

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

3
4 *Running Title: Autophagy & RBPs dyregulation in stress pathology*

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28

29 **Abstract**

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

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52 **Introduction**

53 Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder with a complex
54 pathophysiology and still undefined initiators. Several risk factors have been associated with AD
55 pathology, with recent evidence supporting a detrimental role of lifetime stress¹⁻³. Clinical studies
56 relate distress, high cortisol levels and dysfunction of hypothalamus-pituitary-adrenal (HPA) axis with
57 poor memory scores and earlier disease onset in AD patients highlighting the potential implication of
58 chronic stress and glucocorticoids (GC) in the pathogenesis and/or progression of the disorder⁴⁻⁶. In
59 line with the above clinical evidence, experimental studies have shown that chronic stress and
60 exposure to high GC levels trigger Tau hyperphosphorylation and malfunction leading to its
61 accumulation, formation of neurotoxic Tau aggregates and AD pathology^{1,7,8}. Despite our little
62 knowledge about the molecular mechanisms that underpin stress-driven pathology, experimental
63 evidence suggests that stress/GC reduces Tau turnover⁹, suggesting that stress/GC impact on the
64 chaperones and proteases that regulate Tau levels⁸.

65 Impaired proteostasis is thought to lead to the accumulation of misfolded and aggregated proteins,
66 causing a corresponding increase in neuronal vulnerability and neurodegeneration. One sign of
67 impaired proteostasis is the massive accumulation of autophagic vacuoles and proteins in the
68 autophagic-lysosomal pathway which occurs in brains of AD cases^{10,11}. Divided in different steps,
69 e.g. initiation, elongation and maturation, the (macro)autophagy process critically involves several
70 evolutionarily-conserved molecules such as the microtubule-associated protein light chain 3 (LC3),
71 autophagy receptor p62 and (mammalian target of rapamycin) mTOR¹²; the latter has been
72 suggested as therapeutic target against pathological aggregation of Tau and related AD neurotoxicity
73^{13,14}. Moreover, inhibition of autophagic-lysosome pathway is also shown to impair the degradation
74 and dynamics of stress granules (SGs)¹⁵. SGs are cytoplasmic complexes containing mRNAs and
75 different RNA-binding proteins (RBPs) such as the SG-associated T cell intracellular antigen 1 (TIA1),
76 poly(A)-binding protein (PABP), Fused in Sarcoma protein (FUS) and the stress granule assembly
77 factor, Ras-GTPase-activating protein binding protein 1 (G3BP1)¹⁶. Several SG-associated RBPs
78 have been associated with neurodegenerative diseases with a close link being recently identified

79 between the SGs core-nucleating protein TIA-1 and Tau. Specifically, TIA-1 is shown to bind to Tau
80 contributing to its aggregation and Tau-related neurodegeneration and toxicity found in AD and other
81 Tauopathies¹⁷⁻¹⁹. Based on the above, we hereby monitor the role and potential interplay between
82 autophagy, SG-related proteins and Tau aggregation in stress-driven brain pathology.

83

84 **Methods and Materials**

85 **Animals and stress protocol**

86 6-8 month old P301L-Tau transgenic mice, expressing mutated (P301L) human Tau under the
87 CAMKII promoter (N= 7-9 per group) were used in this study²⁰. All experiments were
88 conducted in accordance with the Portuguese national authority for animal experimentation, Direcção
89 Geral de Veterinária (ID: DGV9457). Animals were kept and handled in accordance with the
90 guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European
91 Parliament and Council. Mice were housed in groups of 4-5 per cage under standard environmental
92 conditions (lights on from 8 a.m. [ZT0] to 8 p.m. [ZT12]; room temperature 22°C; relative humidity of
93 55%, ad libitum access to food and water). Animals were subjected to chronic unpredictable stress
94 protocol over a period of four weeks before the behavioral testing. Experiments were replicated 3
95 times (7-9 mice per group for each experimental replicate). The stress protocol consists of different
96 stressors such as overcrowding, rocking platform, restrain, hair dryer (one stressor per day) that were
97 chosen in a random order to prevent habituation. Stress efficacy was monitored by measurements of
98 daytime serum corticosterone levels (monitored by a radioimmunoassay kit from ICN, Costa Mesa,
99 CA) and body weight. All stressed animals showed significant elevations in daytime serum
100 corticosterone levels ($p<0.05$) and net loss of body weight ($p<0.05$) reflecting the stress efficacy (see
101 Figure 1).

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103

104 **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 (24°C)
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

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

142

143 **Biochemical fractionation and immunoblotting**

144 Hippocampus and medial prefrontal cortex (PFC) tissue was macrodissected (on ice) and immediately
145 stored at -80°C . For detecting insoluble Tau, the sarkosyl-based fractionation protocol was used as
146 previously described^{20,23}. After homogenization in Tris-HCl buffer (10mM Tris, 150mM NaCl including
147 protease and phosphatases inhibitors), lysates were centrifuged at 100.000g. The pellet was re-
148 homogenized in salt/sucrose buffer (0.8M NaCl, 10mM Tris-HCl, 1 EGTA, pH=7.4, 10% sucrose
149 solution including protease and phosphatases inhibitors. After addition of 10% Sarkosyl solution
150 (Sigma, #L-5125), incubation (37°C ; 1h) and centrifugation (150.000g), the resulting pellet was
151 analyzed as sarkosyl-insoluble fraction. As insoluble stress granules (SGs) cannot be detected in
152 sarkosyl-insoluble fractions¹⁸, we separate soluble and insoluble SGs using RIPA buffer (50 mM Tris,
153 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM EGTA, including protease and
154 phosphatase inhibitors) as previously described¹⁹. Homogenates were layered onto 0.32 M sucrose
155 buffer (0.32 M sucrose, 10 mM Tris-HCl, 0.8 M NaCl, 1 mM EGTA) and were centrifuged (20000g, 10
156 min, 4°C). The supernatants were ultracentrifuge at 112.000g (60 min; 4°C). The supernatant (soluble

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

163 The above samples were electrophoresed using SDS-PAGE gels (17%-acrylamide gel for LC3II
164 detection, 10%-acrylamide for the remaining proteins) and semi-dry transferred onto nitrocellulose
165 membranes (Trans-Blot Turbo blotting system, BIORAD). Membranes were blocked with 5% nonfat
166 dry milk in TBST-T buffer and then, incubated with the following antibodies: actin (1:2500; ABCAM,
167 #ab8224), LC3 (1:1000; Novus Biologicals, #100-233), SQM1/p62 (1:1000;Novus Biologicals,
168 #H00008878-M01), S6K total (1:750; Cell Signaling, #2708), p-S6K (1:750; Cell Signaling, #9205),
169 p38 total (1:750; Cell Signaling, #9212) and p-p38 (1:750; Cell Signaling,#4511); eIF4E (1:500, Santa
170 Cruz, #sc-9976), mTOR total (1:500; abcam #32028), p-Ser2448-mTOR (1:200; Cell Signaling,
171 #5536), Tau5 (1:2000; ABCAM, #ab80579), JM (1:1000; kindly gift by Dr A. Takashima, Japan,
172 recognizes human Tau), TLS/FUS (1:500; ABCAM, #84078), EWRS1 (1:1000; ABCAM, #133288),
173 DDX5 (1:1000; ABCAM, #21696), TIA-1 (1:500; ABCAM, #40693), PABP (1:500, ABCAM #21060),
174 tubulin (1:5000, SIGMA #9026), Ac-tubulin (1:1000, ABCAM #24610), Cortactin (1:250, ABCAM
175 #81208), Ac-cortactin (1:10000, Millipore #09881) and HDAC6 (1:100, ABCAM #1440). After
176 incubation with appropriate secondary antibody, antigens were revealed by ECL (Clarity, Bio-Rad),
177 and signal quantification was achieved using a ChemiDoc instrument and and ImageLab software
178 (Bio-Rad). All values were normalized and expressed as a percentage of control values.

179

180

181 **Immunofluorescence and stereological analysis**

182 As previously described²⁰, deeply anesthetized animals were transcardially perfused with saline and
183 PFA (4%). After post-fixation, brains were placed in 30% sucrose and sectioned using vibratome.
184 Sections were exposed to antigen retrieval by citrate buffer, followed by 0.3% Triton X-100 before
185 blocking with solution (5% BSA in TBS-Triton X-100 (0.25%) + 5% FBS) for 30min (RT). Then,
186 incubation with appropriate primary antibody: LC3 (1:100; Novus Biologicals), SQM1(p62) (1:500;
187 Novus Biologicals), TIA-1 (1:200; ABCAM, #40693), PABP (1:100, mouseAb, SantaCruz #166381),
188 PABP (1:200, rabbitAb, ABCAM #21060) and PHF1(1:100, kindly provided by Dr. Peter Davies),
189 followed by incubation with the appropriate secondary antibodies and DAPI staining (1:1000; 10 min).
190 For LC3 and p62, stained cells were counted and densities were quantified and normalized for number
191 of cells using the Olympus BX 51 stereological microscope and the Visiopharma integrator system
192 software. Neuronal densities of hippocampal and PFC areas (DG, CA1 and PrL) were stereologically
193 estimated by counting neurons in cresyl-violet stained serial coronal brain sections, using
194 Neurolucida software (MBF Bioscience, Williston, VT) as previously described^{24,25}.

195

196 **Human AD brain tissue**

197 Temporal cortex tissue from human brain was used for the immunohistochemical studies. Human
198 tissue was sectioned at 20um on a cryostat and stained as free floating sections. To quench
199 autofluorescence, sections were photobleached under a 1500 lumen white LED bulb for a minimum
200 of 72 hours at 4°C while suspended in PBS²⁶. Sections were then washed in PBS followed by TBS
201 with 0.25% Triton-X incubation. Samples were then incubated in 1%w/v sodium borohydride (NaBH₄;
202 Sigma-Aldrich Cat#452882-25G) in PBS for 45 minutes to quench aldehyde autofluorescence which
203 results from the over-fixation of tissue. Then citrate incubation was preformed, followed by solution
204 (Vector Cat#H-3300) at 95°C, blocking with donkey serum in PBS-T; primary antibody incubation with
205 PABP (1:150, ABCAM #21060), DDX6 (1:150, Bethyl Laboratories #A300-460A) and p-Tau-CP13
206 (1:150, kindly provided by Dr. Peter Davies), was followed by incubation with the appropriate
207 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 (37°C and 5%CO₂; all reagents obtained
213 by Invitrogen); for selection purposes, 3ug/mL Blastidicin 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, 10⁻⁵M 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 10⁻⁶M for 48h, as previously described⁹. 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; 4°C), 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

234 (1:2500, Abcam) as described above. For IF analysis, cells were cultured in gelatin-coated glass
235 coverslips and fixed in 4%PFA. After permeabilization with 0.1% TritonX-100/PBS, cells were
236 incubated overnight with primary antibodies: LC3(1:200; Novus Biologicals, #100-233),
237 SQMTS1(p62) (1:200; Novus Biologicals, #H00008878-M01), Tau-5 (1:1000; ABCAM, #ab80579),
238 TLS/FUS (1:300; ABCAM, #84078), G3BP (1:500; ProteinTech, # 13057-2-AP), TIA-1 (1:300;
239 ABCAM, #40693), HDAC6 (1:200; ABCAM, #ab1440). After appropriate fluorescence-conjugated
240 secondary antibodies (RT, 30 min) and DAPI staining, cells were analyzed by laser confocal
241 microscopy (Zeiss LSM 510, Carl Zeiss Microimaging, Goettingen, Germany).

242

243 **Statistical Analysis**

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

247

248 **Results**

249 **Chronic stress triggers accumulation of neurotoxic Tau aggregates in hippocampus and** 250 **prefrontal cortex of P301L-Tau Tg mice.**

251 The hippocampus and the prefrontal cortex (PFC) are some of the brain areas most affected in AD,
252 exhibiting a characteristic accumulation of pathological, aggregated Tau (e.g. hyperphosphorylated,
253 truncated and misfolded); the accumulation of pathological Tau is closely correlated with cognitive
254 impairment^{29,30}. Previous studies have shown that stress and GC trigger aberrant
255 hyperphosphorylation, misfolding and missorting of Tau^{7,9,31,32}. In the current study, we sought to
256 clarify the impact of chronic stress on Tau aggregation mechanism(s) and resulting behavioral deficits.
257 These studies use the P301L-Tau transgenic mice expressing 4R0N human Tau carrying the
258 aggregation-prone P301L-Tau mutation²⁰; these mice were then subjected to chronic unpredictable
259 stress. Stressed P301L-Tau animals exhibited a clear decrease in body-weight in comparison to

260 control (non-stressed) animals (Fig. 1a). They also showed elevated levels of the stress hormone,
261 corticosterone (Fig. 1a), providing confirmation of the stress protocol efficacy. To access
262 hippocampus-dependent cognitive performance, Contextual Fear Conditioning test (CFC) was used.
263 While both groups showed similar freezing levels in pre-training session of context A, stressed animals
264 exhibited lower levels of freezing at the next day (Context A) compared to control animals indicating
265 deficits of associated memory (Fig. 1b). Of note, the stress-driven difference disappeared when both
266 groups were tested in another, non-associated with adverse stimulus, context (context B) (Fig. 1b).
267 Furthermore, animals behavioral flexibility and working memory function were tested using the
268 reversal learning and Y-maze test, respectively. During the reversal learning test, stressed animals
269 exhibited an increase in the time to reach the escaping platform placed at the opposite quadrant in
270 comparison to control animals suggesting cognitive deficits (Fig. 1c). In the Y-maze, stressed mice
271 presented a decrease in the percentage of spontaneous alternations among different arms of the Y-
272 maze apparatus (Fig. 1d), pointing towards a stress-driven impairment in working memory. As non-
273 cognitive neuropsychiatric symptoms, such as anxiety, are frequently observed in AD³³, we also
274 evaluated anxious behavior using open field (OF) and elevated-plus maze (EPM). We found that
275 chronic stress decreased the time that animals spent in the center of the OF arena (Fig. 1e) and
276 reduced both time and entries in the open arms of EPM apparatus (Fig. 1f). Notably, stress did not
277 cause any change in locomotion as assessed by total distance travelled in the OF apparatus (Fig.
278 1e). The above stress-driven behavioral deficits in P301L-Tau Tg animals were accompanied by
279 increased levels of Sarkosyl-insoluble Tau, in both hippocampus and PFC, of stressed animals as
280 measured by Western blot analysis (Fig. 1g). These Tau aggregates are biochemically similar to those
281 found in the neurofibrillary tangles that characterize AD and other Tauopathies³⁰. Furthermore,
282 P301L-Tau aggregates are shown to exhibit a major trigger of neurotoxicity in Tau pathology²⁰, which
283 is in line with the reduction of cell density that stressed P301L-Tau animals exhibited in hippocampus
284 (CA1 and DG) and PFC (prelimbic cortex; PrL) when compared to control P301L-Tau animals (Fig.
285 1h).

286

287 **Stress-driven inhibition of autophagy through mTOR activation**

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

303

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

306 Despite the fact that the detrimental effects of chronic stress on neuronal structure and function are
307 largely attributed to GC ³⁸, and previous work highlights the role of glucocorticoid receptor (GR) in Tau
308 malfunction and pathology ^{7,9}, other studies exclude GC and GR signaling from the effect of stress on
309 Tau ³⁹. Thus, we next tested the impact of prolonged treatment with high GC levels using the synthetic
310 glucocorticoid dexamethasone on a neuronal cell line expressing P301L-Tau tagged with GFP. We
311 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 monitoring 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 (Supplementary 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 SGs¹⁵, 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, EWSR1 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

338 previous work that did not utilize a stress model ^{18,40}. Confirming the above results,
339 immunofluorescence staining demonstrated that chronic stress increased total and cytoplasmic TIA-
340 1 staining in hippocampus together with a striking increase in cytoplasmic co-localization with Tau
341 phosphorylated at amino acids 396 & 404, detected with the PHF1 antibody (Fig. 4c). We also
342 observed an increase in PABP staining in hippocampus after stress, that co-localizes with PHF-1 p-
343 Tau (Fig. 4d) and TIA-1 (Fig. 4e). RNA binding proteins, such as DDX6 and PABP, diffuse around
344 PHF-1 p-Tau inclusions in human AD brain (Fig 4f), although in the human brain the RBPs appear
345 adjacent to the inclusion, which reflects increasing exclusion as the tau pathology consolidates; similar
346 findings are observed in late stage mouse models of tauopathy ¹⁹. Thus, these findings suggest that
347 chronic stress triggers the accumulation of different SG-related proteins which points towards a
348 potential role for RBPs dysregulation in stress-driven Tau pathology.

349 GC exposure in a cell line of SY5Y cells stably expressing P301L-Tau also resulted in increased
350 cytoplasmic levels and inclusions of different SG-related proteins such as TIA-1, TLS/FUS, DDX5 and
351 G3BP (Fig. 5a). IF staining confirmed the GC-induced increased labeling of SG-related proteins TIA-
352 1 and G3BP with increased TIA-1 reactivity and cytoplasmic translocation under GC exposure (Fig.
353 5b-c). For further monitoring the role of SG-related proteins in GC-driven Tau accumulation, we co-
354 treated P301L-Tau cells with GC and either cycloheximide (CHX) or puromycin (PUR); two
355 compounds that are protein synthesis inhibitors, but exhibit opposite effects on SG, promoting (PUR)
356 or inhibiting (CHX) SGs assembly, respectively ¹⁹. We found that GC and PUR co-treatment
357 exacerbated the GC-induced increase of Tau (Fig. 5d), followed by increased levels of TIA-1
358 (Supplementary Fig. S2). In contrast, GC and CHX co-treatment partially attenuated GC-driven
359 accumulation of Tau (Fig. 5e) suggesting a critical role for RBPs accumulation and the related SGs in
360 the cellular mechanisms of GC-driven Tau accumulation.

361

362 **Chronic stress and glucocorticoids impact on HDAC6 and its cytoplasmic downstream**
363 **targets**

364 Importantly, SGs as well as different parts of autophagic process (e.g. autophagosome maturation)
365 rely on microtubule-based networks and cytoskeletal machinery. Histone deacetylase 6 (HDAC6)
366 regulates SGs and autophagy through its influence on different cytoskeletal molecules (e.g. tubulin,
367 cortactin), and corresponding regulation of microtubule-dependent motility^{41,42}. Because HDAC6 is
368 required for the consolidation of cellular complexes related to SGs and autophagy^{41,42}, we proceeded
369 to analyze the impact of GC on HDAC6 and its cytoskeletal targets. WB and IF analyses revealed
370 that HDAC6 levels were increased by chronic stress and GC in P301L-Tau mice and cells (Fig. 6a,
371 6c, 6e) which was accompanied by a decrease in acetylation levels of the cytoskeletal targets of
372 HDAC6, tubulin and cortactin (Fig. 6b, 6d). Matching with the above stress-driven deficits in
373 autophagic and SG-related RBPs, these data suggest a potential cytoplasmic role of HDAC6 in the
374 stress/GC-driven cellular cascades related to imbalanced Tau proteostasis.

375

376 **Pharmacological stimulation of autophagy attenuates GC-driven neurotoxic cascades.**

377 Mounting evidence supports mTOR as an important regulator of protein homeostasis¹³ while the
378 above *in vivo* data suggest that the inhibitory effect of stress on autophagic process is mTOR-related.
379 Thus, we next clarified whether pharmacological inhibition of mTOR could protect against GC-driven
380 Tau-related neurotoxicity. For that purpose, we used a rapamycin analog, Temsirolimus (CCI-779)
381 shown to be safe and recently approved by USA and European Drug authorities⁴³. Co-treatment of
382 CCI-779 with GC attenuated the reduced cell viability caused by GC (Fig. 7a) providing
383 neuroprotection. Furthermore, we found that GC+CCI co-treatment reverted the changes that GC
384 evoked on different autophagy-related molecules (Fig. 7b). Specifically, GC+CCI-779 co-treatment
385 increased LC3II with similar reverse changes in p62 protein levels (Fig. 7b). Additional confirmation
386 was also obtained by LC3 staining, where LC3⁺ puncta in GC+CCI treated cells was increased to
387 control levels (Fig. 7c), suggesting that CCI-779 blocked the GC-evoked impact of autophagic
388 process. Moreover, CCI-779 treatment attenuated the GC-driven elevation of both exogenous (EGF-
389 P301L-Tau) and endogenous (wildtype) Tau, as shown by both WB (Fig. 7e) and IF approaches (Fig.

390 7d), while it also reduced the levels of insoluble Tau aggregates (Fig. 7f); note that CCI alone had no
391 impact on Tau levels (Supplementary Fig 3). Interestingly, CCI-779 blocked the GC-evoked elevation
392 of different RBPs including TIA-1, G3BP, TLS/FUS and DDX5 as assessed by WB (Fig. 8a) and IF
393 analysis (Fig. 8b-c). Note that CCI-779 treatment blocked the GC-triggered cytoplasmic translocation
394 and accumulation (in puncta) of the SGs core-nucleating protein TIA-1 preserving its localization to
395 nucleus (Fig. 8b). These findings suggest that CCI-779-evoked mTOR inhibition can attenuate the
396 GC-evoked neurodegenerative cascades underlying Tau neurotoxicity.

397

398 **Discussion**

399 Understanding the molecular mechanisms that promote Tau misfolding and aggregation are of critical
400 importance because of the key role played by Tau in mediating toxicity and neurodegeneration in AD
401 ^{30,44}. Although multiple preclinical and clinical trials have focused on Tau anti-aggregation strategies
402 with different compounds ^{45,46}, there remains a pressing need for new strategies to prevent disease
403 progression in AD.

404 The putative role of prolonged stress and excessive GC exposure in increasing brain vulnerability to
405 disease pathology could have important implications for treatment of AD. Clinical studies reported a
406 negative correlation between high cortisol levels and memory scores in AD subjects ⁵ and other
407 implicate the activity of the hypothalamus-pituitary-adrenal (HPA) axis in the disease process⁴.
408 Chronic stress is also correlated with earlier age of onset in familial AD ⁶, which further highlights a
409 potential role of chronic stress and GC in the pathogenesis and/or progression of AD. Direct support
410 of the neurodegenerative potential of chronic stress is provided by multiple experimental studies,
411 including ours, that show stress and GC trigger different parameters of Tau pathology such as
412 aberrant hyperphosphorylation, somatodendritic accumulation ^{1,7} and synaptic missorting^{31,32}. In
413 addition, we have recently demonstrated that chronic stress also induces truncation and misfolding of
414 Tau leading to the formation of neurotoxic Tau aggregates⁸.

415 In the current study, we demonstrate that activation of the mTOR pathway is required for the effect of
416 chronic stress and GC on Tau pathology, as the mTOR inhibitor (CCI-779) blocked the GC impact on
417 Tau proteostasis (Fig. 8d). One of the main actions of mTOR inhibition is activation of autophagy.
418 These results expand previous *in vitro* and *in vivo* observations showing that stress/GC reduced Tau
419 turnover⁹ and deregulates molecular chaperones responsible for Tau proteostasis⁸. Autophagy is
420 thought to play a more important role than the proteasome in the catabolism of pathological Tau^{47,48}
421 because the engulfing mechanism used by the autolysosomal system is more capable than the
422 proteasome at degrading large macromolecular structures¹⁰. The potential role of autophagy is
423 strongly supported by studies highlighting a specific relationship between autophagy deficits and Tau
424 pathology in brains of AD patients and animals models of Tauopathies^{49,50}.

425 Autophagy is a highly-regulated process that is initiated by changes in phosphorylation states of
426 individual components such as the Unc51-like-kinase (ULK) complex, which is mainly regulated by
427 mTOR. Interestingly, mTOR signaling is altered in AD^{51,52}, with the levels of mTOR and its
428 downstream targets, including p70S6K, being increased in AD human brains¹³. Accordingly, our
429 current findings demonstrate that chronic stress and GC increase markers of mTOR activation and
430 autophagic inhibition. We observed increased levels of phosphorylated mTOR and S6K, indicators
431 of active mTOR signaling, as well as a reduced LC3II/LC3I ratio and an accumulation of p62. These
432 findings suggest that chronic stress activates the mTOR pathway, which inhibits autophagy. The
433 putative role of mTOR is supported by prior studies showing that chronic stress triggers mTOR
434 activation in the hippocampus⁵³, and that mTOR activation is associated with increased total Tau
435 levels in AD brains⁵⁴. We further demonstrate that the use of the mTOR inhibitor, CCI-779, blocks the
436 GC-driven Tau-related neurotoxicity and induction of aggregation-related cascades which is
437 consistent with prior studies that show a decrease of mTOR signaling reverts Tau pathology^{27,55}.
438 These cumulative results point to a critical role for mTOR-dependent autophagy in the cellular
439 mechanisms through which GC may trigger accumulation of Tau and its aggregates.

440 Recent studies from AD and FTDP human brains as well Tau Tg mice suggest that RBPs
441 dysregulation and SGs represent a novel pathway that may contribute the development and
442 progression of Tau pathology^{16,19}. SGs are cytoplasmic complexes composed of phase separated
443 complexes containing mRNAs and RNA-binding proteins (RBPs), which form to sequester non-
444 essential transcripts in response to cellular stress. SGs are thought to constitute a protective
445 mechanism against cellular stress by directing ribosomal system to translate mRNA transcripts coding
446 for cytoprotective proteins¹⁶. The process of liquid-liquid phase separation and reversible aggregation
447 that underlie SG formation is meant to be a transient event, but with prolonged SG induction is
448 hypothesized to become pathological and neurotoxic^{18,19}. SGs have been suggested to accelerate
449 Tau aggregation while, in a vicious cycle, Tau stimulates SG¹⁹. Factors known to stimulate Tau
450 aggregation, including hyperphosphorylation and aggregation-prone mutations, also enhance the
451 interactions between SGs and Tau¹⁹.

452 The current study suggests a novel role for RBPs in the stress/GC-driven neuronal pathology. We
453 demonstrate for the first time that chronic stress increase the cytoplasmic levels of different RBPs
454 and SG-associated markers (e.g. TIA-1, PABP, G3BP, FUS, DDX5) leading to its insoluble
455 inclusion/accumulation. While SG biology involves the action of multiple different proteins leading to
456 RBPs accumulation which is necessary for SGs⁵⁶⁻⁵⁸, recent evidence demonstrates that TIA-1 possess
457 a primary role in the SG-related mechanism of Tau aggregation. Specifically, TIA-1 is transported
458 from nucleus (where is predominantly found) to cytoplasm where it interacts directly with Tau to
459 stimulate its aggregation and insoluble accumulation/inclusion of other RBPs such as PABP and
460 EWSR1^{17,19,59}. This is of particular importance as our current findings show that, under stressful
461 conditions, TIA-1 is translocated from nucleus to cytoplasm accompanied by insoluble accumulation of
462 different SGs-associated proteins and Tau aggregates; future work should further study whether and
463 how this RBP dysregulation and insolubilization correlates to altered SG formation. Furthermore, we
464 have recently shown that Tau missorting and dendritic accumulation are also part of chronic stress/GC
465 hippocampal pathology^{31,32} while missorting of Tau are hypothesized to facilitate formation of SGs as

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

470 The interface between autophagy and RBPs dysregulation and insolubilization that we highlight in this
471 manuscript is supported by studies suggesting that inhibition of autophagy may impact in the
472 dynamics and removal of SGs ^{15,60}. Whether this occurs in the brain and in particular disease models,
473 was not previously known. The cytoskeletal machinery is known to facilitate the aggregation of RBPs
474 to form SGs, contribute to maturation of autophagic vesicles and autophagosome/lysosome fusion
475 ^{61,62}. One of the molecules involved in these processes is histone deacetylase 6 (HDAC6), which,
476 through the deacetylation of tubulin, reduces microtubule-dependent motility and thereby promotes
477 the consolidation of cellular complexes such as SGs and autophagosomes ^{41,42,63}. Mounting evidence
478 implicates HDAC6 in the formation of SGs in AD brain as HDAC6 seems to co-localize and interact
479 with SG proteins under cellular stress; interestingly, HDAC6 is a SGs component interacting with
480 G3BP ^{64,65}. Additionally, pharmacological inhibition or genetic ablation of HDAC6 abolished SG
481 formation ⁶⁶, while the expression of HDAC6 significantly increases in the hippocampus and other
482 brain regions of AD patients as well as in animal models of the disease ^{67,68}. Our findings show that
483 stress and GC increased HDAC6 levels, resulting in reduced acetylated levels of HDAC6's
484 cytoskeletal targets e.g. tubulin. Reduced acetylation of tubulin is associated with microtubule
485 instability, which is also suggested to cause enlargement of SGs ⁶⁹. Interestingly, recent reports in
486 mice show that HDAC6 inhibition increases resilience to stress ^{70,71}. While these findings point to
487 HDAC6 as a potential regulator in the stress-driven Tau pathology (see Fig. 8d), future studies should
488 clarify its specific role in different cellular pathways evoked by chronic stress and GC.

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

492

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510

511 **CONFLICT OF INTEREST**

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

514

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704

705 **Fig. 1. Chronic stress evokes accumulation of neurotoxic Tau aggregates causing cognitive**
706 **and emotional deficits in P301L-Tau Tg mice. a** P301L-Tg mice exhibited reduced body weight
707 ($p=0.005$) and increased corticosterone ($p<0.001$) levels after chronic environmental stress. **b** In
708 contrast to pre-training session ($p=0.171$), stressed animals exhibited a significant decrease in
709 percentage of freezing time in test section (context A) of CFC in comparison to control animals
710 indicating hippocampus-dependent associative memory impairment ($p<0.001$); note that both animal
711 groups exhibit similar freezing levels in context B which is not non-associated with the adverse stimuli
712 animals received in context A ($p=0.640$). **c** Chronic stress increased the time that animals swam to
713 reach the new (opposite) place of the escaping platform indicating PFC-dependent deficits of
714 behavioral flexibility ($p=0.046$). **d** Stress also reduced percentage of spontaneous alternations in the
715 arms of a Y-maze as compared with control animals pointing to deficits of working memory ($p=0.007$).
716 **e-f** Whereas no different in total distance travelled by animals in OF apparatus ($p=0.988$), stressed
717 animals exhibited a decrease in time spent in the center of the OF arena ($p=0.003$) (e) followed by
718 reduced time ($p=0.004$) and entries ($p=0.001$) that animals spend in the open arms of EPM apparatus
719 (f); these behavioral parameters suggest increased levels of anxiety in stressed animals compared
720 with controls. **g-h** Chronic Stress elevated the levels of Tau in sarkosyl-insoluble in both hippocampus
721 and PFC of P301LTau mice (Hipp: $p=0.028$; PFC: $p<0.001$) (g); an effect that was accompanied by
722 decreased cell density in PFC (prelimbic cortex; PrL: $p<0.001$) and hippocampus (DG: $p<0.001$; CA1:
723 $p<0.001$) (h). All numeric data represent mean \pm SEM, * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

724
725 **Fig. 2. Prolonged exposure to environmental stress inhibits autophagy in a mTOR-dependent**
726 **manner. a** Schematic representation of autophagy highlighting the role of mTOR, LC3 and p62 in this
727 cellular process. **b-e** Stressed animals exhibited reduced LC3 (PFC: $p=0.003$; HIPP: $p=0.026$) and
728 increased p62 (PFC: $p=0.029$; HIPP: $p=0.004$) protein levels as assessed by WB analysis (b); these
729 findings were confirmed by corresponding changes in LC3+ ($p=0.044$) and p62+ ($p=0.044$)
730 fluorescence intensity/cell number (c-d) an decrease in their co-localization (e), indicating a stress-
731 driven inhibition of autophagic process. **f** In line with the above findings, the levels of phospho S6K
732 ($p=0.014$), p38 ($p=0.030$) and mTOR ($p<0.001$) proteins were increased by stress, with decrease
733 levels of eIF4E ($p=0.024$), which are indicative of mTOR activation. All numeric data represent mean
734 \pm SEM, * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

735
736 **Fig. 3. Exposure to glucocorticoids causes Tau accumulation and autophagy inhibition *in***
737 ***vitro*. a** GC treatment (10^{-6} M; 48hr) of EGF-P301L-hTau SHSY cells decreased cell viability
738 ($p=0.004$). **b-d** Glucocorticoid (GC) treatment triggered cytoplasmic accumulation of exogenously
739 expressed mutated human Tau (EGF-P301L-Tau) and endogenous human Tau (WT-Tau) as

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

745

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

756

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

766

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

774

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

785

786 **Fig. 8. The CCI-779 inhibitor of mTOR attenuated GC-induced dysregulation of RBPs.**

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

798