Dysregulation of autophagy and stress granule-related proteins in stress-driven Tau pathology

Running Title: Autophagy & RBPs dyregulation in stress pathology

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Abstract

Imbalance of neuronal proteostasis associated with misfolding and aggregation of Tau protein is a common neurodegenerative feature in Alzheimer's disease (AD) and other Tauopathies. Consistent with suggestions that lifetime stress may be an important AD precipitating factor, we previously reported that environmental stress and high glucocorticoid (GC) levels induce accumulation of aggregated Tau; however, the molecular mechanisms for such process remain unclear. Herein, we monitor a novel interplay between RNA-binding proteins (RBPs) and autophagic machinery in the underlying mechanisms through which chronic stress and high GC levels impact on Tau proteostasis precipitating Tau aggregation. Using molecular, pharmacological and behavioral analysis, we demonstrate that chronic stress and high GC trigger a mTOR-dependent inhibition of autophagy, leading to accumulation of Tau aggregates and cell death in P301L-Tau expressing mice and cells. In parallel, we found that environmental stress and GC disturb cellular homeostasis and trigger the insoluble accumulation of different RBPs, such as PABP, G3BP1, TIA-1, and FUS, shown to form Stress granules(SGs) and Tau aggregation. Interestingly, an mTOR-driven pharmacological stimulation of autophagy attenuates the GC-driven accumulation of Tau and SG-related proteins as well as the related cell death, suggesting a critical interface between autophagy and the response of the SG-related protein in the neurodegenerative potential of chronic stress and GC. These studies provide novel insights into the RNA-protein intracellular signaling regulating the precipitating role of environmental stress and GC on Tau-driven brain pathology.

Introduction

Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder with a complex pathophysiology and still undefined initiators. Several risk factors have been associated with AD pathology, with recent evidence supporting a detrimental role of lifetime stress [1–3]. Clinical studies relate distress, high cortisol levels and dysfunction of hypothalamus-pituitary-adrenal (HPA) axis with poor memory scores and earlier disease onset in AD patients highlighting the potential implication of chronic stress and glucocorticoids (GC) in the pathogenesis and/or progression of the disorder [4–6]. In line with the above clinical evidence, experimental studies have shown that chronic stress and exposure to high GC levels trigger Tau hyperphosphorylation and malfunction leading to its accumulation, formation of neurotoxic Tau aggregates and AD pathology [1,7,8]. Despite our little knowledge about the molecular mechanisms that underpin stress-driven pathology, experimental evidence suggests that stress/GC reduces Tau turnover [9], suggesting that stress/GC impact on the chaperones and proteases that regulate Tau levels [8].

Impaired proteostasis is thought to lead to the accumulation of misfolded and aggregated proteins, causing a corresponding increase in neuronal vulnerability and neurodegeneration. One sign of impaired proteostasis is the massive accumulation of autophagic vacuoles and proteins in the autophagic-lysosomal pathway which occurs in brains of AD cases [10,11]. Divided in different steps, e.g. initiation, elongation and maturation, the (macro)autophagy process critically involves several evolutionarily-conserved molecules such as the microtubule-associated protein light chain 3 (LC3), autophagy receptor p62 and (mammalian target of rapamycin) mTOR [12]; the latter has been suggested as therapeutic target against pathological aggregation of Tau and related AD neurotoxicity [13,14]. Moreover, inhibition of autophagic-lysosome pathway is also shown to impair the degradation and dynamics of stress granules (SGs) [15]. SGs are cytoplasmic complexes containing mRNAs and different RNA-binding proteins (RBPs) such as the SG-associated T cell intracellular antigen 1 (TIA1), poly(A)-binding protein (PABP), Fused in Sarcoma protein (FUS) and the stress granule assembly factor, Ras-GTPase-activating protein binding protein 1 (G3BP1) [16]. Several SG-associated RBPs have been associated with neurodegenerative diseases with a close link being recently identified

between the SGs core-nucleating protein TIA-1 and Tau. Specifically, TIA-1 is shown to bind to Tau contributing to its aggregation and Tau-related neurodegeneration and toxicity found in AD and other Tauopathies [17–19]. Based on the above, we hereby monitor the role and potential interplay between autophagy, SG-related proteins and Tau aggregation in stress-driven brain pathology.

Methods and Materials

Animals and stress protocol

6-8 month old P301L-Tau transgenic mice, expressing mutated (P301L) human Tau under the CAMKII promoter (N= 7-9 per group) were used in this were used in this study[20]. 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 22ºC; relative humidity of 55%, ad libitum access to food and water). Animals were subjected to chronic unpredictable stress protocol over a period of four weeks before the behavioral testing. Experiments were replicated 3 times (7-9 mice per group for each experimental replicate). The stress protocol consists of different stressors such as overcrowding, rocking platform, restrain, hair dryer (one stressor per day) that were chosen in a random order to prevent habituation. Stress efficacy was monitored by measurements of daytime serum corticosterone levels (monitored by a radioimmunoassay kit from ICN, Costa Mesa, CA) and body weight. All stressed animals showed significant elevations in daytime serum corticosterone levels ($p<0.05$) and net loss of body weight ($p<0.05$) reflecting the stress efficacy (see Figure 1).

Behavior Testing

105 Open Field (OF) test was conducted in an arena (43.2 cm43.2 cm) with transparent acrylic walls and
106 white floor (Med Associates Inc., St. Albans, VT, USA). Mice were placed in the center of the arena
107 and their movement was automatically monitored over a period of 5 minutes with the aid of two 16-
108 beam infrared arrays. Time spent in the center of the arena was used as an index of anxious behavior.
109 Total distance traveled was used as an indicator of locomotor activity.
110 Elevated-Plus Maze(EPM) test was used to access anxious behavior. Briefly, animals were placed in
111 the center of the EPM apparatus and entries as well as time spent in open and closed arm were
112 measured for 7 min as previously described [21]. Data were collected using a CCD camera by the use
113 of NIH Image program (http://rsb.info.nih.gov/nih-image/) and were analyzed using customized
114 software based on Matlab (version 7.2, Mathworks Co Ltd, CA) with image analysis tool box
115 (Mathworks Co Ltd, CA).
116 Y-Maze test was used to assess PFC-dependent memory based on spontaneous alternation task.
117 Briefly, animals were placed in the center of the Y-maze apparatus (33cm x 7cm x 15cm) and allowed
118 to freely move for 8 minutes. The number and order of arm entries was recorded. Spontaneous
119 alternations were calculated as the ratio of number of triads (sequence of three consecutive arm
120 entries) and total arm entries.
121 Reversal Learning test was performed in swimming circular pool (1m diameter) filled with water (24ºC)
122 made opaque with a white bio-safe dye. The cylinder contained a slightly submerged transparent
123 escape platform and placed in a room with landmark (reference) objects. Learning trials (3 trials/day;
124 60-s trial period) start by gently placing mice on the water surface close to the cylinder wall. After
125 subjected to probe test, animals were tested (4 trials) for reversal learning task where the platform
126 was moved to the opposite quadrant of swimming pool. Swim path of each animal was recorded by a
127 CCD camera and analyzed using customized software based on Matlab (version 7.2, Mathworks Co
128 Ltd, CA) with an image analysis tool box (Mathworks) [20,22].
129 Contextual Fear Conditioning (CFC) test was conducted CFC sound- and light- proof chambers (Med
130 Associates, St. Albans, VT). On day 1, mice were placed in the conditioning white chamber (Context

A) and received 3 pairings of light (20 sec) and a co-terminating electrical shock (2 sec, 0.5 mA). The chambers were cleaned with 10% ethanol between animal trials. On day 2, animals were placed in the same context (context A) in the absence of the light-shock stimuli. After it, the animals returned to their home cage. Two hours later, the animals were placed in a new context (context B) for 3 min. The context B trial was different from context A in several ways: i) the floor and walls of the chamber were covered by black plastic inserts; ii) the chamber was scented with vanilla; iii) the chamber ventilation fan was turned on; iv) the experimenter wore a different style and color of gloves, mask and lab coat; v) mice were kept in a different holding room before testing and transported in a different cage; vii) the lights of the experimental room were turned on. Mice behavior was recorded by CCD camera and freezing behavior was manually scored using Kinoscope software (http://sourceforge.net/projects/kinoscope/).

Biochemical fractionation and immunoblotting

Hippocampus and medial prefrontal cortex (PFC) tissue was macrodissected (on ice) and immediately stored at –80ºC. For detecting insoluble Tau, the sarkosyl-based fractionation protocol was used as previously described [20,23]. After homogenization in Tris-HCl buffer (10mM Tris, 150mM NaCl including protease and phosphatases inhibitors), lysates were centrifuged at 100.000g. The pellet was re-homogenized in salt/sucrose buffer (0.8M NaCl, 10mM Tris-HCl, 1 EGTA, pH=7.4, 10% sucrose solution including protease and phosphatases inhibitors. After addition of 10% Sarkosyl solution (Sigma, #L-5125), incubation (37ºC; 1h) and centrifugation (150.000g), the resulting pellet was analyzed as sarkosyl-insoluble fraction. As insoluble stress granules (SGs) cannot be detected in sarkosyl-insoluble fractions [18], we separate soluble and insoluble SGs using RIPA buffer (50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM EGTA, including protease and phosphatase inhibitors) as previously described [19]. Homogenates were layered onto 0.32 M sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl, 0.8 M NaCl, 1 mM EGTA) and were centrifuged (20000g, 10 min, 4ºC). The supernatants were ultracentrifuge at 112.000g (60 min; 4ºC). The supernatant (soluble

fraction) was collected and stored at -20ºC. The remaining pellet was dissolved in 1% SDS/RIPA buffer and centrifuged at 112000g (60 min; 4ºC; 2 times) followed by 1% SDS/TBS buffer and ultracentrifugation (60 min; 20ºC). The final pellet was dissolved in 70% formic acid and centrifuged at 20000g (10 min; 20ºC). The supernatant was concentrated in speed vacuum and the resulting pellet dissolved in sample buffer and neutralized with 1.5M Tris-HCl providing the insoluble SG fraction.

The above samples were electrophoresed using SDS-PAGE gels (17%-acrylamide gel for LC3II detection, 10%-acrylamide for the remaining proteins) and semi-dry transferred onto nitrocellulose membranes (Trans-Blot Turbo blotting system, BIORAD). Membranes were blocked with 5% nonfat dry milk in TBST-T buffer and then, incubated with the following antibodies: actin (1:2500; ABCAM, #ab8224), LC3 (1:1000; Novus Biologicals, #100-233), SQMTS1/p62 (1:1000;Novus Biologicals, #H00008878-M01), S6K total (1:750; Cell Signaling, #2708), p-S6K (1:750; Cell Signaling, #9205), p38 total (1:750; Cell Signaling, #9212) and p-p38 (1:750; Cell Signaling,#4511); eIF4E (1:500, Santa Cruz, #sc-9976), mTOR total (1:500; abcam #32028), p-Ser2448-mTOR (1:200; Cell Signaling, #5536), Tau5 (1:2000; ABCAM, #ab80579), JM (1:1000; kindly gift by Dr A. Takashima, Japan, recognizes human Tau), TLS/FUS (1:500; ABCAM, #84078), EWRS1 (1:1000; ABCAM, #133288), DDX5 (1:1000; ABCAM, #21696), TIA-1 (1:500; ABCAM, #40693), PABP (1:500, ABCAM #21060), tubulin (1:5000, SIGMA #9026), Ac-tubulin (1:1000, ABCAM #24610), Cortactin (1:250, ABCAM #81208), Ac-cortactin (1:10000, Millipore #09881) and HDAC6 (1:100, ABCAM #1440). After incubation with appropriate secondary antibody, antigens were revealed by ECL (Clarity, Bio-Rad), and signal quantification was achieved using a ChemiDoc instrument and and ImageLab software (Bio-Rad). All values were normalized and expressed as a percentage of control values.

Immunofluorescence and stereological analysis

As previously described [20], deeply anesthetized animals were transcardially perfused with saline and PFA (4%). After post-fixation, brains were placed in 30% sucrose and sectioned using vibratome. Sections were exposed to antigen retrieval by citrate buffer, followed by 0.3% Triton X-100 before blocking with solution (5% BSA in TBS-Triton X-100 (0.25%) + 5% FBS) for 30min (RT). Then, incubation with appropriate primary antibody: LC3 (1:100; Novus Biologicals), SQMTS1(p62) (1:500; Novus Biologicals), TIA-1 (1:200; ABCAM, #40693), PABP (1:100, mouseAb, SantaCruz #166381), PABP (1:200, rabbitAb, ABCAM #21060) and PHF1(1:100, kindly provided by Dr. Peter Davies), followed by incubation with the appropriate secondary antibodies and DAPI staining (1:1000; 10 min). For LC3 and p62, stained cells were counted and densities were quantified and normalized for number of cells using the Olympus BX 51 stereological microscope and the Visiopharma integrator system software. Neuronal densities of hippocampal and PFC areas (DG, CA1 and PrL) were stereologically estimated by counting neurons in cresyl-violet stained serial coronal brain sections, using Neurolucida software (MBF Bioscience, Williston, VT) as previously described [24,25].

Human AD brain tissue

Temporal cortex tissue from human brain was used for the immunohistochemical studies. Human tissue was sectioned at 20um on a cryostat and stained as free floating sections. To quench autofluorescence, sections were photobleached under a 1500 lumen white LED bulb for a minimum of 72 hours at 4°C while suspended in PBS[26]. Sections were then washed in PBS followed by TBS with 0.25% Triton-X incubation. Samples were then incubated in 1%w/v sodium borohydride (NaBH4; Sigma-Aldrich Cat#452882-25G) in PBS for 45 minutes to quench aldehyde autofluorescence which results from the over-fixation of tissue. Then citrate incubation was preformed, followed by solution (Vector Cat#H-3300) at 95°C, blocking with donkey serum in PBS-T; primary antibody incubation with PABP (1:150, ABCAM #21060), DDX6 (1:150, Bethyl Laboratories #A300-460A) and p-Tau-CP13 (1:150, kindly provided by Dr. Peter Davies), was followed by incubation with the appropriate secondary. All imaging was done using a Zeiss LSM 700 confocal microscope.

208

209 **P301L-Tau-SHSY5Y cells, treatments and analysis**

210 These studies used SH-SY5Y cells stably transfected with P301LhTau-EGFP (2N4R) (kind gift from
211 Professor Juergen Gotz, University of Queensland, Australia). Cells were cultured in DMEM
212 supplemented with 10% FBS 1% G-max and 1% antibiotic (37ºC and 5%CO_2; all reagents obtained
213 by Invitrogen); for selection purposes, 3ug/mL Blasticidin S hydrochloride (Sigma, #15205) was added
214 in the medium. For all experiments, cells were placed on gelatin-coated plates and differentiated for
215 6-7 days with differentiation medium [DMEM, 1% FBS, 1% antibiotics, 1% glutamax, 10^{-5}M all-trans
216 retinoic acid (Sigma)]. Each experiment has 3-4 biological replicates per condition, and experiments
217 were repeated at least 3 times. Dexamethasone (DEX; Fortecortin®, Merck, Darmstadt, Germany)
218 was used at a final concentration of 10^{-6}M for 48h, as previously described[9]. For mTOR inhibition,
219 Temsirolimus (CCI-779, LC Laboratories; 100uM; 48 hrs) was used; CCI-779 exhibits no toxicity in
220 this concentration [27,28]. Cell viability was assessed by MTS assay (CellTiter 96®, Promega, WI, USA)
221 based on manufacturer instructions by the use of ELISA reader (BioRad); triplicates of each condition
222 were used and experiment were repeated three times. For puromycin and cycloheximide treatment,
223 cells were incubated with 5ug/ml of puromycin (Sigma #P8833) or 10ug/mL with cycloheximide
224 (Millipore, #CAS-66-81-9) 6h before the end of the 48h DEX incubation period. For Bafilomycine
225 treatment, cells were incubated with 10nM Bafilomycine A1(Bafilomycin A1 Santa Cruz #CAS-88899-
226 55-2) for 4hrs for analysis of autophagy flux. For WB analysis, cell homogenates were prepared in
227 RIPA buffer (50mM Tris HCl, 2mM EDTA, 250mM NaCl, 10% glycerol, proteinase and phosphatase),
228 After sonication and centrifugation (15min; 14.000rpm; 4ºC), supernatant samples were analyzed by
229 WB as described above. Total and sarkosyl-resistant Tau aggregates of cell homogenates were
230 detected after sample microfiltration. Briefly, 60 ug of samples were diluted in 200 ul of PBS with or
231 without 1% (wt/vol) Sarkosyl and boiled for 5 min. Samples were filtered through a cellulose acetate
232 membrane (0.2 μm, Whatman), pre-equilibrated in PBS, using a Bio-Dot SF microfiltration apparatus
233 (Bio-Rad). The membrane was washed twice with PBS before being probed with Tau-5 antibody

(1:2500, Abcam) as described above. For IF analysis, cells were cultured in gelatin-coated glass coverslips and fixed in 4%PFA. After permeabilization with 0.1% TritonX-100/PBS, cells were incubated overnight with primary antibodies: LC3(1:200; Novus Biologicals, #100-233), SQMTS1(p62) (1:200; Novus Biologicals, #H00008878-M01), Tau-5 (1:1000; ABCAM, #ab80579), TLS/FUS (1:300; ABCAM, #84078), G3BP (1:500; ProteinTech, # 13057-2-AP), TIA-1 (1:300; ABCAM, #40693), HDAC6 (1:200; ABCAM, #ab1440). After appropriate fluorescence-conjugated secondary antibodies (RT, 30 min) and DAPI staining, cells were analyzed by laser confocal microscopy (Zeiss LSM 510, Carl Zeiss Microimaging, Goettingen, Germany).

Statistical Analysis

Numerical data is expressed as group mean±SEM. All data were evaluated by Student's *t*-test or one-way ANOVA (followed by poshoc Sidak test) when appropriate, using GraphPad 6.0; differences were considered to be significant if $p< 0.05$.

Results

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

The hippocampus and the prefrontal cortex (PFC) are some of the brain areas most affected in AD, exhibiting a characteristic accumulation of pathological, aggregated Tau (e.g. hyperphosphorylated, truncated and misfolded); the accumulation of pathological Tau is closely correlated with cognitive impairment [29,30]. Previous studies have shown that stress and GC trigger aberrant hyperphosphorylation, misfolding and missorting of Tau [7,9,31,32]. In the current study, we sought to clarify the impact of chronic stress on Tau aggregation mechanism(s) and resulting behavioral deficits. These studies use the P301L-Tau transgenic mice expressing 4R0N human Tau carrying the aggregation-prone P301L-Tau mutation[20]; these mice were then subjected to chronic unpredictable stress. Stressed P301L-Tau animals exhibited a clear decrease in body-weight in comparison to

control (non-stressed) animals (Fig. 1a). They also showed elevated levels of the stress hormone, corticosterone (Fig. 1a), providing confirmation of the stress protocol efficacy. To access hippocampus-dependent cognitive performance, Contextual Fear Conditioning test (CFC) was used. While both groups showed similar freezing levels in pre-training session of context A, stressed animals exhibited lower levels of freezing at the next day (Context A) compared to control animals indicating deficits of associated memory (Fig. 1b). Of note, the stress-driven difference disappeared when both groups where tested in another, non-associated with adverse stimulus, context (context B) (Fig. 1b). Furthermore, animals behavioral flexibility and working memory function were tested using the reversal learning and Y-maze test, respectively. During the reversal learning test, stressed animals exhibited an increase in the time to reach the escaping platform placed at the opposite quadrant in comparison to control animals suggesting cognitive deficits (Fig. 1c). In the Y-maze, stressed mice presented a decrease in the percentage of spontaneous alternations among different arms of the Y-maze apparatus (Fig. 1d), pointing towards a stress-driven impairment in working memory. As non-cognitive neuropsychiatric symptoms, such as anxiety, are frequently observed in AD [33], we also evaluated anxious behavior using open field (OF) and elevated-plus maze (EPM). We found that chronic stress decreased the time that animals spent in the center of the OF arena (Fig. 1e) and reduced both time and entries in the open arms of EPM apparatus (Fig. 1f). Notably, stress did not cause any change in locomotion as assessed by total distance travelled in the OF apparatus (Fig. 1e). The above stress-driven behavioral deficits in P301L-Tau Tg animals were accompanied by increased levels of Sarkosyl-insoluble Tau, in both hippocampus and PFC, of stressed animals as measured by Western blot analysis (Fig. 1g). These Tau aggregates are biochemically similar to those found in the neurofibrillary tangles that characterize AD and other Tauopathies [30]. Furthermore, P301L-Tau aggregates are shown to exhibit a major trigger of neurotoxicity in Tau pathology [20], which is in line with the reduction of cell density that stressed P301L-Tau animals exhibited in hippocampus (CA1 and DG) and PFC (prelimbic cortex; PrL) when compared to control P301L-Tau animals (Fig. 1h).

Stress-driven inhibition of autophagy through mTOR activation

A growing body of research has connected autophagy to neurodegenerative pathology while autophagic clearance has been suggested to exhibit selectivity for the degradation of Tau aggregates [11]. Accordingly, we have monitored autophagy focusing on LC3 and p62, the most widely used indicators of autophagic flux (Fig. 2a). Molecular analysis of hippocampus and PFC of P301L-Tau animals showed that chronic stress reduced the levels of LC3 and increased levels of p62 (Fig. 2b) in hippocampus and PFC; this was further confirmed by parallel shifts in the IF intensity of LC3 and p62 (Fig. 2c-e). As many studies describe an essential role for mTOR in protein homeostasis through its involvement in the initiation of autophagic process [34], we assessed mTOR activity by analyzing mTOR and some of its downstream targets (Fig. 2f). We found that chronic stress increased the levels of phosphorylated mTOR and the stress-activated protein kinase, p38; the later is suggested to be involved in both Tau pathology and mTOR activation[35–37]. Furthermore, the phosphorylated levels of downsteam mTOR target, S6K was also elevated by stress while the protein levels of the downstream target, eIF4E were decreased by chronic stress (Fig. 2f). Altogether, the above findings suggest that exposure to chronic stress may inhibit autophagy, probably at the level of mTOR-mediated induction of autophagy (Fig. 2a).

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

Despite the fact that the detrimental effects of chronic stress on neuronal structure and function are largely attributed to GC [38], and previous work highlights the role of glucocorticoid receptor (GR) in Tau malfunction and pathology [7,9], other studies exclude GC and GR signaling from the effect of stress on Tau [39]. Thus, we next tested the impact of prolonged treatment with high GC levels using the synthetic glucocorticoid dexamethasone on a neuronal cell line expressing P301L-Tau tagged with GFP. We found that GC treatment (10^{-6}M, 48hr) caused increase of GFP-labelled P301L-Tau protein as well as

312 total Tau levels (Fig. 3b-c) in parallel to decreased cell viability (Fig. 3a). In addition, WB analysis
313 revealed that GC impacted both on wild-type (human)Tau and exogenously-expressed (P301L-Tau)
314 human Tau protein as well as insoluble levels of Tau (Fig. 3c-d). Using both IF and WB analysis, we
315 found that autophagic markers such as LC3, p62 are also affected by GC treatment. In line with our
316 *in vivo* findings in P301L-Tau mice, GC treatment of P301L-Tau cells led to an increase in p62 levels
317 accompanied by a reduction in LC3II measured by WB analysis (Fig. 3e). Accordingly, we also
318 observed a decrease in $LC3^{+}$ puncta after GC exposure (Fig. 3f). Furthermore, in another experiment
319 moniroting autophagic flux by bafilomycin A1, a known inhibitor of the late phase of autophagy, GC
320 led to accumulation of p62 in parallel to a decrease of LC3II levels compatible with an inhibition of
321 autophagy (Supplementay Fig. S1). Altogether, the above results indicate that GC induced a blockage
322 of autophagic clearance and accumulation and aggregation of Tau.
323
324 **Chronic stress and glucocorticoids trigger accumulation of Stress granule-related proteins**
325 The cellular stress response under pathological conditions includes the translational stress response,
326 which results in the dysregulation of RBPs and consequent SG formation. These SGs have also
327 recently been shown to stimulate the accumulation of aggregated Tau [16,18]. As inhibition of autophagic-
328 lysosome pathway was recently shown to impair the degradation and dynamics of SGs [15], we next
329 monitored different SG-related proteins as well as their insoluble accumulation in P301L-Tau animals
330 under control and stressful conditions.

331 Our analysis showed the presence of different RBPs and SGs markers in soluble and insoluble
332 fractions of homogenates of P301L-Tau mice under control (non-stressed) conditions (Fig. 4), which
333 is in line with previous studies that show the association of many SG-related proteins with pathological
334 Tau [18,19]. In addition, we observed that chronic stress significantly elevated cytoplasmic localization
335 of TIA-1, and also increased the soluble levels of RBPs proteins TIA-1, DDX5, EWRS1 and TLS/FUS
336 (Fig. 4a); similar increases of insoluble levels of TLS/FUS, DDX5 and PABP were observed with
337 chronic stress (Fig. 4b). Notably, TIA-1 was not detected in the insoluble fraction in contrast with

previous work that did not utilize a stress model [18,40]. Confirming the above results, immunofluorescence staining demonstrated that chronic stress increased total and cytoplasmic TIA-1 staining in hippocampus together with a striking increase in cytoplasmic co-localization with Tau phosphorylated at amino acids 396 & 404, detected with the PHF1 antibody (Fig. 4c). We also observed an increase in PABP staining in hippocampus after stress, that co-localizes with PHF-1 p-Tau (Fig. 4d) and TIA-1 (Fig. 4e). RNA binding proteins, such as DDX6 and PABP, diffuse around PHF-1 p-Tau inclusions in human AD brain (Fig 4f), although in the human brain the RBPs appear adjacent to the inclusion, which reflects increasing exclusion as the tau pathology consolidates; similar findings are observed in late stage mouse models of tauopathy [19]. Thus, these findings suggest that chronic stress triggers the accumulation of different SG-related proteins which points towards a potential role for RBPs dysregulation in stress-driven Tau pathology.

GC exposure in a cell line of SY5Y cells stably expressing P301L-Tau also resulted in increased cytoplasmic levels and inclusions of different SG-related proteins such as TIA-1, TLS/FUS, DDX5 and G3BP (Fig. 5a). IF staining confirmed the GC-induced increased labeling of SG-related proteins TIA-1 and G3BP with increased TIA-1 reactivity and cytoplasmic translocation under GC exposure (Fig. 5b-c). For further monitoring the role of SG-related proteins in GC-driven Tau accumulation, we co-treated P301L-Tau cells with GC and either cycloheximide (CHX) or puromycin (PUR); two compounds that are protein synthesis inhibitors, but exhibit opposite effects on SG, promoting (PUR) or inhibiting (CHX) SGs assembly, respectively [19]. We found that GC and PUR co-treatment exacerbated the GC-induced increase of Tau (Fig. 5d), followed by increased levels of TIA-1 (Supplementary Fig. S2). In contrast, GC and CHX co-treatment partially attenuated GC-driven accumulation of Tau (Fig. 5e) suggesting a critical role for RBPs accumulation and the related SGs in the cellular mechanisms of GC-driven Tau accumulation.

Chronic stress and glucocorticoids impact on HDAC6 and its cytoplasmic downstream targets

Importantly, SGs as well as different parts of autophagic process (e.g. autophagosome maturation) rely on microtubule-based networks and cytoskeletal machinery. Histone deacetylase 6 (HDAC6) regulates SGs and autophagy through its influence on different cytoskeletal molecules (e.g. tubulin, cortactin), and corresponding regulation of microtubule-dependent motility [41,42]. Because HDAC6 is required for the consolidation of cellular complexes related to SGs and autophagy [41,42], we proceeded to analyze the impact of GC on HDAC6 and its cytoskeletal targets. WB and IF analyses revealed that HDAC6 levels were increased by chronic stress and GC in P301L-Tau mice and cells (Fig. 6a, 6c, 6e) which was accompanied by a decrease in acetylation levels of the cytoskeletal targets of HDAC6, tubulin and cortactin (Fig. 6b, 6d). Matching with the above stress-driven deficits in autophagic and SG-related RBPs, these data suggest a potential cytoplasmic role of HDAC6 in the stress/GC-driven cellular cascades related to imbalanced Tau proteostasis.

Pharmacological stimulation of autophagy attenuates GC-driven neurotoxic cascades.

Mounting evidence supports mTOR as an important regulator of protein homeostasis [13] while the above *in vivo* data suggest that the inhibitory effect of stress on autophagic process is mTOR-related. Thus, we next clarified whether pharmacological inhibition of mTOR could protect against GC-driven Tau-related neurotoxicity. For that purpose, we used a rapamycin analog, Temsirolimus (CCI-779) shown to be safe and recently approved by USA and European Drug authorities [43]. Co-treatment of CCI-779 with GC attenuated the reduced cell viability caused by GC (Fig. 7a) providing neuroprotection. Furthermore, we found that GC+CCI co-treatment reverted the changes that GC evoked on different autophagy-related molecules (Fig. 7b). Specifically, GC+CCI-779 co-treatment increased LC3II with similar reverse changes in p62 protein levels (Fig. 7b). Additional confirmation was also obtained by LC3 staining, where $LC3^{+}$ puncta in GC+CCI treated cells was increased to control levels (Fig. 7c), suggesting that CCI-779 blocked the GC-evoked impact of autophagic process. Moreover, CCI-779 treatment attenuated the GC-driven elevation of both exogenous (EGF-P301L-Tau) and endogenous (wildtype) Tau, as shown by both WB (Fig. 7e) and IF approaches (Fig.

7d), while it also reduced the levels of insoluble Tau aggregates (Fig. 7f); note that CCI alone had no impact on Tau levels (Supplementary Fig 3). Interestingly, CCI-779 blocked the GC-evoked elevation of different RBPs including TIA-1, G3BP, TLS/FUS and DDX5 as assessed by WB (Fig. 8a) and IF analysis (Fig. 8b-c). Note that CCI-779 treatment blocked the GC-triggered cytoplasmic translocation and accumulation (in puncta) of the SGs core-nucleating protein TIA-1 preserving its localization to nucleus (Fig. 8b). These findings suggest that CCI-779-evoked mTOR inhibition can attenuate the GC-evoked neurodegenerative cascades underlying Tau neurotoxicity.

Discussion

Understanding the molecular mechanisms that promote Tau misfolding and aggregation are of critical importance because of the key role played by Tau in mediating toxicity and neurodegeneration in AD [30,44]. Although multiple preclinical and clinical trials have focused on Tau anti-aggregation strategies with different compounds [45,46], there remains a pressing need for new strategies to prevent disease progression in AD.

The putative role of prolonged stress and excessive GC exposure in increasing brain vulnerability to disease pathology could have important implications for treatment of AD. Clinical studies reported a negative correlation between high cortisol levels and memory scores in AD subjects [5] and other implicate the activity of the hypothalamus-pituitary-adrenal (HPA) axis in the disease process[4]. Chronic stress is also correlated with earlier age of onset in familial AD [6], which further highlights a potential role of chronic stress and GC in the pathogenesis and/or progression of AD. Direct support of the neurodegenerative potential of chronic stress is provided by multiple experimental studies, including ours, that show stress and GC trigger different parameters of Tau pathology such as aberrant hyperphosphorylation, somatodendritic accumulation [1,7] and synaptic missorting[31,32]. In addition, we have recently demonstrated that chronic stress also induces truncation and misfolding of Tau leading to the formation of neurotoxic Tau aggregates[8].

In the current study, we demonstrate that activation of the mTOR pathway is required for the effect of chronic stress and GC on Tau pathology, as the mTOR inhibitor (CCI-779) blocked the GC impact on Tau proteostasis (Fig. 8d). One of the main actions of mTOR inhibition is activation of autophagy. These results expand previous *in vitro* and *in vivo* observations showing that stress/GC reduced Tau turnover [9] and deregulates molecular chaperones responsible for Tau proteostasis[8]. Autophagy is thought to play a more important role than the proteosome in the catabolism of pathological Tau [47,48] because the engulfing mechanism used by the autolysosomal system is more capable than the proteasome at degrading large macromolecular structures[10]. The potential role of autophagy is strongly supported by studies highlighting a specific relationship between autophagy deficits and Tau pathology in brains of AD patients and animals models of Tauopathies [49,50].

Autophagy is a highly-regulated process that is initiated by changes in phosphorylation states of individual components such as the Unc51-like-kinase (ULK) complex, which is mainly regulated by mTOR. Interestingly, mTOR signaling is altered in AD[51,52], with the levels of mTOR and its downstream targets, including p70S6K, being increased in AD human brains[13]. Accordingly, our current findings demonstrate that chronic stress and GC increase markers of mTOR activation and authophagic inhibition. We observed increased levels of phosphorylated mTOR and S6K, indicators of active mTOR signaling, as well as a reduced LC3II/LC3I ratio and an accumulation of p62. These findings suggest that chronic stress activates the mTOR pathway, which inhibits autophagy. The putative role of mTOR is supported by prior studies showing that chronic stress triggers mTOR activation in the hippocampus[53], and that mTOR activation is associated with increased total Tau levels in AD brains[54]. We further demonstrate that the use of the mTOR inhibitor, CCI-779, blocks the GC-driven Tau-related neurotoxicity and induction of aggregation-related cascades which is consistent with prior studies that show a decrease of mTOR signaling reverts Tau pathology [27,55]. These cumulative results point to a critical role for mTOR-dependent autophagy in the cellular mechanisms through which GC may trigger accumulation of Tau and its aggregates.

Recent studies from AD and FTDP human brains as well Tau Tg mice suggest that RBPs dysregulation and SGs represent a novel pathway that may contribute the development and progression of Tau pathology[16,19]. SGs are cytoplasmic complexes composed of phase separated complexes containing mRNAs and RNA-binding proteins (RBPs), which form to sequester non-essential transcripts in response to cellular stress. SGs are thought to constitute a protective mechanism against cellular stress by directing ribosomal system to translate mRNA transcripts coding for cytoprotective proteins [16]. The process of liquid-liquid phase separation and reversible aggregation that underlie SG formation is meant to be a transient event, but with prolonged SG induction is hypothesized to become pathological and neurotoxic [18,19]. SGs have been suggested to accelerate Tau aggregation while, in a vicious cycle, Tau stimulates SG[19]. Factors known to stimulate Tau aggregation, including hyperphosphorylation and aggregation-prone mutations, also enhance the interactions between SGs and Tau [19].

The current study suggests a novel role for RBPs in the stress/GC-driven neuronal pathology. We demonstrate for the first time that chronic stress increase the cytosplasmic levels of different RBPs and SG-associated markers (e.g. TIA-1, PABP, G3BP, FUS, DDX5) leading to its insoluble inclusion/accumulation. While SG biology involves the action of multiple different proteins leading to RBPs accumulation which is necessary for SGs[56–58], recent evidence demonstrates that TIA-1 pocess a primary role in the SG-related mechanism of Tau aggregation. Specifically, TIA-1 is transported from nucleus (where is predominantly found) to cytospasm where it interacts directly with Tau to stimulate its aggregation and insoluble accumulation/inclusion of other RBPs such as PABP and EWSR1 [17,19,59]. This is of particular importance as our current findings show that, under stressful conditions, TIA-1 is traslocated from nucleus to cytosplam accompanied by insoluble accumulation of different SGs-associated proteins and Tau aggregates; future work should further study whether and how this RBP dysregulation and insolubilization correlates to altered SG formation. Furthermore, we have recently shown that Tau missorting and dendritic accumulation are also part of chronic stress/GC hippocampal pathology [31,32] while missorting of Tau are hypothesized to facilitate formation of SGs as

part of the translational stress response [19]. While the temporal profile and precise mechanisms underlying the stress/GC-evoked dysregulation of RBPs and SG-associated cascade remain to be elucidated, the working model suggested in this study (see Fig. 8d) opens a novel window of research and therapeutic exploration focusing on biology of RNA-protein interplay in stress-related pathologies.

The interface between autophagy and RBPs dysregulation and insolubilization that we highlight in this manuscript is supported by studies suggesting that inhibition of autophagy may impact in the dynamics and removal of SGs [15,60]. Whether this occurs in the brain and in particular disease models, was not previously known. The cytoskeletal machinery is known to facilitate the aggregation of RBPs to form SGs, contribute to maturation of autophagic vesicles and autophagosome/lysosome fusion [61,62]. One of the molecules involved in these processes is histone deacetylase 6 (HDAC6), which, through the deacetylation of tubulin, reduces microtubule-dependent motility and thereby promotes the consolidation of cellular complexes such as SGs and autophagosomes [41,42,63]. Mounting evidence implicates HDAC6 in the formation of SGs in AD brain as HDAC6 seems to co-localize and interact with SG proteins under cellular stress; interestingly, HDAC6 is a SGs component interacting with G3BP [64,65]. Additionally, pharmacological inhibition or genetic ablation of HDAC6 abolished SG formation [66], while the expression of HDAC6 significantly increases in the hippocampus and other brain regions of AD patients as well as in animal models of the disease [67,68]. Our findings show that stress and GC increased HDAC6 levels, resulting in reduced acetylated levels of HDAC6's cytoskeletal targets e.g. tubulin. Reduced acetylation of tubulin is associated with microtubule instability, which is also suggested to cause enlargement of SGs [69]. Interestingly, recent reports in mice show that HDAC6 inhibition increases resilience to stress [70,71]. While these findings point to HDAC6 as a potential regulator in the stress-driven Tau pathology (see Fig. 8d), future studies should clarify its specific role in different cellular pathways evoked by chronic stress and GC.

In conclusion, the current study adds to our limited knowledge about how chronic stress increases brain vulnerability to disease aiming to illuminate novel fundamental molecular mechanisms through which stress and GC may damage neuronal homeostasis precipitating Tau-driven neurodegeneration.

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CONFLICT OF INTEREST

BW is co-founder and chief scientific officer of Aquinnah Pharmaceutics Inc. The other authors declare no conflict of interest.

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Fig. 1. Chronic stress evokes accumulation of neurotoxic Tau aggregates causing cognitive and emotional deficits in P301L-Tau Tg mice. a P301L-Tg mice exhibited reduced body weight (p=0.005) and increased corticosterone (p<0.001) levels after chronic environmental stress. **b** In contrast to pre-training session (p=0.171), stressed animals exhibited a significant decrease in percentage of freezing time in test section (context A) of CFC in comparison to control animals indicating hippocampus-dependent associative memory impairment (p<0.001); note that both animal groups exhibit similar freezing levels in context B which is not non-associated with the adverse stimuli animals received in context A (p=0.640*)*. **c** Chronic stress increased the time that animals swum to reach the new (opposite) place of the escaping platform indicating PFC-dependent deficits of behavioral flexibility (p=0.046). **d** Stress also reduced percentage of spontaneous alternations in the arms of a Y-maze as compared with control animals pointing to deficits of working memory (p=0.007). **e-f** Whereas no different in total distance travelled by animals in OF apparatus (p=0.988), stressed animals exhibited a decrease in time spent in the center of the OF arena (p=0.003) (e) followed by reduced time (p=0.004) and entries (p=0.001) that animals spend in the open arms of EPM apparatus (f); these behavioral parameters suggest increased levels of anxiety in stressed animals compared with controls. **g-h** Chronic Stress elevated the levels of Tau in sarkosyl-insoluble in both hippocampus and PFC of P301LTau mice (Hipp: p=0.028; PFC: p<0.001) (g); an effect that was accompanied by decreased cell density in PFC (prelimbic cortex; PrL: p<0.001) and hippocampus (DG: p<0.001; CA1: p<0.001) (h). All numeric data represent mean ± SEM, *P<0.05; **P<0.01; ***P<0.001.

Fig. 2. Prolonged exposure to environmental stress inhibits autophagy in a mTOR-dependent manner. a Schematic representation of autophagy highlighting the role of mTOR, LC3 and p62 in this cellular process. **b-e** Stressed animals exhibited reduced LC3 (PFC: p=0.003; HIPP: p=0.026) and increased p62 (PFC: p=0.029; HIPP: p=0.004) protein levels as assessed by WB analysis (b); these findings were confirmed by corresponding changes in LC3+ (p=0.044) and p62+ (p=0.044) fluorescence intensity/cell number (c-d) an decrease in their co-localization (e), indicating a stress-driven inhibition of autophagic process. **f** In line with the above findings, the levels of phospho S6K (p=0.014), p38 (p=0.030) and mTOR (p<0.001) proteins were increased by stress, with decrease levels of eIF4E (p=0.024), which are indicative of mTOR activation. All numeric data represent mean ± SEM, *P<0.05; **P<0.01; ***P<0.001.

Fig. 3. Exposure to glucocorticoids causes Tau accumulation and autophagy inhibition *in vitro*. a GC treatment (10^{-6}M; 48hr) of EGF-P301L-hTau SHSY cells decreased cell viability (p=0.004). **b-d** Glucocorticoid (GC) treatment triggered cytoplasmic accumulation of exogenously expressed mutated human Tau (EGF-P301L-Tau) and endogenous human Tau (WT-Tau) as

assessed by IF (f) (p=0.044) (b) and WB analysis (c) (WT-Tau: p<0.001; EGF-P301L-Tau: p=0.001), and lead to increased levels of insoluble Tau aggregates (p=0.042) (d). **e-f** GC decreased LC3II (p=0.013) protein levels with parallel increase of p62 (p=0.039) (e); immunofluorescence analysis confirmed the GC-induced reduction in LC3 (p=0.004) puncta (f). All numeric data represent mean ± SEM, *P<0.05; **P<0.01; ***P<0.001.

Fig. 4. Chronic stress evokes dysregulation of RBPs and their insoluble accumulation in P301L-tau Tg mice. a-b Chronic stress triggered an increase in the protein levels of several RBPs and SG markers in soluble fraction [TIA-1 (p=0.013), TLS/FUS (p=0.017), DDX5 (p<0.001) and EWRS1 (p=0.005)] (a), as well in insoluble fraction of P301L-Tau mice [DDX5 (p=0.02) and PABP (p=0.011)] (b). **c-d** Chronic stress causes the cytoplasmic accumulation of the SG marker TIA-1 in hippocampus (c) and the accumulation of PABP, a SG marker (d), increasing their co-localization with p-Tau (PHF1). **e** Moreover, chronic stress leads to an increase co-localization of TIA-1 and PABP in perinuclear region of hippocampal neurons, indicating the presence of TIA-1 positive stress granules. **f** Immunofluroresecent analysis of PABP and DDX with p-Tau in human AD brain. All numeric data are represented as mean ± SEM, *p<0.05; **p<0.01; ***p<0.001.

Fig. 5. Dysruption and insoluble accumulation of RBPs in GC-driven Tau accumulation. Treatment of EGF-P301L-hTau SHSY cells with GC (10^{-6}M; 48hr) elevated the cytoplasmic levels of the RBPs, TIA-1 (p=0.043), TLS/FUS (p=0.034), G3BP (p=0.004) and DDX5 (p=0.038) as assessed by WB analysis. **b-c** IF staining of TIA-1 (b) and G3BP (c) demonstrate that GC triggered their accumulation and cytoplasmic appearance in EGF-P301L-hTau SHSY cells. **d** Co-treatment of GC and puromycin (PUR), a well-known SG inducer, aggravate levels of EGF-P301L-Tau (p=0.013) and WT-Tau (p=0.023) when compared to GC treatment. **e** While co-treament of GC and CHX (the later inhibits SG formation) blocked the GC-driven Tau increase (p<0.001). All numeric data are represented as mean ± SEM, *p<0.05; **p<0.01; ***p<0.001.

Fig. 6. Stress and glucocorticoids induce HDAC6 reducing the acetylation levels of its cytoplasmic targets. a-b Chronic stress elevated HDAC6 levels (p=0.004) (a) and reduced acetylation of tubulin (p=0.016) and cortactin (p=0.048), two cytoskeletal targets of HDAC6, in P301L-Tau mice (b). **c-e** GC treatment increased HDAC6 (p<0.001) as analyzed by WB (c) and elevated HDAC6 staining in P301LTau cells (p=0.041) (e), in parallel with decreased levels of acetylated forms of tubulin (p=0.004) and cortactin (p<0.001), two cytoskeletal targets of HDAC6 (d). All numeric data are represented as mean ± SEM, *p<0.05; **p<0.01; ***p<0.001.

Fig. 7. mTOR-driven pharmacological stimulation of autophagy blocked GC-triggered Tau accumulation. a-b Combined treatment of GC with CCI-779 (Temsirolimus), a rapamycin analog, attenuated the GC-driven decrease of cell viability (p=0.002) in EGF-P301LTau SHSY5Y cells (a) and reversed the GC effect on autophagy markers increasing protein levels of LC3II (p<0.001) with parallel decrease of p62 levels (p=0.001) (b). **c** IF staining of LC3 confirmed the CCI-779-evoked blockage of GC-driven reduction of LC3II puncta (p=0.0012). **d-f** CCI-779 treatment reduced the GC-driven elevated IF levels of GFP-P301LTau (p=0.002) (d); WB analysis revealed that CCI-779 attenuated the GC-driven accumulation of both EGF-P301L-Tau (p<0.005) and WT-Tau (p<0.001) levels (e), also leading to decrease in insoluble Tau levels (f). All numeric data are represented mean ± SEM, *p<0.05; **p<0.01; ***p<0.0001.

Fig. 8. The CCI-779 inhibitor of mTOR attenuated GC-induced dysregulation of RBPs.
a-c GC-driven increase of different RBPs [TLS/FUS G3BPand DDX5] was attenuated by co-treatment with CCI-779 (p=0.022; p=0.007; p=0.045, respectively) (a); IF staining of TIA-1 (b) and G3BP (c) confirmed the blockage of GC-driven induction of RBPs by CCI-779. **c** Working/hypothetical model integrating autophagy inhibition and dysregulation of RBPs in the cellualr mechanisms through which chronic stress and/or high levels of stress hormones, glucocorticoids (GC), precipitate Tau pathology. Chronic exposure to environmental stress and/or prolonged signaling of GC receptors (GR) evoke the activation of mTOR signaling and the induction of Histone deacetylase 6 (HADC6) and subsequently, reduce acetylation of proteins related to cytoskeletal instability. These cellular events lead to inhibition of autophagic process that, together with the dysregulation and insolubilization of RBPs and the potential formation of SGs, may contribute to the accumulation of Tau and its neurotoxic aggregates causing cell death and cognitive deficits.