone[3], Osborne F.X. Almeida[3], Christoph W. Turck[3], Nuno Sousa[1,2], Ioannis Sotiropoulos[1,2] and Michaela D. Filiou[3]

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga, Portugal, [2]ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães 4710-057, Portugal, [3]Max Planck Institute of Psychiatry, 80804 Munich, Germany and [4]Current address: Sandoz Biopharmaceuticals, 82041 Oberhaching, Germany

Address correspondence to Michaela D. Filiou, PhD, Max Planck Institute of Psychiatry, Kraepelinstr. 2, 80804, Munich, Germany. Email: mfiliou@psych.mpg.de; Ioannis Sotiropoulos, PhD, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal. Email: ioannis@ecsaude.uminho.pt

Abstract

Tau protein in dendrites and synapses has been recently implicated in synaptic degeneration and neuronal malfunction. Chronic stress, a well-known inducer of neuronal/synaptic atrophy, triggers hyperphosphorylation of Tau protein and cognitive deficits. However, the cause–effect relationship between these events remains to be established. To test the involvement of Tau in stress-induced impairments of cognition, we investigated the impact of stress on cognitive behavior, neuronal structure, and the synaptic proteome in the prefrontal cortex (PFC) of Tau knock-out (Tau-KO) and wild-type (WT) mice. Whereas exposure to chronic stress resulted in atrophy of apical dendrites and spine loss in PFC neurons as well as significant impairments in working memory in WT mice, such changes were absent in Tau-KO animals. Quantitative proteomic analysis of PFC synaptosomal fractions, combined with transmission electron microscopy analysis, suggested a prominent role for mitochondria in the regulation of the effects of stress. Specifically, chronically stressed animals exhibit Tau-dependent alterations in the levels of proteins involved in mitochondrial transport and oxidative phosphorylation as well as in the synaptic localization of mitochondria in PFC. These findings provide evidence for a causal role of Tau in mediating stress-elicited neuronal atrophy and cognitive impairment and indicate that Tau may exert its effects through synaptic mitochondria.

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

Introduction

Chronic stress has a significant impact on the prefrontal cortex (PFC) leading to impairments of executive functions such as working memory, cognitive flexibility, and decision-making (Cerqueira, Mailliet et al. 2007; Sotiropoulos, Cerqueira et al. 2008; Sousa and Almeida 2012; McEwen and Morrison 2013). The structural–functional correlates of these stress-induced behavioral impairments include neuronal atrophy and synaptic loss. A large body of evidence indicates that these changes presumably reflect the actions of glucocorticoids (GCs) and, in turn, altered glutamatergic activity and disrupted Ca^{2+} homeostasis (Cerqueira, Mailliet et al. 2007; Sousa and Almeida 2012)

that ultimately interfere with the stability of cytoskeletal proteins (Cereseto et al. 2006). We previously demonstrated that chronic stress triggers the hyperphosphorylation of the cytoskeletal protein Tau with concomitant cognitive deficits (Sotiropoulos et al. 2011); while previous work implicated synaptic loss in the latter (Cerqueira, Taipa et al. 2007), the cellular and molecular underpinnings of these events are largely unknown. Hyperphosphorylated Tau is strongly associated with synaptic malfunction and loss as well as neuronal degeneration in Alzheimer's disease (AD) (Kimura et al. 2007; Ittner et al. 2010). Tau protein, which plays a key role in microtubule stabilization and intracellular cargo trafficking, has recently been shown to be important for the regulation of synaptic plasticity (Ittner et al. 2010; Frandemiche et al. 2014), albeit through mechanisms that are still poorly understood. Here, we tested the hypothesis that Tau mediates the actions of stress on prefrontocortical structure and function by exposing mice lacking the Tau protein (Tau-KO) (Dawson et al. 2001) to a chronic unpredictable stress (CUS) paradigm. We subsequently used ^{15}N metabolic labeling-based quantitative proteomics (Filiou and Turck 2012) to explore the underlying molecular correlates of these effects in PFC synapses. Our data suggest that stress produces a decline in PFC-dependent memory by altering the proteome and density of mitochondria in synapses and, subsequently, dendritic and synaptic atrophy in a Tau-dependent manner.

Materials and Methods

Animals

Tau knock-out (Tau-KO) and wild-type (WT) mice (C57BL/6J background; 4–5 month-old males) (Dawson et al. 2001) were used in this study. All experiments were conducted in accordance with the Portuguese national authority for animal experimentation, *Direcção Geral de Veterinária* (ID: DGV9457). Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and Council. Mice were housed in groups of 4–5 per cage under standard environmental conditions (lights on from 8 a.m. [ZT0] to 8 p.m. [ZT12]; room temperature 22°C; relative humidity of 55%, ad libitum access to food and water).

Stress Paradigm

Animals of both genotypes were submitted to a 6-week protocol of CUS, consisting of 4 different stressors: restraint, vibrating platform, overcrowding, and exposure to a hot air stream. Animals were exposed to one stressor per day for 3 h, during the daily period of illumination. The order of stressors as well as the time of the day that the stressor was applied were randomly chosen for each week as previously described (Cerqueira, Mailliet et al. 2007; Sotiropoulos et al. 2011). Control (non-stressed; CON) mice remained undisturbed in their home cages; mice exposed to CUS are hereinafter referred to as the stressed (STR) group. Animal body weight and serum corticosterone (CORT) levels were recorded as indicators of stress efficacy. At the end of the stress protocol, blood was collected from all animals at 8 p.m. (peak time point of the circadian cycle), serum was isolated by centrifugation and CORT levels were measured using a radioimmunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. For CORT measurement after acute stressor (8 a.m.), blood was collected before and at 2 different time points after exposure to acute stressor (4 min restraint).

Behavioral Analysis

Y-maze

For assessing PFC-dependent working memory, spontaneous alternations in the Y-maze arms were monitored using the Y-maze apparatus (33 cm × 7 cm × 15 cm). Animals (n = 10–12 per group) were placed in the center of the Y-maze apparatus and were allowed to explore freely for 8 min; the number and order of arm entries was recorded. Spontaneous alternations were calculated as the ratio of number of triads (sequence of 3 consecutive arm entries) and total arm entries.

Novel Place Recognition

To assess recognition memory we used the novel place recognition test. Briefly, animals (n = 10–12 per group) were habituated to an open arena (33 cm × 33 cm × 33 cm) for 3 days. Each animal was then allowed to explore 2 identical objects for 10 min. One hour later, mice were returned to the arena, with one of the objects being placed to a new position. Recognition index was calculated based on the following formula [time in novel place / (novel place + familial place) - time in familial place / (novel place + familial place)] x 100. Sessions were videotaped and evaluated using Kinoscope software (http://sourceforge.net/projects/kinoscope/).

Open Field

For monitoring locomotor activity we used an open field apparatus (square arena [43.2 cm × 43.2 cm]) surrounded by tall perspex walls (Med Associates Inc., St. Albans City, VT). Mice (n = 10 per group) were placed in the center and allowed to explore the area for 10 min. Infrared beams and manufacturer's software were used to automatically register animals' movements.

Neuronal Structure Analysis

For 3D morphometric analysis, animals (n = 5 per group) were transcardially perfused with 0.9% saline under deep anesthesia (ketamine hydrochloride [150 mg/kg] plus medetomidine [0.3 mg/kg]). Brains were immersed in a Golgi-Cox solution for 14 days and then transferred to a 30% sucrose solution. Vibratome coronal sections (200 μm thick) were collected in 6% sucrose and dried onto gelatin-coated microscope slides. Sections were then alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed, dehydrated, and mounted. Dendritic arborization was analyzed in the layer II/III of the infralimbic division of PFC. Per experimental group, 22–23 neurons were studied. For each selected neuron, all branches of the dendritic tree were reconstructed at ×600 (oil) magnification using a motorized Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany) and Neurolucida software (Microbrightfield, Williston, VT) and dendritic length was automatically calculated. Dendritic spine density (number of spines/dendritic length) was determined in distal branches of apical dendrites that were either parallel or at acute angles to the coronal surface of the section. For Sholl analysis (index of dendritic complexity and degree of arborization), the number of dendritic intersections with concentric spheres positioned at radial intervals of 20 μm from the soma was accessed using NeuroExplorer software (Microbrightfield) as previously described (Cerqueira, Mailliet et al. 2007; Bessa et al. 2009).

Transmission Electron Microscopy Analysis

To monitor synaptic density and mitochondria by transmission electron microscopy (TEM), animals (n = 3 per group) were transcardially perfused with 4% PFA in microtubule stabilization

buffer (65 mM PIPES, 25 mM HEPES, 10 mM EGTA, 3 mM $MgCl_2$, pH = 6.9), as previously described (David et al. 2005). Isolated brains were post-fixed in 4% PFA, 0.8% glutaraldehyde in microtubule stabilization buffer (overnight; RT), transferred to 4% PFA, 0.8% glutaraldehyde in 0.1 M of phosphate buffer (pH = 7.4) for 2 h and then to 0.1 M phosphate buffer (4°C). The medial PFC area was carefully dissected out from vibratome-cut coronal sections (300 μm) and embedded in Epon resin along the superficial-to-deep axis. Ultrathin sections (500 Å) were cut onto nickel grids. Images were obtained using JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan) and Orious Sc1000 digital camera. Approximately, 40–42 nonoverlapping TEM (×30 000) images of counterstained ultrathin sections per animal group were analyzed by an experimenter blind to the samples.

Statistical Analysis of Animal Data

Unless otherwise specified, animal data were analyzed using 2-way analysis of variance (ANOVA) and Tukey post hoc comparisons (GraphPad Prism v 5.0, La Jolla, CA); differences were considered statistically significant when $P < 0.05$. Results are presented as mean ± SEM.

Proteomic Sample Preparation

C57BL/6 mice were in vivo metabolically labeled with a bacteria-based ^{15}N-labeled diet in the animal facility of the Max Planck Institute of Psychiatry as previously described (Frank et al. 2009) and a whole brain from a ^{15}N-labeled C57BL/6 adult mouse was used as internal labeled standard for quantitative proteomic comparisons. PFC from individual animals ($n = 5$ per group) and the whole brain from the ^{15}N-labeled mouse brain were homogenized in buffer A (0.32 M sucrose, 4 mM HEPES, complete protease cocktail inhibitor tablets [Roche Diagnostics, Mannheim, Germany]) and centrifuged twice for 10 min at 1000 × g at 4°C. For each animal, the 2 supernatants were combined. Five hundred micrograms from each PFC homogenate per group were combined (2500 μg in total) and were mixed at a 1:1 (w/w) ratio with the ^{15}N-labeled internal standard homogenate. Synaptosomes were then enriched from the combined WT CON/^{15}N, WT STR/^{15}N, Tau-KO CON/^{15}N, and Tau-KO STR/^{15}N PFC homogenate mixtures according to Filiou et al. 2010. Protein content in the synaptosomal fraction was estimated by Bradford Assay. One hundred micrograms of each WT CON/^{15}N, WT STR/^{15}N, Tau-KO CON/^{15}N, and Tau-KO STR/^{15}N synaptosomal fractions were loaded on a SDS gel. Each lane was cut in 4 mm slices. In-gel digestion and peptide extraction were performed as previously described (Frank et al. 2009). Peptide extracts were lyophilized and dissolved in 0.1% HCOOH for mass spectrometry (MS) analysis.

Mass Spectrometry (MS)

Peptide extracts from each gel slice were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) using a nanoflow HPLC-2D system (Eksigent, Dublin, CA) coupled online to an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, Bremen, Germany). Samples were loaded onto an in house packed fused silica 3 μm RP-C18 column (Maisch, Monheim, Germany) followed by a 20 min wash with 0.1% HCOOH and elution with a 95% AcN/0.1% HCOOH gradient from 2% to 45% over 90 min at a flow rate of 200 nL/min. All other MS parameters were as previously described (Filiou et al. 2010).

Proteomic Data Analysis

MS raw files were analyzed as previously described (Filiou et al. 2012). In brief, data were searched twice against a concatenated decoy Swiss Prot mouse database v 3.46 using BioWorks v 3.3.1 (ThermoFisher Scientific, San Jose, CA) and SEQUEST v 28 (ThermoFisher Scientific). Parameters for SEQUEST search were as described previously (Filiou et al. 2011). DTA select v 1.9 was used to filter and assemble peptides into proteins. Ion chromatograms were extracted and peptide/protein ratios were calculated by ProRata v 1.0 (Pan et al. 2006). Non-synaptosomal protein contaminants with altered protein levels and proteins inaccurately quantified were not considered. Proteins with fold change >1.3 and $P < 0.05$ (corrected for multiple testing) were considered differentially expressed. All MS quantification results are provided in Supplementary Table 1.

Results

Tau Deletion Does not Interfere with Peripheral Biomarkers of the Stress Response

The hypothalamus–pituitary–adrenal (HPA) axis, including the secretion of corticosteroids, comprises the physiological response to stress; chronic stress is characterized by hyperactivity of the HPA axis and body weight loss. We subjected Tau-KO and WT mice to a 6-week CUS protocol (Fig. 1A) and body weight and serum CORT levels were monitored at the end of the paradigm, just before behavioral testing. Two-way ANOVA showed a highly significant effect of *Stress* on body weight ($F_{1,75} = 21.83$, $P < 0.0001$); body weight was significantly decreased in stressed WT animals (WT STR; $P = 0.013$) and stressed Tau-KO animals (Tau-KO STR; $P = 0.004$) compared with control animals of the same genotype (Tau-KO CON and WT CON, respectively) (Fig. 1B). Furthermore, we found an overall effect of *Stress* on basal circulating CORT levels at the onset of the dark period (ZT12/8 p.m.; $F_{1,43} = 18.68$, $P < 0.0001$) (Fig. 1C); post hoc analysis revealed a significant increase in circulating CORT levels in both WT STR ($P = 0.041$) and Tau-KO STR ($P = 0.009$) mice when compared with the corresponding non-stressed animals. To monitor HPA axis function, we assessed CORT levels at different time points after exposure of mice to an acute stressor (restraint, 4 min at 8 a.m.; Fig. 1D). We found an overall effect of *Stress* at 30 min after this stressor ($F_{1,45} = 4.352$, $P = 0.043$). Notably, there was no *Genotype* effect in any of the above comparisons, indicating that the effects of stress on these parameters were not influenced by the absence of Tau.

Absence of Tau Confers Resilience to Stress-Induced Deficits in Memory and Neuronal Atrophy in the Prefrontal Cortex (PFC)

Chronic stress is known to impair working memory, a function that requires PFC integrity and the intactness of its connections with other brain areas (Cerqueira, Mailliet et al. 2007; Sotiropoulos, Cerqueira et al. 2008). Here, we monitored spontaneous alternations in the arms of the Y maze as a measure of working memory (Sarnyai et al. 2000) in WT and Tau-KO mice that were exposed to CUS. Statistical analysis (2-way ANOVA) of correct alternations in the Y-maze showed a clear interaction between *Stress* and *Genotype* ($F_{1,38} = 4.677$, $P = 0.037$) (Fig. 1E), with post hoc analysis revealing significant differences between stressed and control WT ($P = 0.041$), but not Tau-KO ($P = 0.998$), animals. Similarly, using the novel place recognition test, we found that stress decreased the (%) recognition index of WT ($P = 0.048$) but not of Tau-KO

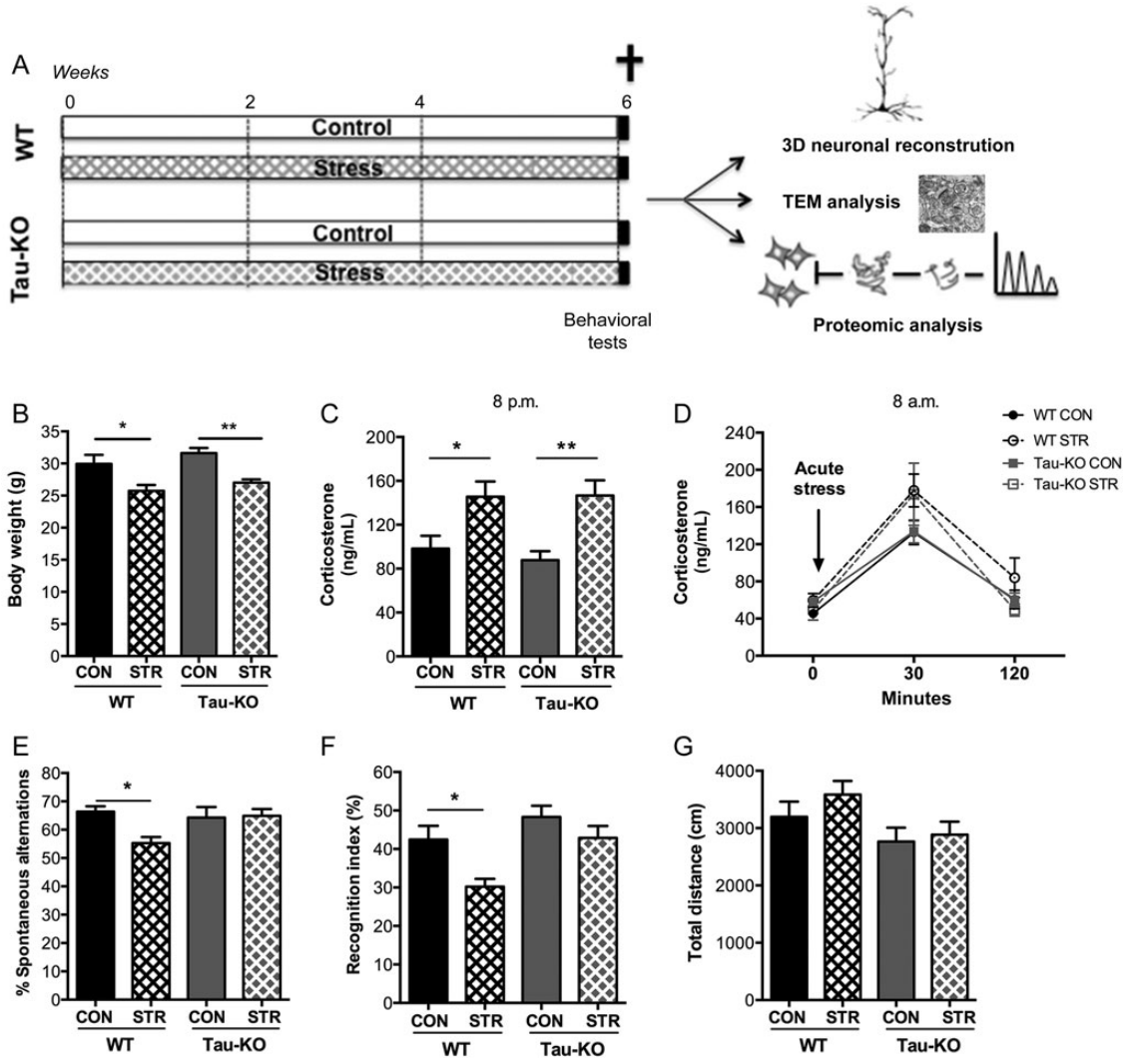

Figure 1. Tau deletion prevents stress-induced decline in working memory without interfering with endocrine response to stress. (*A*) The experimental design is represented schematically. Control and stressed WT and Tau-KO mice were used. Analysis included behavioral, neurostructural, TEM, and quantitative proteomic approaches. (*B*) Exposure of WT and Tau-KO animals to 6 weeks of chronic stress (Stress; STR) resulted in decrease of body weight, as compared with non-stressed (Control; CON) counterparts. (*C*) Chronic stress resulted in elevated circulating CORT levels in both WT and Tau-KO animals (measured at usual peak of CORT secretion, ZT12/8 p.m.). (*D*) Previously stressed animals of both genotypes (WT and Tau-KO) respond similarly to an acute stressor; both groups showed peak responses 30 min after the stressor, and displayed basal levels of CORT secretion after 120 min. (*E*) WT STR animals exhibited a reduced percentage of spontaneous alternations in the arms of a Y-maze, as compared with WT CON animals. This deficit in working memory was not observed in Tau-KO animals. (*F*) Similarly, stress reduced % recognition index in WT but not in Tau-KO animals indicating deficits of short-term memory only in WT mice. (*G*) Total distance traveled in open field apparatus did not differ in animal groups of both genotypes under stress and control conditions. All numerical data are shown as mean ± SEM. (*$P < 0.05$; **$P < 0.01$). WT CON, wild-type control; WT STR, wild-type stressed; Tau-KO CON, Tau knock-out control; Tau-KO STR, Tau knock-out stressed; TEM, transmission electron microscopy.

($P = 0.548$) animals (Fig. 1F). These findings indicate that Tau protein is essential for stress to elicit cognitive deficits. Furthermore, we found no changes in locomotor activity in animals exposed to stress from both genotypes as assessed by total distance traveled in open field (Fig. 1G).

Neuronal atrophy and synaptic loss are robust correlates of impaired cognitive behavior, including that induced by chronic stress (Cerqueira, Mailliet et al. 2007; Sousa and Almeida 2012). Using unbiased 3D morphometric analysis of Golgi-impregnated pyramidal neurons, we observed a significant interaction between *Stress* and *Genotype* when total dendritic length of PFC neurons was analyzed ($F_{1,77} = 4.918$, $P = 0.030$); strikingly, stress resulted in a reduction in total dendritic length in WT ($P = 0.019$) but not Tau-KO ($P = 0.998$) animals (Fig. 2A,F); no difference was found between control WT and Tau-KO animals ($P = 0.627$). Further analysis showed that atrophy was confined to apical dendrites, again in a manner reflecting an interaction between *Stress* and *Genotype* ($F_{1,74} = 4.512$, $P = 0.037$) and with stress only causing dendritic shrinkage in WT animals ($P = 0.040$) (Fig. 2B; cf. data for basal dendrites in Fig. 2C); both apical and basal dendritic lengths were also similar between control animals of both genotypes ($P = 0.892$ and $P = 0.971$, respectively). Similarly, hippocampal CA3 neurons of WT, but not Tau-KO, animals displayed a significant reduction of dendritic length after exposure to chronic stress (Supplementary Table 2). The above stress-induced neuronal atrophy was confirmed by Sholl analysis which provided data on the number of dendritic intersections as a function of their distance from the soma (Cerqueira, Mailliet et al. 2007) (Fig. 2E,F). Two-way ANOVA analysis revealed an interaction between *Stress* and *Genotype* for the number of intersections

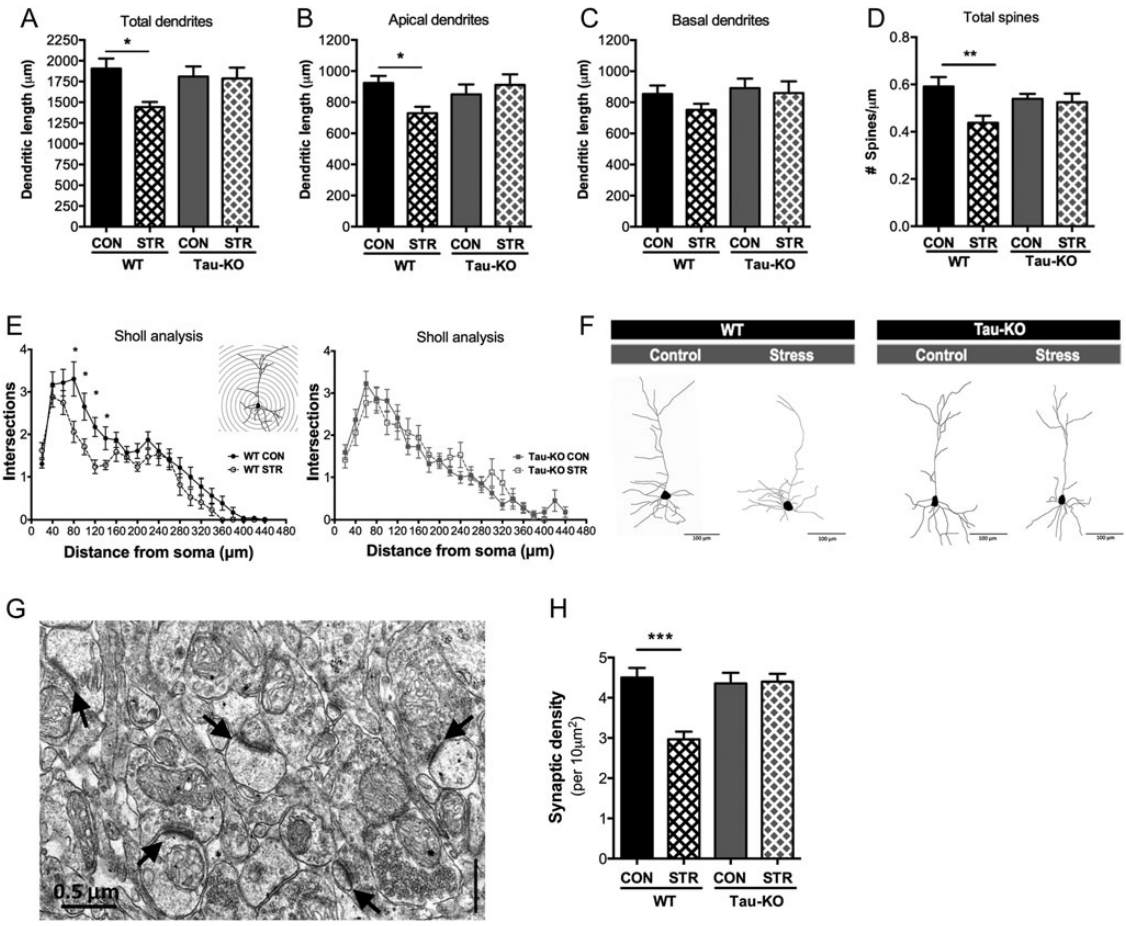

Figure 2. Stress-induced neuronal atrophy and synaptic loss depends on the presence of Tau protein. (*A–C*) Exposure to chronic stress resulted in reduced total dendritic length in PFC neurons of WT, but not Tau-KO, animals (*A*); atrophy was pronounced in apical (*B*) but not basal (*C*) dendrites. (*D*) Spine density was reduced in WT STR but not in Tau-KO STR mice, as compared with their respective unstressed controls. (*E*) Sholl analysis demonstrated a reduction of dendritic arborization at the proximal part of neurons of WT STR compared with WT CON mice; no marked differences were found between neurons of Tau-KO STR and Tau-KO CON mice. (*F*) 3D reconstruction of neurons from CON and STR WT and Tau-KO animals. (*G*) TEM image of medial PFC layers II and III; each arrow marks a post-synaptic density (PSD). (*H*) Chronic stress decreases synaptic density in WT, but not Tau-KO animals as monitored in TEM images. All numerical data are shown as mean ± SEM. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). WT CON, wild-type control; WT STR, wild-type stressed; Tau-KO CON, Tau knock-out control; Tau-KO STR, Tau knock-out stressed; PSD, post-synaptic density.

at a distance of 80 µm ($F_{1,75} = 4.039$, $P = 0.04$), 120 µm ($F_{1,75} = 4.008$, $P = 0.04$), 140 µm ($F_{1,75} = 5.326$, $P = 0.02$), 300 µm ($F_{1,75} = 4.627$, $P = 0.03$) and 320 µm ($F_{1,75} = 5.432$, $P = 0.02$) from the soma while an overall effect of *Stress* was found at 100 µm ($F_{1,75} = 6.323$, $P = 0.01$). Additional analysis showed decreased branching at 80, 100, 120, 140 µm in PFC neurons from stressed WT animals as compared with neurons from control WT mice ($P < 0.05$ in all cases).

A significant interaction between *Stress* and *Genotype* ($F_{1,88} = 4.991$, $P = 0.028$) was found when spine density in Golgi-impregnated PFC neurons was analyzed. While neurons of WT CON and Tau-KO CON animals exhibited similar spine densities ($P = 0.649$), spine density was significantly lower in WT STR mice vs. WT CON ($P = 0.008$); stress did not have a significant effect on spine density in Tau-KO mice ($P = 0.986$) (Fig. 2*D*). The Golgi-based findings of spine loss in stressed WT animals were confirmed by measurements of synaptic density in TEM images. As shown in Figure 2*G*,*H*, chronic stress reduced synaptic density in PFC of WT ($P < 0.0001$), but not Tau-KO, mice ($P > 0.99$) (*Stress* × *Genotype* interaction $F_{1,\ 162} = 11.00$; $P = 0.001$); synaptic densities did not differ between WT and Tau-KO controls ($P = 0.966$). The behavioral and neurostructural data from this study suggest that, while absence of Tau does not lead to any particular phenotype (Harada et al. 1994; Ke et al. 2012), it abolishes the detrimental effects of chronic stress.

Tau Deletion Effects on PFC Synaptosomal Protein Expression

Synaptic malfunction and atrophy represent a core feature of stress-driven neuroplasticity underlying stress-related pathologies. To investigate the molecular underpinnings of the differential neuroplastic responses of WT and Tau-KO mice to stress, we compared the synaptic proteomes of control and stressed Tau-KO and WT animals. The following comparisons were performed for PFC synaptosomes using a proteomic workflow based on in vivo ^{15}N metabolic labeling and quantitative mass spectrometry (Frank et al. 2009; Filiou and Turck 2012; Filiou 2013): Tau-KO CON versus WT CON, WT STR versus WT CON, Tau-KO STR versus Tau-KO CON and Tau-KO STR versus WT STR. At first, we compared the synaptic proteomes of control Tau-KO and WT mice (Tau-KO CON versus WT CON). We found that the expression of 3 proteins, tubulin β-4A, tenascin-R and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase, was significantly decreased in Tau-KO CON mice (Supplementary Table 3). Interestingly, these proteins

are functionally related to cytoskeletal components; tubulin β-4A is a core cytoskeletal protein, tenascin-R induces actin-rich microprocesses and branches along neurite shafts (Zacharias et al. 2002), and 2′, 3′-cyclic-nucleotide 3′-phosphodiesterase is a microtubule-associated protein (Bifulco et al. 2002). These findings show that the expression of other cytoskeleton-related proteins is altered when Tau is absent, adding to our limited knowledge regarding the influence of Tau in regulating overall cytoskeletal integrity (Takei et al. 2000; Ke et al. 2012).

Chronic Stress Alters Expression of Cytosolic Proteins in WT PFC Synaptosomes

To investigate the effect of stress in WT animals, we compared PFC synaptosomes from WT STR versus WT CON mice and found 8 proteins which were differentially expressed; 7 of these are cytosolic/membrane proteins and one (Aco2) is of mitochondrial localization (Supplementary Table 4). We observed significantly decreased expression levels of Na^+/K^+- and Ca^{2+}-transporting ATPase proteins (Atp1a1, Atp2b2, both involved in ion homeostasis). We also found increased expression levels of creatine kinase B (Ckb, involved in intracellular energy transfer), clathrin heavy chain 1 and Rab GDP dissociation inhibitor α (Cltc and Gdi1, respectively, implicated in synaptic vesicle recycling and intracellular receptor trafficking), ubiquitin-like modifier-activating enzyme 1 (Uba1, involved in degradation regulation), and heterogeneous nuclear ribonucleoprotein A2/B1 (Hnrnpa2b1, an RNA-binding protein implicated in transport of dendrite-localized mRNAs in activated synapses).

Stress-Driven Alterations in Mitochondrial Protein Expression in Tau-KO PFC Synaptosomes

Chronic stress resulted in the differential expression of 23 proteins in PFC synaptosomes of Tau-KO STR versus Tau-KO CON mice (Table 1). All of these proteins were expressed at higher levels in Tau-KO STR compared with Tau-KO CON mice and are all mitochondrial. It should be noted that there is no overlap with the differentially expressed proteins identified when WT animals were exposed to stress. The differentially expressed proteins were assigned to the following categories, based on their known functions:

Table 1 Stress-evoked protein expression changes in PFC synaptosomes of Tau-KO mice (Tau-KO STR versus Tau-KO CON)

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

Electron Transport Chain
Increased expression of electron transport chain (ETC) subunits of complexes I (Mtnd4, Ndufs1), III (Cyc1, Uqcrc2, Uqcrfs1), IV (Cox6c), and V (Atp5a1, Atp5b) was observed in Tau-KO STR mice.

Mitochondrial Transport
Increased expression of 6 members of the mitochondrial carrier family Slc25 was found in Tau-KO STR mice, including a phosphate carrier (Slc25a3), ADP/ATP translocases (Slc25a4, Slc25a5), glutamate transporters (Slc25a12, Slc25a22) and a mitochondrial carrier with largely unknown function (Slc25a46). Increased expression of 2 members of the iron transporting sideroflexin family (Sfxn1, Sfxn3) was also observed.

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

In addition, to investigate how the absence of Tau affects stress response, we compared the PFC synaptosome proteomes of Tau-KO STR versus WT STR mice. We found 29 differentially expressed proteins (Table 2), 23 of which are located in mitochondria and are mainly classified in 2 functional categories:

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

Mitochondrial Transport
Five differentially expressed proteins in Tau-KO STR compared with WT STR mice belong to the Slc25 mitochondrial carrier family, including Slc25a3, Slc25a4, Slc25a5, Slc25a22 (which were also found in higher levels in Tau-KO STR compared with Tau-KO CON PFC synaptosomes), as well as the 2-oxoglutarate/malate carrier protein Slc25a11. The Sfxn1 and Sfxn5 sideroflexin family members were also higher expressed.

Results of the comparative proteomic analysis are summarized in Table 3. All differentially expressed proteins across group comparisons are categorized according to subcellular localization. The levels of mitochondrial proteins are prominently increased in synaptosomes of stressed Tau-KO mice both when compared with unstressed Tau-KO and stressed WT mice.

Tau Deletion Abrogates Stress-Induced Depletion of Synaptic Mitochondria

In light of previous reports that Tau deletion protects against disruption of mitochondrial motility in dendrites (Zempel et al. 2013) and axons (Vossel et al. 2010, 2015), we next monitored mitochondrial density in PFC synapses using TEM (Fig. 3A). While total mitochondria density was similar among all groups (Fig. 3C), the density of synaptic mitochondria was differentially affected by stress in WT and Tau-KO animals (Fig. 3B). Specifically, 2-way ANOVA analysis revealed a *Stress* × *Genotype* interaction ($F_{1,162} = 8.641$, $P = 0.004$), with stressed Tau-KO, but not WT, animals exhibiting increased mitochondrial density in the post-synaptic compartment ($P = 0.045$ and $P = 0.432$, respectively), when compared with their control littermates; synaptic mitochondrial density did not differ between WT CON and Tau-KO CON mice ($P = 0.932$). This observation indicates that stress leads to an enrichment of mitochondria in the synapses of Tau-KO mice, consistent with our findings from the PFC synaptosome proteomic analysis.

Discussion

Tau is a cytoskeletal protein implicated in various neuronal processes, including microtubule stabilization and axonal maintenance and transport; recent studies have also suggested that Tau may be critical for synaptic signaling and structure (Gotz et al. 2013). Despite the essential role of Tau in regulating cytoskeletal assembly and dynamics, young/adult animals with a conventional deletion of Tau fail to display obvious behavioral, neurostructural or axonal abnormalities (Dawson et al. 2001; Yuan et al. 2008; Vossel et al. 2010; Ke et al. 2012; Morris et al. 2013; Ma et al. 2014; Lopes et al. 2016). Accordingly, our current behavioral analysis shows no cognitive or locomotor deficits in young adult animals and no structural differences in PFC neuronal structure as assessed by 3D neuronal reconstruction and TEM analyses. However, it should be noted that, the absence of an obvious phenotype in Tau-KO animals may reflect the recruitment of compensatory mechanisms, for example, through altered expression of other cytoskeletal proteins (Harada et al. 1994; Dawson et al. 2001; Ma et al. 2014). The latter view was supported by our proteomic data which demonstrate that adult Tau-KO mice display an altered pattern of expression of other cytoskeleton-related proteins, highlighting the suggested close interrelationship between Tau and other proteins involved in cytoskeletal dynamics and integrity (Takei et al. 2000).

Previous evidence suggests that Tau ablation has a neuroprotective role against neurotoxic insults such as amyloid beta (Aβ) (Rapoport et al. 2002; Roberson et al. 2007). The present study shows that, although WT and Tau-KO mice do not differ in their overt responses to chronic stress (endocrine, body weight), Tau-KO mice are protected against the well-known central effects of stress, namely, impairment of working memory and dendritic remodeling and synaptic loss in the PFC (Cerqueira, Mailliet et al. 2007; Sousa and Almeida 2012; McEwen and Morrison 2013). Furthermore, the altered levels of PFC synaptic mitochondrial proteins between Tau-KO STR and WT STR animals provide novel insights into the molecular and cellular events that underlie stress-triggered neuronal atrophy and cognitive dysfunction. With the exception of a few studies (Cereseto et al. 2006), the effects of stress on cytoskeletal proteins have received little attention, despite the fact that the cytoskeleton is important for maintaining neuronal architecture and function (Morris et al. 2011). Extending our previous demonstration that chronic stress and/or GCs lead to the abnormal hyperphosphorylation of Tau protein (Sotiropoulos, Catania et al. 2008; Sotiropoulos et al. 2011), the present findings show that Tau protein plays a key role in stress-induced dendritic remodeling, synaptic loss and cognitive impairment. Recently, localization of the Tau protein at the synapses has been reported where Tau is suggested to be involved in the N-methyl-D-aspartate (NMDA) signaling (Ittner et al. 2010; Gotz et al. 2013). These new data on the relationship between Tau protein and NMDA receptors support the results of earlier work suggesting the essential role of NMDA, but not AMPA, receptors in stress-triggered morphofunctional alterations in neurons (Magarinos and McEwen 1995a; 1995b; Pawlak et al. 2005; Martin and Wellman 2011). Notably, GluN2B-contaning NMDA receptors are enriched in stress-sensitive apical dendrites (Rudolf et al. 1996) whereas AMPA receptors are clustered in basal dendrites and soma (Vickers et al. 1993), consistent with previously reported selective neuroremodeling by chronic stress of apical, but not basal, dendrites of pyramidal neurons in the PFC (Cerqueira, Taipa et al. 2007).

While chronic stress is known to lead to dendritic remodeling and synaptic atrophy in the PFC of WT mice (Cerqueira, Mailliet

Table 2 Stress-evoked protein expression changes between Tau-KO and WT mice (Tau-KO STR versus WT STR)

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

Table 3 Summary of differentially expressed proteins across all group comparisons

Protein name	Tau-KO CON versus WT CON	WT STR versus WT CON	Tau-KO STR versus Tau-KO CON	Tau-KO STR versus WT STR
Mitochondria-related proteins				
Atp5a1			↑	↑
Atp5b			↑	↑
Ckmt1			↑	↑
Cox6c			↑	
Cyc1			↑	↑
Endogl1			↑	
Gpd2			↑	↑
Hspd1				↓
Mtnd4			↑	↑
Ndufa5				↑
Ndufa9				↑
Ndufs1			↑	↑
Ndufs2				↑
Ndufs6				↑
Ndufv2				↑
Phb			↑	
Phb2			↑	
Sfxn1			↑	↑
Sfxn3			↑	
Sfxn5				↑
Slc25a11				↑
Slc25a12			↑	
Slc25a22			↑	↑
Slc25a3			↑	↑
Slc25a4			↑	↑
Slc25a46			↑	
Slc25a5			↑	↑
Uqcrc2			↑	↑
Uqcrfs1			↑	↑
Vdac1			↑	
Vdac3			↑	
Aco2		↑		↓
Cytosol/membrane-related proteins				
Anxa5				↑
Atp1a1		↓		
Atp1a2				↑
Atp2b2		↓		
Ckb		↑		↓
Cltc		↑		
Gdi1		↑		
Hnrnpa2b1		↑		↓
Slc3a2				↑
Uba1		↑		
Cytoskeleton-related proteins				
Cnp	↓			
Mtap2				↓
Tnr	↓			
Tubb4	↓			

Proteins are listed according to their subcellular localization.

et al. 2007; McEwen and Morrison 2013), there is a conspicuous lack on information on the contributing pathways and mechanisms. The present quantitative proteomic analysis of PFC synaptosomes now reveals that stress results in the differential expression of a number of cytosolic/membrane proteins located within synapses. We observed decreased expression of 2 membrane proteins, ATPases Atp1a1 and Atp2b2, which are implicated in synaptic homeostasis, neurotransmission, and buffering of neurons against Ca^{2+}-dependent excitotoxic damage. Decreased ATPase levels are involved in synaptic pathology related to the neurotoxic peptide Aβ and Ca^{2+} in AD patients and mouse models of the disease (Chauhan et al. 1997; Vitvitsky et al. 2012), while ATPases have been suggested to offer neuroprotection against AD pathology (Lu et al. 2014). Here, it is pertinent to recall that previous studies revealed that chronic stress and stress hormones (GCs) increase production of Aβ, which is related to synaptic malfunction and atrophy (Catania et al. 2009). Reduced levels or absence of processed Atp1a1 have been associated with hypersecretion of CORT (Moseley et al. 2007; Mozhui et al. 2010). Among the proteins with increased expression in WT STR compared with WT CON mice, Rab GDP dissociation inhibitor alpha (Gdi1) has been reported as a candidate marker of stress reactivity in a chronic mild stress rat model of depression (Bisgaard et al. 2012) whereas increased creatine kinase B-type (Ckb) levels were found in acutely stressed rats (Yang et al. 2014). Generation of overexpressing or knock-out mouse models for the proteins with altered expression levels identified here and their subsequent characterization would shed light on the functional interdependencies of these proteins on synaptic atrophy and malfunction.

Our results demonstrate that Tau is essential for the manifestation of the effects of stress on dendritic and synaptic atrophy in the PFC. In order to obtain an insight into the underlying molecular processes, we compared the PFC synaptosomes of stressed and control Tau-KO animals using a state-of-the-art comparative proteomic approach that provides high quantification accuracy (Filiou and Turck 2012; Filiou 2013). Synaptosomes are artificially isolated synapses that also include cytosol, membranes, and mitochondria (Schrimpf et al. 2005). The specificity of the synaptosome enrichment protocol has been previously addressed (Filiou et al. 2010). We observed that while chronic stress alters the expression levels of mainly cytosolic proteins in the synaptosomal fraction of WT mice, chronic stress only affected mitochondrial proteins in the synaptosomes of Tau-KO mice (Table 3). This finding strongly suggests that stress-induced neuronal damage and behavioral impairments depend on an interaction between Tau and mitochondrial proteins. Oxidative phosphorylation (ETC complexes I, III, IV, and V) and mitochondrial transport (Slc25 and sideroflexin family) were the main processes affected by chronic stress in Tau-KO mice. Interestingly, these 2 processes were also affected when stressed Tau-KO and WT mice were compared. Mitochondrial dysfunction and alterations in ETC complexes have been linked to pathological oxidative stress and apoptotic pathways (Einat et al. 2005; Szego et al. 2010) and are implicated in psychiatric disorders in which stress is a main etiopathogenic factor (Shao et al. 2008; Sousa and Almeida 2012). Indeed, altered expression of proteins involved in oxidative phosphorylation and mitochondrial transport have been reported in mice selectively bred for high versus low anxiety (Filiou et al. 2011), transgenic mice with schizophrenia-like symptoms (Otte et al. 2011; Filiou et al. 2012) and in patients suffering from major psychiatric disorders (Gardner et al. 2003; Rezin et al. 2009).

We previously showed that chronic stress increases the generation of Aβ and results in Tau hyperphosphorylation in WT mice (Catania et al. 2009; Sotiropoulos et al. 2011) while other authors reported that both, Aβ and Tau hyperphosphorylation interfere with mitochondria mobility (Vossel et al. 2010; Shahpasand et al. 2012). Interestingly, while genetic or shRNA-driven Tau deletion does not interfere with mitochondrial trafficking, Tau ablation prevents Aβ-induced defects in the motility of mitochondria in dendrites (Zempel et al. 2013) and axons (Vossel et al.

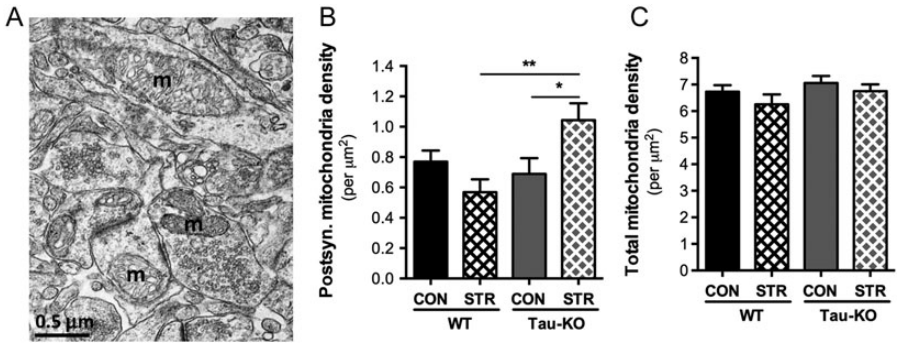

Figure 3. Chronic stress increases the number of mitochondria in synapses of Tau-KO, but not WT, animals. (*A*) TEM image showing the presence of mitochondria (m) in a synapse of a medial PFC neuron. (*B*,*C*) Exposure of Tau-KO but not WT animals to chronic stress increases the density of mitochondria in the post-synaptic compartment (*B*) whereas total mitochondria density was not different among groups (*C*). All numerical data are shown as mean ± SEM. (*P < 0.05; **P < 0.01).

2010, 2015). Our TEM-based findings show that exposure of Tau-KO, but not WT, mice to stress leads to an increase in the density of mitochondria in the post-synaptic compartment. Based on this, it is highly plausible that stress-induced Tau hyperphosphorylation in WT animals affects mitochondrial localization at the synapses whereas absence of Tau blocks this stress-damaging effect on mitochondria motility leading to dendritic and synaptic protection against stress (MacAskill et al. 2009). Notably, Tau malfunction-associated neuronal damage has been suggested to involve mitochondria (Atlante et al. 2008; McInnes 2013) and a causal link between mitochondria, abnormal Tau phosphorylation, and impaired synaptic plasticity in AD pathology has been previously proposed (Lee et al. 2012; Schulz et al. 2012). The present findings add support to previous indications that Tau reduction or prevention of Tau malfunction could be neuroprotective (Roberson et al. 2007; Vossel et al. 2010) and they suggest a close link between Tau and synaptic mitochondria in the mechanisms underlying stress-induced synaptic damage and neuronal malfunction. Indeed, it is proposed that mitochondria play a key role in conserving synapses (Cho et al. 2009). Disruption of mitochondrial proteins has been shown to reduce dendritic arborization (without major changes in axon morphology) (Chihara et al. 2007), indicating a critical role of mitochondria in neuronal architecture. Furthermore, besides their role in generating energy necessary for synaptic maintenance and activity, mitochondria are also involved in Ca^{2+} homeostasis and signaling, play a role in cytoprotection and neuronal plasticity (Toescu and Verkhratsky 2004; Cheng et al. 2010) and are dynamically regulated by GCs (Du et al. 2009). Taken together, the above findings suggest a potential association between the absence of synaptic loss and changes in mitochondrial localization and protein expression in the synapses of stressed Tau-KO animals.

In summary, the current study demonstrates a lack of stress-induced neuronal atrophy and cognitive deficits in the absence of Tau protein, providing a solid proof that Tau plays an essential role in the stress-directed orchestration of cellular cascades involved in dendritic and synaptic atrophy/loss and subsequent cognitive deficits. The implication of mitochondria in the mediation of stress effects via Tau protein encourages exploration of the potential importance of mitochondrial pathways in the search for means to prevent, delay or treat neurodegenerative conditions; it is worth noting that mitochondrial pathways have already been shown to be amenable to pharmacological manipulation in various disease settings (Fulda et al. 2010; Edeas and Weissig 2013), including stress-related pathologies (Nussbaumer et al. 2015).

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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