

1 **Endoplasmic reticulum stress induces tau pathology and forms a**
2 **vicious cycle: Implication in Alzheimer’s disease pathogenesis**

3

4 Yuen-Shan Ho¹, Xifei Yang^{1,5}, Jeffery Chi-Fai Lau¹, Clara Hui-Ling Hung¹,
5 Suthicha Wuwongse^{1,4}, Qishan Zhang¹, Jianzhi Wang⁶, Larry Baum⁷, Kwok-Fai

6

So^{1,2,3}

7

and Raymond Chuen-Chung Chang^{1,2,3*}

8

9 ¹Laboratory of Neurodegenerative Diseases, Department of Anatomy, LKS Faculty of
10 Medicine;

11

²Research Centre of Heart, Brain, Hormone and Healthy Aging;

12

³State Key Laboratory of Brain and Cognitive Sciences;

13

⁴Department of Psychiatry, LKS Faculty of Medicine;

14

The University of Hong Kong, Pokfulam, Hong Kong SAR, CHINA

15

⁵Shenzhen Centre of Disease Control and Prevention, Shenzhen, CHINA;

16

⁶Department of Pathophysiology, Tongji Medical College, Huazhong University of
17 Science and Technology, Wuhan, CHINA

18

⁷School of Pharmacy, Faculty of Medicine, The Chinese University of Hong Kong,
19 Shatin, Hong Kong SAR, CHINA

20

21 **Running Title:** ER-stress and tau pathology

22 **Keywords:** ER-stress; tau phosphorylation; thapsigargin; okadaic acid; Alzheimer’s
23 disease

24

25

1

2

3 ***Correspondence address:**

4 Dr. Raymond C. C. Chang, Department of Anatomy, Faculty of Medicine,

5 The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong SAR,

6 CHINA

7 Tel: (+852) 2819-9127; Fax: (+852) 2817-0857; E-mail: rccchang@hku.hk

8

1 Abstract

2 Accumulation of unfolded proteins can disturb the functions of the
3 endoplasmic reticulum (ER), leading to ER-stress or unfolded protein responses
4 (UPR). Recent data have shown that activation of UPR can be found in post-mortem
5 brains of Alzheimer's disease (AD) patients; and biological markers for activation of
6 UPR are abundant in neurons with diffuse phosphorylated tau. Although these
7 observations suggest a linkage between ER-stress and tau pathology, little is known of
8 their relationship. In this study, we found that high levels of phosphorylated PKR-like
9 ER-resident kinase (p-PERK) and phosphorylated eukaryotic initiation factor 2 alpha
10 (p-eIF2 α) as markers for activation of UPR in the hippocampus of aged P301L
11 mutant tau transgenic mice. The immunoreactivity of p-PERK was found to co-
12 localize with that of phosphorylated tau. We then hypothesized that phosphorylation
13 of tau could induce ER-stress and vice versa in promoting AD-like pathogenesis. By
14 using the protein phosphatase 2A (PP2A) inhibitor okadaic acid (OA) as an inducer
15 for phosphorylation of tau, we found that primary cultures of rat cortical neurons
16 treated with OA triggered UPR as indicated by increased levels of p-PERK and p-
17 eIF2 α , splicing of mRNA for xbp-1 and elevated levels of mRNA for GADD153. On
18 the other hand, thapsigargin (Tg) as an ER-stress inducer stimulated phosphorylation
19 of tau at Thr231, Ser262 and Ser396. Tg also induced activation of caspase-3 and
20 cleavage of tau. These findings suggested that ER-stress and hyperphosphorylation of
21 tau could be induced by each other to form a vicious cycle to propagate AD-like
22 neurodegeneration. (Word count: 246)

23

1 INTRODUCTION

2 Alzheimer's disease (AD) is an aging-associated neurodegenerative disease
3 leading to progressive cognitive impairment and memory loss. Among various
4 pathological changes in the development of AD, accumulation of senile plaques and
5 neurofibrillary tangles (NTFs) has long been considered to be two distinct
6 pathological hallmarks. Senile plaques are extracellular aggregates of β -amyloid ($A\beta$)
7 protein; NTFs are intracellular filamentous inclusions which mainly consist of
8 abnormally hyperphosphorylated and aggregated tau protein [1]. Both $A\beta$ and tau are
9 found in misfolded and aggregated forms in AD, suggesting that impairment of
10 protein folding machinery is involved in the pathogenesis of AD.

11

12 In the eukaryotic cell, the endoplasmic reticulum (ER) is responsible for
13 protein synthesis, correction of protein folding, post-translational modification and
14 transport. In physiological circumstances, there is strict control of protein folding
15 machinery, with misfolded proteins either retained in the ER for further correction of
16 their folding or subjected to degradation [2]. Disturbance in the ER integrity or
17 folding machinery by accumulation of misfolded proteins or changes of calcium
18 homeostasis in the ER can trigger ER-stress responses, or the unfolded protein
19 response (UPR) [3]. Shortly after perturbation of ER homeostasis, UPR can serve as
20 an adaptation process to prevent further disturbance of cellular functions. Three ER-
21 stress signaling pathways, including activation transcription factor 6 (ATF6), double-
22 stranded RNA-dependent protein kinase (PKR)-like ER-resident kinase (PERK) and
23 inositol requiring 1 (IRE1), are activated. These signaling cascades lead to increase
24 production of ER-resident chaperones and foldases such as BiP/GRP78 to enhance
25 efficiency of protein folding; the activation of eukaryotic initiation factor 2 alpha

1 (eIF2 α) to shut down global protein translation for newly transcribed genes [4]; and
2 the upregulation of ER-associated degradation to promote clearance of misfolded
3 proteins [5]. Hence, UPR is considered to be initially protective.

4
5 However, sustained activation of the UPR can induce apoptotic responses that
6 would finally cause cell death [6]. Although the involvement of ER-stress in
7 neurodegenerative diseases such as AD and Parkinson's disease has been widely
8 reported [7-9], the mechanisms by which ER-stress participates in neurodegenerative
9 processes are not fully understood. Many studies reported the direct activation of both
10 intrinsic and extrinsic apoptotic pathways by ER-stress [10-12]. Apart from apoptosis,
11 it has also been suggested that ER-stress would alter cellular responses and sensitize
12 neurons to A β toxicity, hence enhancing A β -induced neurodegeneration [13].

13

14 In postmortem AD brains, the levels of UPR markers such as BiP/GRP78 and
15 p-PERK were found to be increased in the cortex and hippocampus [14,15],
16 suggesting the involvement of ER-stress in AD pathogenesis. It is important to
17 understand the role of ER-stress in the pathogenesis of AD and the related signaling
18 pathways for the development of promising therapeutic interventions [16]. In familial
19 AD (FAD), mutation of presenilin-1 (PS1) can alter signaling of UPR sensitizing
20 neurons to be more vulnerable to stress. Mutant PS1 might increase the vulnerability
21 of neurons to ER-Golgi toxin-induced apoptosis [17]; mutation of PS1 can also
22 deregulate the expression of PERK, IRE1 and ATF-6 under ER-stress [8]. However,
23 most AD cases are sporadic and lack PS1 mutations. As accumulation of aggregated
24 A β and hyperphosphorylated tau have been considered to be the significant features in
25 AD, it is reasonable to speculate that A β or tau are responsible for the induction of

1 ER-stress observed in AD. Previously, our laboratory had reported that extracellular
2 accumulation of A β peptide in both fibrillar and oligomeric forms was not able to
3 induce the UPR in primary cultures of cortical or hippocampal neurons [18,19]. In
4 combination with the results showing that UPR was activated in pre-tangle neurons in
5 AD brain [14], we speculated that hyperphosphorylated tau, instead of A β peptide,
6 might play an important role in inducing ER-stress in AD. In the last two years,
7 several studies had been conducted to investigate the effects of ER-stress on tau
8 protein. The results, however, were controversial [14,20], and it is necessary to further
9 clarify the relationship between ER-stress and tau.

10

11 In this study, we hypothesized that hyperphosphorylation of tau can induce
12 ER-stress and vice versa in promoting pathogenesis of AD. Both transgenic mouse
13 models and *in vitro* models were used to investigate the relationship between ER-
14 stress and tau pathology. We found increased levels of p-PERK and p-eIF2 α in the
15 hippocampus of aged P301L mutant tau transgenic mice. Immunoreactivity of these
16 two phospho-proteins co-localized with hyperphosphorylated tau. In primary cultures
17 of cortical neurons, we observed that hyperphosphorylation of tau was induced by
18 okadaic acid. Furthermore, we found that thapsigargin-induced ER-stress could
19 trigger hyperphosphorylation as well as tau cleavage of tau. Our results suggested that
20 hyperphosphorylation of tau and ER-stress can promote the initiation of each other
21 and form a vicious cycle contributing to AD-like pathology.

22

1 MATERIAL AND METHODS

2 *Materials and chemicals*

3 Materials for cell culture, including minimum essential medium (MEM),
4 NeurobasalTM medium, B-27 supplement, penicillin and streptomycin, were
5 purchased from Gibco-BRL (Burlington, Ont., Canada). Other chemicals were
6 obtained from the following companies: Thapsigargin (Tg) and okadaic acid (OA)
7 were from Calbiochem, Inc. (La Jolla, CA, USA). 4-Phenyl butyric acid (4-PBA),
8 protease inhibitor cocktail, phosphatase inhibitor cocktail, 4'6-Diamidino-2-
9 phenylindole (DAPI), mouse monoclonal anti- β -actin antibody and mouse
10 monoclonal anti- α -tubulin antibody were obtained from Sigma-Aldrich, Inc. (St.
11 Louis, MO, USA). Mouse monoclonal antibody against cleaved-Tau (Asp421) was
12 purchased from Upstate (Lake Placid, New York, USA). Rabbit polyclonal antibody
13 against phosphorylated eIF2 α (Ser51), tau pS396 against tau phosphorylated at
14 Ser396, tau pT231 against tau phosphorylated at Thr231, tau pS262 against tau
15 phosphorylated at Ser262 and mouse polyclonal antibody tau-1 against non-
16 phosphorylated tau at Ser198/199/202 were purchased from BioSource International
17 (Hopkinton, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit and goat
18 anti-mouse antibodies were from DAKO (Glostrup, Denmark). Rabbit monoclonal
19 antibody against phosphorylated PERK (Thr980), rabbit monoclonal antibody against
20 total PERK, rabbit polyclonal antibody against anti-BiP, total eIF2 α , phosphorylated-
21 GSK3 β (Ser9) and cleaved-caspase-3 were purchased from Cell Signaling
22 Technology (Beverly, MA, USA). Mouse polyclonal antibody anti-GSK3 β (pY216)
23 to detect phosphorylated GSK3 β at Tyr216, antibody Tau-5 to detect total tau and
24 antibody against total GSK3 β were from BD Transduction Laboratories (CA, USA).
25 Polyvinylidene difluoride (PVDF) membrane was from Bio-Rad (Richmond, CA,

1 USA). Biomax X-ray film was from GE Healthcare (New York, USA). Enhanced
2 chemiluminescence (ECL) detection kit was from Amersham (Buckinghamshire, UK).
3 Anti-mouse or anti-rabbit Alexa fluor-488 or -568, Anti-Fade Gold mounting medium
4 and Fluo-4AM were from Molecular Probes, Invitrogen. Anti-PHF-tau antibody
5 clones AT180 against phosphorylated tau at Thr231 and Ser235 was from Thermo
6 Scientific (Rockford, USA). Rabbit polyclonal antibody against human-tau (K9JA/
7 pan-tau, recognize the total tau at C-terminal part, amino acids 243–441) was from
8 DAKO (USA).

9

10 *Primary cultures of hippocampal and cortical neurons and drug treatment*

11 Primary cultures of rat hippocampal and cortical neurons were prepared from
12 embryonic day 17 Sprague-Dawley rat embryos (The Laboratory Animal Unit, The
13 University of Hong Kong) via the method described previously [21,22]. Briefly,
14 cerebral cortices or hippocampus were dissected in 1X phosphate-buffered saline
15 (PBS) supplemented with glucose (18 mM). Neurons were then mechanically
16 dissociated. Hippocampal neurons were seeded onto glass coverslips pre-coated with
17 poly-L-lysine (25 µg/ml) at a density of 0.7×10^5 cells/coverslip. They were cultured
18 with Neurobasal™ medium containing 2% B-27, L-glutamine (2 mM), penicillin (50
19 U/ml) and streptomycin (50 µg/ml). Cortical neurons were seeded onto 6-well plates
20 pre-coated with poly-L-lysine (25 µg/ml) at a density of 1.2×10^6 cells/well. They
21 were cultured with minimum essential medium (MEM) supplemented with 5% heat
22 inactivated fetal bovine serum, glucose (18 mM), L-glutamine (2 mM), insulin (5
23 µg/ml), progesterone (0.02 µM), putrescine (100 µM), selenium (30 pM), penicillin
24 (50 U/ml) and streptomycin (50 µg/ml). All the neurons were maintained at conditions
25 of 37°C in a humidified 5% CO₂ atmosphere. Deoxyfluorouridine (final concentration

1 1 μ M) were added into cultures on DIV 2 to remove glial cells. All experiments were
2 performed on DIV 7.

3

4 To induce hyperphosphorylation of tau, the protein phosphatase 2A (PP2A)
5 inhibitor OA prepared as a 0.2 mM stock solution in DMSO was added into cultures
6 at a final concentration of 50 nM for the indicated duration. For control experiments,
7 DMSO was added at the final concentration present in the OA-treated cultures
8 (0.025%).

9

10 To induce ER-stress, Tg prepared as a 10 mM stock solution in DMSO was
11 added into cultures at final concentrations of 4 or 10 nM for the indicated duration.
12 For control experiments, DMSO was added at the final concentration present in the
13 Tg-treated cultures (0.0001%). In some experiments, the chemical chaperone 4-
14 phenyl butyric acid (PBA) 1 mM was added at the same time as Tg. The cultures were
15 then incubated for 6 h before they were harvested for Western blot analysis.

16

17 *Brain samples of transgenic mice expressing P301L human tau*

18 The transgenic mice (JNPL3 mice) expressing human mutant P301L tau
19 (proline to leucine mutation at amino acid 301 in exon 10) have been described
20 previously [23]. These TgTau^{P301L} mice develop tau pathologies (tau
21 hyperphosphorylation and aggregation) starting from age 3-months and increasing in
22 severity with age [24]. Male wild type and TgTau^{P301L} mice were sacrificed at age 21
23 months, and the brains were quickly excised and processed according to the following
24 procedures. For Western blot analysis, brains were micro-dissected for the
25 hippocampus and snap-frozen immediately in liquid nitrogen and stored at -80°C

1 before use. For histological and immunohistochemical analyses, all mouse brains
2 were fixed with 0.1 M phosphate buffer (pH 7.6) containing 4% paraformaldehyde
3 and embedded in paraffin. Each group contained at least 4 animals (n=4) for both
4 immunohistochemical staining and Western blot analysis. In this study, we only focus
5 on the hippocampus because it is the region mostly relevant to the development of AD,
6 especially in the early stage. Since we did not keep the mice, other brain regions had
7 been used by other investigators.

8

9 *Immunohistochemical staining*

10 For immunohistochemical analysis, consecutive 4- μ m-thick coronal mouse
11 brain sections were cut. After dewaxing and rehydration, the sections were treated
12 with 0.01 M citrate buffer (pH 6.0) with 0.1% Tween-20 at 90°C for 15 min for
13 antigen retrieval. The sections were then blocked with 10% normal goat serum in PBS
14 for 1 h. Sections were co-incubated with primary antibody AT180 (1:200) and
15 phosphorylated-PERK (1:200) at 4°C overnight. After washing with PBS, the sections
16 were stained with secondary antibody, Alexa fluor-488 or -568 for 2 h and finally
17 stained with DAPI for revealing the nuclei. Sections were then mounted with Anti-
18 Fade Gold mounting medium. The sections were examined with a BioRad Radiance
19 2100 confocal microscopic system.

20

21 *Western blot analysis*

22 Total lysate was collected from cell culture or mouse brain samples as
23 described elsewhere [25]. In brief, cultured neurons or brain samples were lysed in
24 ice-cold lysis buffer (10 mM Tris-HCl (pH 7.4), 1 mM NaCl, 20 mM Na₄P₂O₇, 2 mM
25 Na₃VO₄, 1% Triton-X-100, 10% glycerol, 0.5% deoxycholate, 0.1% SDS, 1 mM

1 phenylmethylsulfonyl fluoride, protease inhibitor cocktail and phosphatase inhibitor
2 cocktail). After homogenization and centrifugation, the supernatant was collected.
3 The sample were subjected to SDS 10% or 12.5% polyacrylamide gels electrophoresis
4 and transferred to PVDF membranes. Membranes were blocked with 5% w/v non-fat
5 milk in TBST (TBS containing 0.1% Tween-20) for 1 h to prevent non-specific
6 binding. Primary antibodies were diluted in TBST and incubated with the membrane
7 as described follow: cleaved caspase-3 (1:2000), cleaved-tau (Asp421) (1:1000), tau
8 pS396 (1:4000), tau pT231 (1:4000), tau pS262 (1:2000), tau-1 (1:30,000), pan-tau
9 (1:4000), phosphorylated PERK (1:1000), total PERK (1:1000), phosphorylated
10 eIF2 α (1:1000), total eIF2 α (1:2000), BiP (1:1000), p35/p25 (1:2000), CDK5
11 (1:2000), p-GSK3 β (Ser9) (1:4000), p-GSK3 β (pY216) (1:6000). After washing, the
12 membranes were incubated with horseradish peroxidase-conjugated secondary
13 antibodies (1:2000) for 1 h and subsequently developed by using the ECL or ECL-
14 plus Western blotting detection kit. The membranes were then stripped with stripping
15 buffer (50 mM glycine, 2% SDS, pH 2.0) and re-probed with monoclonal anti- β -actin
16 antibody (1:10,000, 1 h) and goat anti-mouse HRP secondary antibody. Optical
17 density of the blots was measured with Image J software (National Institutes of Health,
18 USA).

19

20 *Analysis of splicing in x-box binding protein (xbp-1) mRNA*

21 Splicing in xbp-1 mRNA was investigated by RT-PCR and PstI restriction
22 enzyme digestion as in our previous studies [18,19]. After treatment with OA,
23 extraction of RNA and reverse transcription were carried out. cDNA of Xbp-1 was
24 amplified using PCR (Invitrogen) with xbp-1 specific primers (Table 1) in a
25 RoboCycler 96 (Stratagene). The PCR cycles consisted of denaturation at 94°C for 1

1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min, for 33 cycles. PstI
2 (New England Biolabs) restriction digestion of the 600 bp PCR products was done at
3 37°C for 16 h. The restriction fragments were separated on a 1% (w/v) agarose gel
4 with ethidium bromide and visualized under UV illumination.

5

6 *Reverse Transcription (RT)-PCR*

7 After exposure to OA [50 nM] for different times, total RNA from neurons
8 was extracted with NucleoSpin[®] RNA II (Macherey-Nagel, Germany). Total RNA
9 yields were measured by A260/280 using GenQuant II (Pharmacia Biotech). 1 µg
10 RNA was reverse-transcribed into cDNA with Superscript[™] III first strand synthesis
11 system (Invitrogen) for RT-PCR. PCR was done in a RoboCycler 96 (Stratagene)
12 with *Taq* polymerase (Invitrogen). Target genes for amplification and their primer sets
13 and thermal conditions are listed in Table 1.

14

15 *Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$)*

16 Cultured cortical neurons were incubated with Fluo-4AM [1 µM] in cell
17 culture medium as indicated above for 30 min at 37°C. After incubation, the loading
18 medium was removed, and cells were washed with 1X HBSS and maintained in 0.5
19 ml of the same solution in dark. The fluorescence signals were recorded at 488 nm
20 (excitation wavelength) and 538 nm (emission wavelength) with a Bio-Rad Radian
21 2100 confocal microscope system. Upon measurement, the first 120 s were recorded
22 to serve as the baseline of $[Ca^{2+}]_i$ before any drug application. At 120 s, Tg was added
23 into the culture dish to a final concentration of 10 nM or 20 µM. The $[Ca^{2+}]_i$ was
24 recorded for another 400 s. At least 4 independent experiments were performed, and
25 the readings of 9 neurons were recorded from each individual experiment. Data were

1 represented as fold of control [fold of control was calculated as follows = reading at
2 particular time point/ mean of reading of baseline].

3

4 *Statistical analysis*

5 Data were expressed as mean \pm standard error (SE) from at least 3
6 independent experiments. The significance of the differences among different groups
7 was determined by unpaired *t*-test or one-way ANOVA, followed by Student
8 Newman-Keuls as post-hoc test. $P < 0.05$ was considered to be statistically significant.

9

1 **Results**

2 *Increased levels of UPR markers were observed in the hippocampus of aged P301L* 3 *tau transgenic mice*

4 In postmortem AD brains, immunoreactivity of p-PERK was found to be
5 increased and associated with phosphorylated tau [14]. To explore the relationship
6 between ER-stress and tau pathology, we examined the levels of p-eIF2 α and p-
7 PERK in 21-month-old TgTau^{P301L} mice. Levels of p-eIF2 α in hippocampus of
8 TgTau^{P301L} mice were 3.2 ± 0.4 fold those of age-matched controls (Fig. 1A).
9 Activation (phosphorylation) of eIF2 α can lead to inhibition of protein translation,
10 and eIF2 α can be phosphorylated by up-stream kinases such as double-stranded
11 RNA-dependent protein kinase (PKR), PERK, amino acids-regulated eIF2 α kinase
12 (GCN2) or heme-regulated eIF2 α kinase (HRI) [26]. Among these kinases, the role of
13 PERK during ER-stress has been well-reported; therefore, we further examined the
14 immunoreactivity of p-PERK in brain sections of these transgenic mice. We found
15 that the immunoreactivity of p-PERK was markedly increased in the hippocampus of
16 TgTau^{P301L} mice compared to age-matched controls. Furthermore, the elevated
17 immunoreactivities of p-PERK were found to be co-localized with AT180
18 immunoreactivity, which detects tau phosphorylated at Thr231 and Ser235 (Fig. 1B).
19 Thus, ER-stress was increased in aged TgTau^{P301L} mice.

20

21 *Induction of tau hyperphosphorylation led to ER-stress in primary cultures of cortical* 22 *neurons*

23 In the previous section, we had demonstrated that aged TgTau^{P301L} mice had
24 increased levels of p-PERK, which was co-localized with AT180 staining. Our results
25 suggested that there might be a direct linkage between phosphorylation of tau and ER-

1 stress. We then asked whether the UPR was induced by hyperphosphorylation of tau.
2 OA is a well-reported inducer for hyperphosphorylation of tau; it acts by inhibiting
3 protein phosphatase 2A (PP2A) that is responsible for dephosphorylation of tau
4 [27,28]. OA was applied to primary cultures of cortical neurons to induce
5 hyperphosphorylation of tau. As shown in Fig. 2A-E, 50 nM OA induced the
6 hyperphosphorylation of tau on Thr231, Ser396 and Ser198/199/202 at different time
7 points. Except for phosphorylated tau at Ser396, which decreased after 16 h, the other
8 phosphorylation sites of tau showed persistent increases in their immunoreactivity. It
9 had been reported that changes in some tau epitopes induced by OA were more
10 persistent than others; hence our results were consistent with previous findings [29].
11 The level of total tau (as detected by the Tau-5 and K9JA antibodies) was decreased
12 in neurons exposed to OA (Fig. 2A and Supplementary Fig. 3A), which has been
13 previously reported by another group [30]. It has been speculated that the reduction in
14 total tau indicates either an enhanced clearance of aggregated tau (due to
15 hyperphosphorylation) or an ineffectiveness of detection for aggregated tau by
16 Western blot analysis. Nevertheless, OA clearly induced hyperphosphorylation of tau.
17 To further confirm our observation, we performed immunocytochemical staining on
18 primary cultures of hippocampal neurons; and we found that OA could also induce
19 tau hyperphosphorylation in these cells (Supplementary Fig.1). Since we observed
20 similar phenomenon between the two cell types, and there were limited availability of
21 primary hippocampal neurons, we used cortical neurons for most of our experiments.

22 We then detected the presence of ER-stress by OA. We found that
23 immunoreactivity of p-PERK significantly increased from 6 to 24 h while that of p-
24 eIF2 α exhibited a tendency toward an increase at all time-points (though statistically
25 significance was only detected at 24 h) (Fig. 2F, G, I). The level of BiP was

1 unchanged at all tested time-points. To further confirm the presence of UPR in
2 neurons after treatment of OA, we detected splicing of mRNA for xbp1 and the
3 mRNA level of GADD153 (growth arrest-and DNA damage-inducible gene 153).
4 UPR activates IRE1 to function as an endoribonuclease to splice mRNA for xbp-1 by
5 cutting off a 26-nucleotide intron [31]. This would prevent the digestion of mRNA for
6 xbp-1 (~600bp) by Pst1 as the Pst1 restriction enzyme cutting site is within the
7 excised 26-nucleotide sequence. Fig. 2J shows that it was about 600 bp of mRNA for
8 xbp-1 before Pst1 digestion in all treatment groups. After Pst1 digestion (Fig. 2K),
9 fragments of about 300 bp were only found in controls. In neurons exposed to OA for
10 16 or 24 h or to DTT [1mM] for 24 h (a positive control), the digestion of xbp-1 by
11 Pst1 to yield a 300 bp fragment was attenuated, indicating the splicing of mRNA for
12 xbp-1. Our results suggested that OA induced UPR and activation of IRE1.
13 GADD153 is a highly inducible gene responsive to ER-stress; its expression increases
14 during UPR [32]. As shown in Fig. 2L, the level of mRNA for GADD153
15 significantly increased after exposure of neurons to OA for 16 and 24 h. Taken
16 together, our data showed that OA-induced hyperphosphorylation of tau could trigger
17 UPR.

18

19 *Induction of ER-stress by thapsigargin led to increased phosphorylation of tau in*
20 *primary cultures of cortical neurons*

21 Our *in vivo* and *in vitro* data suggested that hyperphosphorylation of tau could
22 induce ER-stress. It has well been reported that prolonged ER-stress can lead to
23 apoptosis. Besides inducing neuronal cell death as late and final events, ER-stress
24 may also induce other pathological changes which are involved in the
25 neurodegenerative process of AD. We, therefore, examined the effects of thapsigargin

1 (Tg) as a typical ER-stress inducer on an *in vitro* model of primary cultures of cortical
2 neurons. Tg is a specific inhibitor of intracellular Ca^{2+} -transport ATPases. It blocks
3 the activity of the enzyme to induce perturbation of intracellular calcium homeostasis,
4 resulting in ER-stress. As shown in Fig. 3A-D, 10 nM Tg induced an elevation of
5 immunoreactivities of UPR markers including p-PERK and BiP in a dose-dependent
6 manner. We then detected the levels of phosphorylated tau. The band intensity of
7 phosphorylated tau at Thr231, Ser262 and Ser396 was found to increase at 6 and 12 h
8 time points (Fig. 3E-H). These elevated immunoreactivities of phosphorylated tau
9 were then reduced at 18 h and became similar to levels of the controls. The
10 immunoreactivity of phosphorylated tau (Thr231 and Ser396) continued to decrease
11 and became markedly lower than the controls at 24 h (Fig. 3F and H). The
12 immunoreactivity of Tau-5 and K9JA (both detected total tau level) among all the
13 treatment groups was similar without significant change,

14

15 *Truncation of tau was induced by ER-stress*

16 It has been reported that Tg-induced ER-stress can lead to activation of
17 caspase-3 and apoptosis [33]. Tau is a substrate of caspase-3, and its cleaved form is
18 pro-apoptotic [34] and is more prone to form aggregates [35]. We therefore examined
19 the levels of caspase-3 and cleaved tau in our Tg-treated cortical neurons. We found
20 that the immunoreactivity of cleaved caspase-3 showed a trend toward increase after
21 12 h exposure to 10 nM Tg. After 18 or 24 h incubation, the immunoreactivity of
22 cleaved caspase-3 significantly increased to nearly 3-fold of the control. The increase
23 also showed a dose-dependent pattern. At the same time, the immunoreactivity of
24 cleaved-tau at Asp421 was also elevated in a pattern similar to that of caspase-3 (Fig.

1 4). At 18 h, the immunoreactivity of cleaved-tau reached the highest levels and was
2 more than 7-fold greater than the control (Fig. 4B).

3

4 *Induction of tau phosphorylation by Tg [10 nM] was due to the accumulation of*
5 *misfolded/unfolded protein*

6 To confirm whether Tg-induced hyperphosphorylation of tau was a result of
7 UPR, we co-treated cortical neurons with Tg and 4-phenyl butyric acid (4-PBA), a
8 chemical chaperone which can enhance the adaptive capacity of the ER [36]. As
9 shown in Fig. 5A-D, 1 mM 4-PBA significantly reduced the immunoreactivity of
10 phosphorylated tau induced by Tg. There was little effect of 4-PBA itself on
11 immunoreactivities of phosphorylated tau at Thr231 and Ser 262, but 4-PBA did
12 increase the immunoreactivity of phosphorylated tau at Ser396. Our results confirmed
13 the role of ER-stress in Tg-induced tau phosphorylation.

14

15 *Exposure of cortical neurons to Tg [10 nM] did not induce acute increase of $[Ca^{2+}]_i$*

16 Tg can disturb intracellular calcium homeostasis and cause a transient increase
17 in $[Ca^{2+}]_i$ [37]. Previous study has shown that a transient increase in $[Ca^{2+}]_i$ can result
18 in prolonged phosphorylation of some tau epitopes [38]. To investigate the
19 downstream causative factors that mediated phosphorylation of tau in our Tg-treated
20 cultures, we measured the levels of $[Ca^{2+}]_i$. Our data showed that 10 nM Tg did not
21 trigger an acute and transient increase in $[Ca^{2+}]_i$. There was a mild increase in $[Ca^{2+}]_i$
22 only for 1.06 ± 0.02 fold of control. There were only gradual changes in $[Ca^{2+}]_i$
23 induced by 10 nM Tg. As a positive control, we also treated cortical neurons with 20
24 μ M Tg, and we found that this concentration of Tg could induce a 1.43 ± 0.08 fold
25 increase in $[Ca^{2+}]_i$. As shown in Fig. 6, 20 μ M Tg induced a typical calcium influx

1 pattern, in which $[Ca^{2+}]_i$ increased immediately after loading of Tg, peaked at about
2 240 s, then decreased gradually.

3

4 *Tg did not induce significant change in the level of p-GSK3 β*

5 To investigate possible upstream kinases affected by ER-stress and tau
6 phosphorylation, we detected the levels of p-GSK3 β , by Western-blot analysis (Fig.
7 7A). GSK-3 β can be activated by dephosphorylation at Ser 9 or phosphorylation at
8 Tyr 216. We found that the immunoreactivity of p-GSK3 β (Ser9) showed a trend
9 toward increase at 6 h and remained the same as the controls at other detected time
10 points (Fig. 7B). The immunoreactivity of p-GSK3 β (pY216) remained the same as
11 that of the controls at all detected time points (Fig. 7C). There was no significant
12 change in the protein levels of total GSK3 β (data not shown).

13

1 **Discussion**

2 ER-stress can trigger UPR, which is an adaptive response to cope with the
3 accumulation of misfolded proteins. It is believed that ER-stress is linked to a number
4 of neurodegenerative diseases including AD [3]. In postmortem AD brain, activation
5 of the UPR was found in neurons with diffuse phosphorylated tau [14,15]; yet the
6 temporal sequence of the events is not clear. Our study aims to investigate the
7 causative relationship between ER-stress and phosphorylation of tau. By using three
8 models, both *in vivo* and *in vitro*, we provide evidence that ER-stress and tau
9 phosphorylation are closely linked.

10

11 Our initial hypothesis is to prove that abnormal tau can lead to ER-stress and
12 activation of UPR. To study the chronic effects of tau pathology on ER-stress,
13 transgenic mice which display phosphorylated and aggregated tau would be an
14 appropriate experimental model as they can mimic the chronic disease development
15 process. The first model we used was therefore aged TgTau^{P301L} transgenic mice.
16 These mice expressed the P301L mutant version of the longest form of human tau
17 (4R0N), which can cause frontotemporal lobe dementia with parkinsonism linked to
18 chromosome 17 (FTDP-17). They developed pre-tangles and NFTs with increasing
19 age [23]. Tau pathology can be observed in different regions of the P301L mice
20 including the cortex, hippocampus, midbrain and cerebellum. Since we only focused
21 on the hippocampus in this study, we are not sure if similar phenomenon on ER stress
22 can be observed in other regions. The change of cognitive functions in P301L mice
23 has been studied by other groups. Although there was a report showing normal
24 cognitive function of these mice [39], another study found that these mice had poorer
25 cognitive performance, which was associated with increased number of

1 phosphorylated tau containing neurons in cerebral cortex and hippocampus [40].
2 Another group also reported that the P301L mice showed decline in cognitive
3 functions evaluated by Morris-water maze test [41]. Hence, TgTau^{P301L} transgenic
4 mice can be an appropriate animal model for studying tau pathology related to ER-
5 stress. In view of all these published behavioral reports of cognitive functions of this
6 line of mice, our study mainly focused on the pathological changes in these mice
7 rather than the behavioral functions. We therefore did not perform cognitive function
8 tests. Our data showed that the levels of p-PERK were elevated in the hippocampus of
9 aged TgTau^{P301L} mice, and the staining was co-localized with phosphorylated tau. The
10 levels of p-eIF2 α were also increased in the hippocampus of these transgenic mice.
11 Our data have proved that ER-stress and UPR occur in aged TgTau^{P301L} mice; and
12 hence ER-stress and tau pathology might have a casual relationship. In fact, a
13 previous report showed that the amount of tau protein was preferentially increased in
14 the membrane of the rough ER in motor neurons of TgTau^{P301L} mice; and
15 hyperphosphorylated tau was associated with the ER membrane in AD brains [42].
16 These findings further support the notion that abnormal tau would affect ER function
17 in the neurodegenerative process. Interestingly, another research group has reported
18 recently that they could not find any evidence of ER-stress or UPR activation in
19 another mutant tau transgenic mice model [20]. In that study, transgenic mice
20 expressing the P301S mutant version of human tau (PS19 transgenic mice) were used.
21 These PS19 transgenic mice gradually developed synaptic loss, neuronal loss and
22 NFTs with increasing age [43]. The reason for the opposite results observed in these
23 two transgenic models is unclear. One may question that the elevation of ER-stress
24 markers in our model may be due to the specific mutant i.e. P301L. Since we do not
25 have the htau mice which overexpress human wild-type tau, we cannot examine the

1 levels of ER-stress in these animals to exclude the possibility. In this study, the use of
2 TgTau^{P301L} mice only serve as an model to show that tau phosphorylation is correlated
3 to ER-stress, and we are not intend to use it as an experimental model of AD.

4
5 With the results in transgenic animals, it is better to further study the
6 mechanisms *in vitro*. We further prove that phosphorylation of tau can lead to ER-
7 stress in an *in vitro* model. Previous findings reported by Kim et al. have shown that
8 OA induced the phosphorylation of eIF2 α ; and hence the translocation of activating
9 transcription factor (ATF4) and induction of apoptosis [44]. Our present study further
10 proved the involvement of ER-stress in OA-induced phosphorylation of tau. Our
11 preliminary data showed that phosphorylation of tau occurs as early as 1 h after OA
12 treatment, while the levels of p-eIF2 α also increased as early as 1 h. It is known that
13 eIF2 α can be phosphorylated under many situations, and its phosphorylation alone is
14 insufficient to prove the presence of ER-stress. Here we reported that, after OA
15 treatment, elevation of the levels of p-PERK was observed only after 6 h; splicing of
16 mRNA for xbp-1 and increased expression of mRNA for GADD153 were observed
17 only at late time points (16 and 24 h). These findings clearly demonstrated activation
18 of the PERK and IRE1 pathways in the UPR; and more importantly, the occurrence of
19 the UPR follows the phosphorylation of tau, suggesting a causal relationship between
20 the two factors. In the later time points i.e. 16 h and 24 h, the levels of phosphorylated
21 tau at Ser 396 decreased (Fig. 2A). Although we did not study the apoptotic signal or
22 apoptotic state in OA-treated neurons as it is not our aim of study, previous report
23 documented that treatment with OA leads to apoptosis in late time points [45]. This
24 decrease in tau phosphorylation may be explained by the entrance of the early phase
25 of apoptosis [46]. It was shown that when neurons were exposed to stress factors such

1 as staurosporine, tau was first hyperphosphorylated transiently and then undergone
2 dephosphorylation and cleavage, followed by apoptosis [47]. We speculate that under
3 OA-treatment, tau may also be dephosphorylated when neurons undergo apoptosis.

4
5 While phosphorylation of tau can induce UPR, induction of ER-stress has
6 been shown to induce phosphorylation of tau. Tg was used to induce ER-stress in our
7 study. In many studies [48,49], high concentrations of Tg at micromolar levels were
8 used to induce ER-stress and apoptosis. Since we aimed to examine the events that
9 occur before the induction of apoptosis, we chose a very low dose of Tg (10 nM) to
10 induce ER-stress. It has been shown that treatment of primary cultures of cortical
11 neurons with Tg [10 nM] for 24 h did not affect cell viability, and significant cell
12 death was observed at 48 h [48]. We demonstrated here that this low concentration of
13 Tg could induce the UPR as early as 6 h; and at the same time, there was a transient
14 increase in the phosphorylation of tau. Interestingly, when we investigated the kinases
15 that may be responsible for Tg-induced tau phosphorylation, we found that GSK3 β
16 was unlikely to be involved. The levels of p-GSK-3 β (Ser9), which is the inactivated
17 form, were even increased after Tg treatment. This contradicts to a recent report
18 suggesting that Tg-induced phosphorylation of tau could be attenuated by inhibiting
19 activity of GSK3 β [50]. Since we used primary cultures of cortical neurons as our
20 model system, while they used a neuronal cell line and animals in their study, it is
21 difficult to have a direct comparison between the results. Another possibility for
22 causing the discrepancy would be the dosage of Tg in the experiments. The
23 concentration of Tg in our study was very low. As shown in Fig. 6, 10 nM Tg did not
24 induce even a sharp and transient increase in $[Ca^{2+}]_i$, yet this dosage of Tg was
25 sufficient to induce ER-stress as shown in Fig. 3. It has been shown that Tg at even

1 subnanomolar concentration is effective at inhibiting Ca^{2+} -ATPase [51]. Our data
2 suggested that tau was very sensitive to environmental stress, and post-translational
3 modification of tau can be altered by a slight change in intracellular homeostasis. In
4 Tg-treated neurons, apart from phosphorylation, truncation of tau was also observed
5 together with activation of caspase-3 at late time points (Fig. 4). Caspase-cleaved tau
6 in immortalized cortical neurons was found to increase neuronal susceptibility to ER-
7 stress [52]. Hence, it is possible that the cleaved tau generated after treatment of Tg
8 can further promote UPR leading to neurodegeneration. It is worth to note that in the
9 late time points (18 and 24 h), the levels of UPR markers continued to increase while
10 that of phosphorylated-tau decreased. Under the exposure of Tg, it is not surprised
11 that ER-stress response would continue in the later time points. The prolonged ER-
12 stress displayed activated caspase-3, the observed tau dephosphorylation and cleavage
13 may therefore represent the entrance of the execution phase of apoptosis [53]. Since
14 the phosphorylation of tau can be affected by multiple factors during prolonged stress,
15 this makes it more difficult to observe the causal relation between ER stress and tau
16 hyperphosphorylation. Nevertheless, we showed that the chemical chaperone 4-PBA
17 reduced the levels of phosphorylated-tau in Tg-treated neurons in early time point (6
18 h). This gives evidence that the level of ER stress is correlated to the changes of
19 phosphorylated tau.

20 While GSK3 β is unlikely to be involved in Tg-induced phosphorylation of tau
21 in our system, we also detected other possible candidates involved in this process. We
22 found that there were no changes in the levels of p-PP2A (Tyr 307) (data not shown);
23 but there were dose-dependent decreases in levels of p35, suggesting the possible
24 involvement of CDK5 (data not shown). Since we did not find a clear increased level
25 of p25, which is a critical factor for activation of CDK5, the role of CDK5 in Tg-

1 induced tau pathology will be further investigated. Previous findings suggested that
2 dysregulation of CDK5 is involved in ER-stress mediated apoptosis [54].
3 Nevertheless, detailed mechanistic linkage between ER-stress and phosphorylation of
4 tau in relation to CDK5 will be further examined.

5

6 Our findings provide evidence showing that ER-stress and phosphorylation of
7 tau can induce each other. We propose that ER-stress can play an important role in
8 AD-like pathogenesis since ER-stress can induce phosphorylation of tau and form a
9 vicious cycle. This close relationship between ER-stress and tau pathology might be
10 one of the common pathways to explain the multi-factorial nature of AD. Many risk
11 factors for AD are found to be associated with ER-stress. For example, prolonged
12 light illumination of mice to deprive them of sleep has been shown to induce AD-like
13 pathology and ER-stress [55]; hyperhomocysteinemia and stroke can induce ER-stress
14 [13,56,57]. Abnormal phosphorylation of tau is always found in these situations
15 [21,55,58,59]. Given that the consequence of ER-stress responses will be translated
16 into hyperphosphorylation of tau protein, and ER-stress is very sensitive to
17 environmental changes (even without perturbation of ER calcium, as demonstrated in
18 our study), it is therefore understandable that phosphorylation of tau was observed in
19 different pathological conditions. Understanding the role of ER-stress and its
20 relationship with tau protein would certainly aid the development of promising
21 therapeutic interventions for AD.

22

1 Acknowledgement

2 This work is supported by the HKU Alzheimer's Disease Research Network,
3 Azalea (1972) Endowment Fund, General Research Grant (755206M & 761609M),
4 NSFC/RGC Joint Research Scheme (N_HKU707/07M) from Research Grant Council
5 and HKU Seed Funding for Basic Science Research (200911159082) to RCCC.
6 CHLH, SW and QSZ are supported by the Graduate School; YSH is supported by a
7 Postdoctoral Fellowship, The University of Hong Kong.

8

9

1 Figure legends

2 **Fig. 1** Increased levels of markers for UPR and hyperphosphorylation of tau were
3 observed in the hippocampus of aged TgTau^{P301L} mice. (A) Western blots show the
4 levels of p-eIF2 α and total eIF2 α in the hippocampi of 21-month-old TgTau^{P301L} mice
5 and age-matched wild type mice (upper panel). Quantitative analysis of the
6 immunoreactivity of p-eIF2 α / total eIF2 α was performed by Image J (lower panel).
7 Data represent mean \pm SE from at least 3 independent experiments. Statistical
8 analysis was performed by unpaired *t*-test. **p*<0.05 compared to WT mice. (B)
9 AT180 and p-PERK staining of the hippocampi of 21-month-old TgTau^{P301L} mice and
10 age-matched wild type mice. CA3 region is shown in the photos (40X). There was
11 little staining of AT180 in WT mice, but AT180 immunoreactivity increased
12 markedly in the TgTau^{P301L} mice and was colocalized with p-PERK immunoreactivity.

13

14 **Fig. 2** OA-induced hyperphosphorylation of tau led to ER-stress in primary cultures
15 of cortical neurons. Neurons were exposed to OA [50 nM] for 6, 16 or 24 h. (A to E)
16 The levels of total tau and p-Tau at Thr231, Ser262, and Ser198/199/202 were
17 detected by Western blot analysis. (F to I) The levels of p-PERK, total PERK, p-
18 eIF2 α and total eIF2 α were detected by Western blot analysis. β -Actin was used as
19 internal control. Quantitative analysis of the immunoreactivity of the detected proteins
20 was performed by Image J. Data represent mean \pm SE from at least 3 independent
21 experiments. Statistical analysis was performed by unpaired *t*-test. (J) RT-PCR was
22 performed to amplify mRNA for xbp-1, (K) followed by PstI restriction enzyme
23 digestion. DTT [1mM] served as a positive control to induce alternative splicing in
24 mRNA for xbp-1, as shown by the absence of ~300 bp digestion fragment after PstI
25 restriction enzyme digestion. (L) Levels of mRNA for GADD153 were detected by

1 PCR. Photos of agarose gel electrophoresis are representatives from three independent
2 experiments. (M) Quantitative analysis of the mRNA levels for GADD153 was
3 performed by Image J. Data represent mean \pm SE from at least 3 independent
4 experiments. Statistical analysis was performed by unpaired *t*-test. * $p < 0.05$ compared
5 to corresponding control.

6

7 **Fig. 3** Tg induced UPR and phosphorylation of tau in primary cultures of cortical
8 neurons. Tg [4 or 10 nM] was added to neuronal cultures and incubated for different
9 durations to induce ER-stress. (A to D) The levels of UPR markers including p-PERK,
10 BiP/GRP78, and p-eIF2 α were detected by Western blot analysis. (E to H) The levels
11 of total tau and p-tau at Thr231, Ser262 and Ser396 at different time-points were
12 detected. α -Tubulin was used as internal control. Quantitative analysis of the
13 immunoreactivity was performed by Image J. Data represent mean \pm SE from at least
14 3 independent experiments. Statistical analysis was performed by one-way ANOVA
15 for multiple comparisons, followed by Student Newman-Keuls as post-hoc test.
16 * $p < 0.05$ compared to corresponding control.

17

18 **Fig. 4** Tg induced activation of caspase-3 and truncation of tau. Tg [4 or 10 nM] was
19 added into neuronal cultures and incubated for different durations to induce ER-stress.
20 (A) The levels of cleaved tau and cleaved caspase-3 were detected by Western blot
21 analysis. α -Tubulin was used as internal control. Quantitative analysis of the
22 immunoreactivity was performed, and the results are shown in (B and C). Data
23 represent mean \pm SE from at least 3 independent experiments. Statistical analysis was
24 performed by one-way ANOVA for multiple comparisons, followed by Student
25 Newman-Keuls as post-hoc test. * $p < 0.05$ compared to corresponding control.

1

2 **Fig. 5** Tg-induced phosphorylation of tau was attenuated by the molecular chaperone
3 4-PBA. Primary cultures of cortical neurons were co-treated with 4-PBA [1 mM] and
4 Tg [10 nM] for 6 h. (A) Total cell lysates were collected for the detection of p-tau at
5 Thr231, Ser262 and Ser396. Representative images of Western blot films were shown.
6 (B to D) Quantitative analysis of the immunoreactivity was performed, and the results
7 were shown. Data represent mean \pm SE from at least 3 independent experiments.
8 Statistical analysis was performed by one-way ANOVA for multiple comparisons,
9 followed by Student Newman-Keuls as post-hoc test. # $p < 0.05$ compared to control.
10 * $p < 0.05$ compared to cultures treated with Tg [10 nM].

11

12 **Fig. 6** Tg [10 nM] did not trigger an acute elevation of $[Ca^{2+}]_i$. Tg-induced changes in
13 $[Ca^{2+}]_i$ were detected by using Fluo-4AM dye. Cortical neurons were exposed to Tg
14 [10 nM or 20 μ M] at time 120 s. The changes of $[Ca^{2+}]_i$ were detected and expressed
15 as fold of control. Data represent mean \pm SE from at least 4 independent experiments.

16

17 **Fig. 7** Tg did not induce activation of GSK3 β but induced the cleavage of p35.
18 Primary cultures of cortical neurons were incubated with Tg [4 or 10 nM] for different
19 durations. (A) Total cell lysates were collected for the detection of p-GSK3 β (Ser9)
20 and p-GSK3 β (pY216) by Western blot analysis. α -Tubulin was used as internal
21 control. (B and C) Quantitative analysis of the immunoreactivity was performed. Data
22 represent mean \pm SE from at least 3 independent experiments. Statistical analysis was
23 performed by one-way ANOVA for multiple comparisons, followed by Student
24 Newman-Keuls as post-hoc test. * $p < 0.05$ compared to corresponding control.

25

1

Table 1

2

Primer sequences and reaction conditions for RT-PCR

Target genes	Sequence	Product size (bp)	Annealing temperature (°C)	No. of cycles
GAPDH	F: 5'- gcaagttcaacggcacag-3' R: 5'- gccgtagactccacgacat-3'	140	62	33
Xbp-1	F: 5'-aaacagagtagcagcgcagactgc-3' R: 5'-ggatctctaaaactagaggcttggtg-3'	600	62	33
GADD153	F: 5'-tcagatgaaattggggcac-3' R: 5'-tttctcgttgagccgctcg-3'	340	60	35

3

4

1 **REFERENCES**

- 2
- 3 [1] Querfurth HW, LaFerla FM (2010) Alzheimer's disease. *N Engl J Med* **362**,
4 329-344.
- 5 [2] Schroder M, Kaufman RJ (2005) ER stress and the unfolded protein response.
6 *Mutat Res* **569**, 29-63.
- 7 [3] Lindholm D, Wootz H, Korhonen L (2006) ER stress and neurodegenerative
8 diseases. *Cell Death Differ* **13**, 385-392.
- 9 [4] Harding HP, Zhang Y, Ron D (1999) Protein translation and folding are
10 coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397**, 271-274.
- 11 [5] Friedlander R, Jarosch E, Urban J, Volkwein C, Sommer T (2000) A
12 regulatory link between ER-associated protein degradation and the unfolded-
13 protein response. *Nat Cell Biol* **2**, 379-384.
- 14 [6] Marciniak SJ, Ron D (2006) Endoplasmic reticulum stress signaling in disease.
15 *Physiol Rev* **86**, 1133-1149.
- 16 [7] Milhavel O, Martindale JL, Camandola S, Chan SL, Gary DS, Cheng A,
17 Holbrook NJ, Mattson MP (2002) Involvement of Gadd153 in the pathogenic
18 action of presenilin-1 mutations. *J Neurochem* **83**, 673-681.
- 19 [8] Katayama T, Imaizumi K, Honda A, Yoneda T, Kudo T, Takeda M, Mori K,
20 Rozmahel R, Fraser P, George-Hyslop PS, Tohyama M (2001) Disturbed
21 activation of endoplasmic reticulum stress transducers by familial Alzheimer's
22 disease-linked presenilin-1 mutations. *J Biol Chem* **276**, 43446-43454.
- 23 [9] Hoozemans JJ, van Haastert ES, Eikelenboom P, de Vos RA, Rozemuller JM,
24 Scheper W (2007) Activation of the unfolded protein response in Parkinson's
25 disease. *Biochem Biophys Res Commun* **354**, 707-711.
- 26 [10] Zong WX, Li C, Hatzivassiliou G, Lindsten T, Yu QC, Yuan J, Thompson CB
27 (2003) Bax and Bak can localize to the endoplasmic reticulum to initiate
28 apoptosis. *J Cell Biol* **162**, 59-69.
- 29 [11] Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D (2000)
30 Coupling of stress in the ER to activation of JNK protein kinases by
31 transmembrane protein kinase IRE1. *Science* **287**, 664-666.
- 32 [12] Rao RV, Castro-Obregon S, Frankowski H, Schuler M, Stoka V, del RG,
33 Bredesen DE, Ellerby HM (2002) Coupling endoplasmic reticulum stress to
34 the cell death program. An Apaf-1-independent intrinsic pathway. *J Biol Chem*
35 **277**, 21836-21842.
- 36 [13] Kim HJ, Cho HK, Kwon YH (2008) Synergistic induction of ER stress by
37 homocysteine and beta-amyloid in SH-SY5Y cells. *J Nutr Biochem* **19**, 754-
38 761.

- 1 [14] Hoozemans JJ, van Haastert ES, Nijholt DA, Rozemuller AJ, Eikelenboom P,
2 Scheper W (2009) The unfolded protein response is activated in pretangle
3 neurons in Alzheimer's disease hippocampus. *Am J Pathol* **174**, 1241-1251.
- 4 [15] Hoozemans JJ, Veerhuis R, van Haastert ES, Rozemuller JM, Baas F,
5 Eikelenboom P, Scheper W (2005) The unfolded protein response is activated
6 in Alzheimer's disease. *Acta Neuropathol* **110**, 165-172.
- 7 [16] Hosoi T, Ozawa K (2010) Endoplasmic reticulum stress in disease:
8 mechanisms and therapeutic opportunities. *Clin Sci (Lond)* **118**, 19-29.
- 9 [17] Terro F, Czech C, Esclaire F, Elyaman W, Yardin C, Baclet MC, Touchet N,
10 Tremp G, Pradier L, Hugon J (2002) Neurons overexpressing mutant
11 presenilin-1 are more sensitive to apoptosis induced by endoplasmic
12 reticulum-Golgi stress. *J Neurosci Res* **69**, 530-539.
- 13 [18] Yu MS, Suen KC, Kwok NS, So KF, Hugon J, Chang RC (2006) Beta-
14 amyloid peptides induces neuronal apoptosis via a mechanism independent of
15 unfolded protein responses. *Apoptosis* **11**, 687-700.
- 16 [19] Lai CS, Preisler J, Baum L, Lee DH, Ng HK, Hugon J, So KF, Chang RC
17 (2009) Low molecular weight Aβ induces collapse of endoplasmic
18 reticulum. *Mol Cell Neurosci* **41**, 32-43.
- 19 [20] Spatara ML, Robinson AS (2010) Transgenic mouse and cell culture models
20 demonstrate a lack of mechanistic connection between endoplasmic reticulum
21 stress and tau dysfunction. *J Neurosci Res* **88**, 1951-1961.
- 22 [21] Ho YS, Yu MS, Yang XF, So KF, Yuen WH, Chang RCC (2010)
23 Neuroprotective effects of polysaccharides from wolfberry, the fruits of
24 *Lycium barbarum*, against homocysteine-induced toxicity in rat cortical
25 neurons. *J Alzheimers Dis.* **19**, 813-827.
- 26 [22] Cheung YT, Zhang NQ, Hung CH, Lai CS, Yu MS, So KF, Chang RCC (2011)
27 Temporal relationship of autophagy and apoptosis in neurons challenged by
28 low molecular weight beta-amyloid peptide. *J. Cell Mol. Med* **15**, 244-257.
- 29 [23] Lewis J, McGowan E, Rockwood J, Melrose H, Nacharaju P, Van SM,
30 Gwinn-Hardy K, Paul MM, Baker M, Yu X, Duff K, Hardy J, Corral A, Lin
31 WL, Yen SH, Dickson DW, Davies P, Hutton M (2000) Neurofibrillary
32 tangles, amyotrophy and progressive motor disturbance in mice expressing
33 mutant (P301L) tau protein. *Nat Genet* **25**, 402-405.
- 34 [24] Sahara N, Lewis J, DeTure M, McGowan E, Dickson DW, Hutton M, Yen SH
35 (2002) Assembly of tau in transgenic animals expressing P301L tau: alteration
36 of phosphorylation and solubility. *J Neurochem* **83**, 1498-1508.
- 37 [25] Ho YS, Yu MS, Yik SY, So KF, Yuen WH, Chang RC (2009)
38 Polysaccharides from wolfberry antagonizes glutamate excitotoxicity in rat
39 cortical neurons. *Cell Mol Neurobiol* **29**, 1233-1244.
- 40 [26] Chang RCC, Yu MS, Lai CS (2006) Significance of molecular signaling for

- 1 protein translation control in neurodegenerative diseases. *Neurosignals* **15**,
2 249-258.
- 3 [27] Kins S, Cramer A, Evans DR, Hemmings BA, Nitsch RM, Gotz J (2001)
4 Reduced protein phosphatase 2A activity induces hyperphosphorylation and
5 altered compartmentalization of tau in transgenic mice. *J Biol Chem* **276**,
6 38193-38200.
- 7 [28] Kim D, Su J, Cotman CW (1999) Sequence of neurodegeneration and
8 accumulation of phosphorylated tau in cultured neurons after okadaic acid
9 treatment. *Brain Res* **839**, 253-262.
- 10 [29] Arendt T, Holzer M, Bruckner MK, Janke C, Gartner U (1998) The use of
11 okadaic acid in vivo and the induction of molecular changes typical for
12 Alzheimer's disease. *Neuroscience* **85**, 1337-1340.
- 13 [30] Vogel J, Anand VS, Ludwig B, Nawoschik S, Dunlop J, Braithwaite SP (2009)
14 The JNK pathway amplifies and drives subcellular changes in tau
15 phosphorylation. *Neuropharmacology* **57**, 539-550.
- 16 [31] Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG,
17 Ron D (2002) IRE1 couples endoplasmic reticulum load to secretory capacity
18 by processing the XBP-1 mRNA. *Nature* **415**, 92-96.
- 19 [32] Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R,
20 Kreibich G, Hendershot LM, Ron D (1996) Signals from the stressed
21 endoplasmic reticulum induce C/EBP-homologous protein
22 (CHOP/GADD153). *Mol Cell Biol* **16**, 4273-4280.
- 23 [33] Takadera T, Fujibayashi M, Kaniyu H, Sakota N, Ohyashiki T (2007)
24 Caspase-dependent apoptosis induced by thapsigargin was prevented by
25 glycogen synthase kinase-3 inhibitors in cultured rat cortical neurons.
26 *Neurochem Res* **32**, 1336-1342.
- 27 [34] Fasulo L, Ugolini G, Visintin M, Bradbury A, Brancolini C, Verzillo V,
28 Novak M, Cattaneo A (2000) The neuronal microtubule-associated protein tau
29 is a substrate for caspase-3 and an effector of apoptosis. *J Neurochem* **75**, 624-
30 633.
- 31 [35] Yin H, Kuret J (2006) C-terminal truncation modulates both nucleation and
32 extension phases of tau fibrillization. *FEBS Lett* **580**, 211-215.
- 33 [36] Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO,
34 Gorgun CZ, Hotamisligil GS (2006) Chemical chaperones reduce ER stress
35 and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science*
36 **313**, 1137-1140.
- 37 [37] Sabala P, Czarny M, Woronczak JP, Baranska J (1993) Thapsigargin: potent
38 inhibitor of Ca²⁺ transport ATP-ases of endoplasmic and sarcoplasmic
39 reticulum. *Acta Biochim Pol* **40**, 309-319.
- 40 [38] Hartigan JA, Johnson GV (1999) Transient increases in intracellular calcium

- 1 result in prolonged site-selective increases in Tau phosphorylation through a
2 glycogen synthase kinase 3beta-dependent pathway. *J Biol Chem* **274**, 21395-
3 21401.
- 4 [39] Morgan D, Munireddy S, Alamed J, DeLeon J, Diamond DM, Bickford P,
5 Hutton M, Lewis J, McGowan E, Gordon MN (2008) Apparent behavioral
6 benefits of tau overexpression in P301L tau transgenic mice. *J Alzheimers Dis*
7 **15**, 605-614.
- 8 [40] Arendash GW, Lewis J, Leighty RE, McGowan E, Cracchiolo JR, Hutton M,
9 Garcia MF (2004) Multi-metric behavioral comparison of APPsw and P301L
10 models for Alzheimer's disease: linkage of poorer cognitive performance to
11 tau pathology in forebrain. *Brain Res* **1012**, 29-41.
- 12 [41] Ramsden M, Kotilinek L, Forster C, Paulson J, McGowan E, SantaCruz K,
13 Guimaraes A, Yue M, Lewis J, Carlson G, Hutton M, Ashe KH (2005) Age-
14 dependent neurofibrillary tangle formation, neuron loss, and memory
15 impairment in a mouse model of human tauopathy (P301L). *J Neurosci* **25**,
16 10637-10647.
- 17 [42] Perreault S, Bousquet O, Lauzon M, Paiement J, Leclerc N (2009) Increased
18 association between rough endoplasmic reticulum membranes and
19 mitochondria in transgenic mice that express P301L tau. *J Neuropathol Exp*
20 *Neurol* **68**, 503-514.
- 21 [43] Yoshiyama Y, Higuchi M, Zhang B, Huang SM, Iwata N, Saido TC, Maeda J,
22 Suhara T, Trojanowski JQ, Lee VM (2007) Synapse loss and microglial
23 activation precede tangles in a P301S tauopathy mouse model. *Neuron* **53**,
24 337-351.
- 25 [44] Kim SM, Yoon SY, Choi JE, Park JS, Choi JM, Nguyen T, Kim DH (2010)
26 Activation of eukaryotic initiation factor-2 alpha-kinases in okadaic acid-
27 treated neurons. *Neuroscience* **169**, 1831-1839.
- 28 [45] Yoon S, Choi J, Yoon J, Huh JW, Kim D (2006) Okadaic acid induces JNK
29 activation, bim overexpression and mitochondrial dysfunction in cultured rat
30 cortical neurons. *Neurosci Lett* **394**, 190-195.
- 31 [46] Kerokoski P, Suuronen T, Salminen A, Soininen H, Pirttila T (2001) The
32 levels of cdk5 and p35 proteins and tau phosphorylation are reduced during
33 neuronal apoptosis. *Biochem Biophys Res Commun* **280**, 998-1002.
- 34 [47] Rametti A, Esclaire F, Yardin C, Terro F (2004) Linking alterations in tau
35 phosphorylation and cleavage during neuronal apoptosis. *J Biol Chem* **279**,
36 54518-54528.
- 37 [48] Linford NJ, Dorsa DM (2002) 17beta-Estradiol and the phytoestrogen
38 genistein attenuate neuronal apoptosis induced by the endoplasmic reticulum
39 calcium-ATPase inhibitor thapsigargin. *Steroids* **67**, 1029-1040.
- 40 [49] Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J (2000)
41 Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and

- 1 cytotoxicity by amyloid-beta. *Nature* **403**, 98-103.
- 2 [50] Fu ZQ, Yang Y, Song J, Jiang Q, Liu ZC, Wang Q, Zhu LQWJZ, Tian Q
3 (2010) LiCl attenuates thapsigargin-induced tau hyperphosphorylation by
4 inhibiting GSK-3beta in vivo and in vitro. *J Alzheimers Dis* **21**, 1107-1117.
- 5 [51] Sagara Y, Inesi G (1991) Inhibition of the sarcoplasmic reticulum Ca²⁺
6 transport ATPase by thapsigargin at subnanomolar concentrations. *J Biol*
7 *Chem* **266**, 13503-13506.
- 8 [52] Matthews-Roberson TA, Quintanilla RA, Ding H, Johnson GV (2008)
9 Immortalized cortical neurons expressing caspase-cleaved tau are sensitized to
10 endoplasmic reticulum stress induced cell death. *Brain Res* **1234**, 206-212.
- 11 [53] Meske V, Albert F, Richter D, Schwarze J, Ohm TG (2003) Blockade of
12 HMG-CoA reductase activity causes changes in microtubule-stabilizing
13 protein tau via suppression of geranylgeranylpyrophosphate formation:
14 implications for Alzheimer's disease. *Eur J Neurosci* **17**, 93-102.
- 15 [54] Saito T, Konno T, Hosokawa T, Asada A, Ishiguro K, Hisanaga S (2007)
16 p25/cyclin-dependent kinase 5 promotes the progression of cell death in
17 nucleus of endoplasmic reticulum-stressed neurons. *J Neurochem* **102**, 133-
18 140.
- 19 [55] Ling ZQ, Tian Q, Wang L, Fu ZQ, Wang XC, Wang Q, Wang JZ (2009)
20 Constant illumination induces Alzheimer-like damages with endoplasmic
21 reticulum involvement and the protection of melatonin. *J Alzheimers Dis* **16**,
22 287-300.
- 23 [56] Althausen S, Paschen W (2000) Homocysteine-induced changes in mRNA
24 levels of genes coding for cytoplasmic- and endoplasmic reticulum-resident
25 stress proteins in neuronal cell cultures. *Brain Res Mol Brain Res* **84**, 32-40.
- 26 [57] Oida Y, Shimazawa M, Imaizumi K, Hara H (2008) Involvement of
27 endoplasmic reticulum stress in the neuronal death induced by transient
28 forebrain ischemia in gerbil. *Neuroscience* **151**, 111-119.
- 29 [58] Zhang CE, Tian Q, Wei W, Peng JH, Liu GP, Zhou XW, Wang Q, Wang DW,
30 Wang JZ (2008) Homocysteine induces tau phosphorylation by inactivating
31 protein phosphatase 2A in rat hippocampus. *Neurobiol Aging* **29**, 1654-1665.
- 32 [59] Wen Y, Yang S, Liu R, Simpkins JW (2004) Transient cerebral ischemia
33 induces site-specific hyperphosphorylation of tau protein. *Brain Res* **1022**, 30-
34 38.
35
36

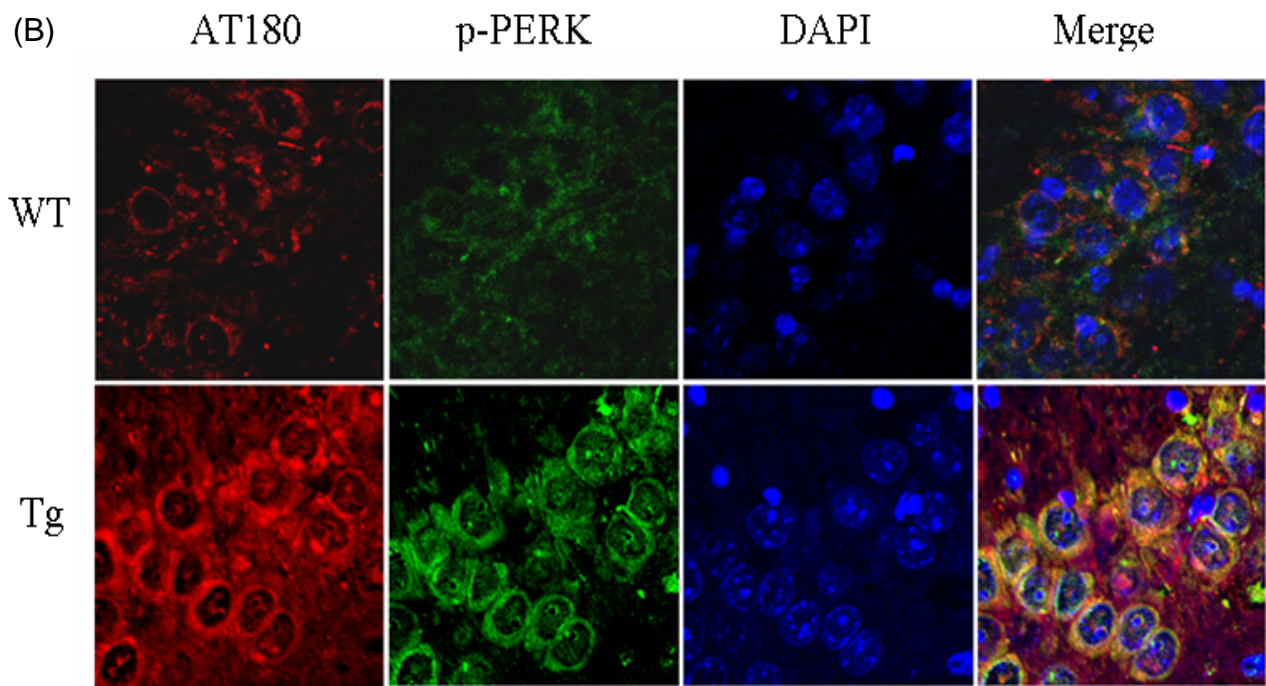
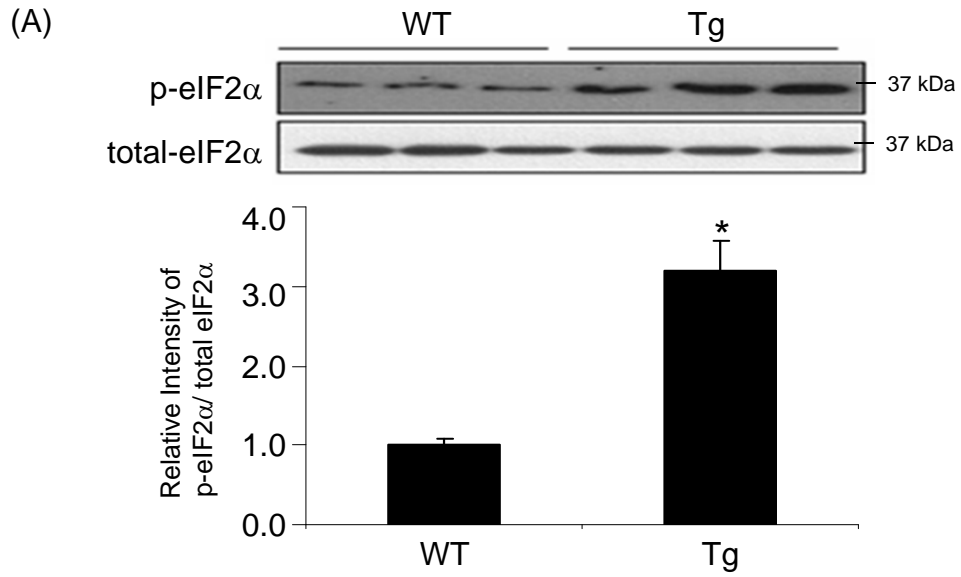


Fig. 1

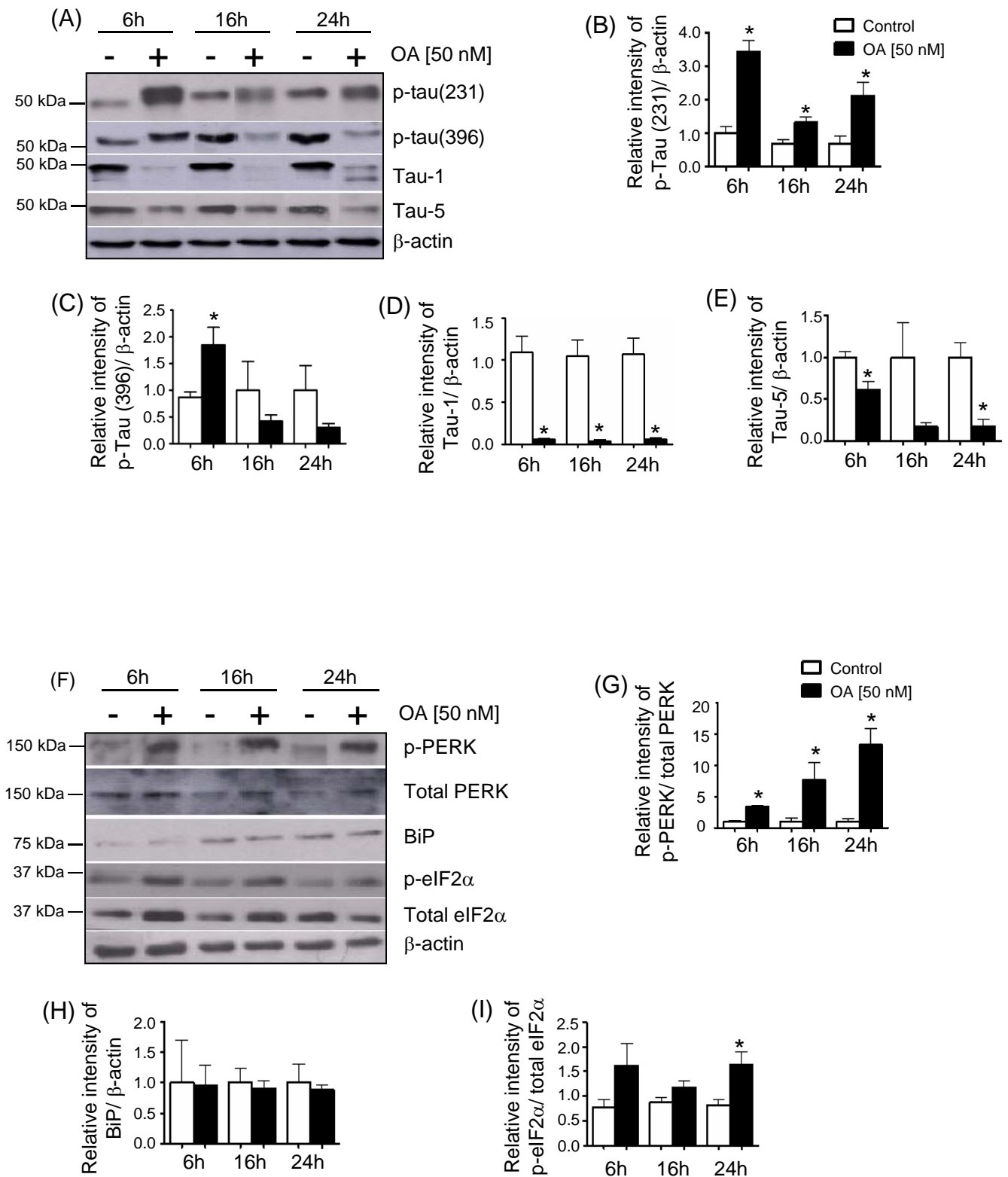


Fig. 2

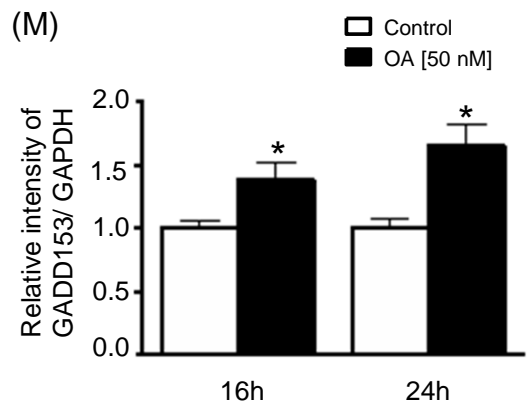
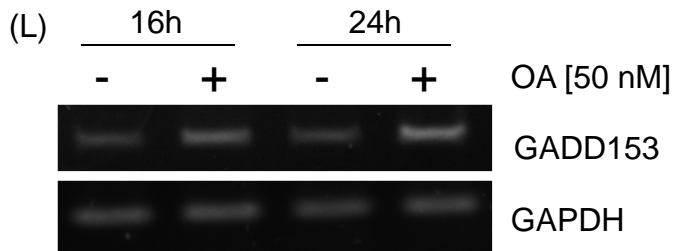
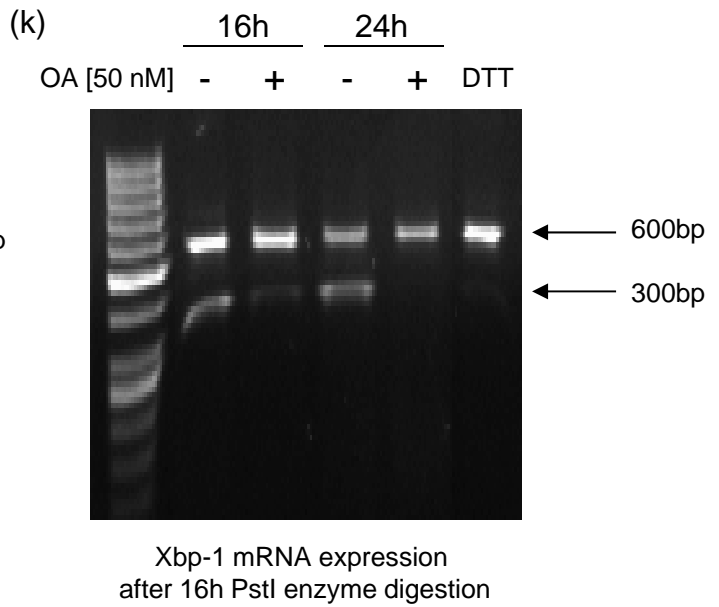
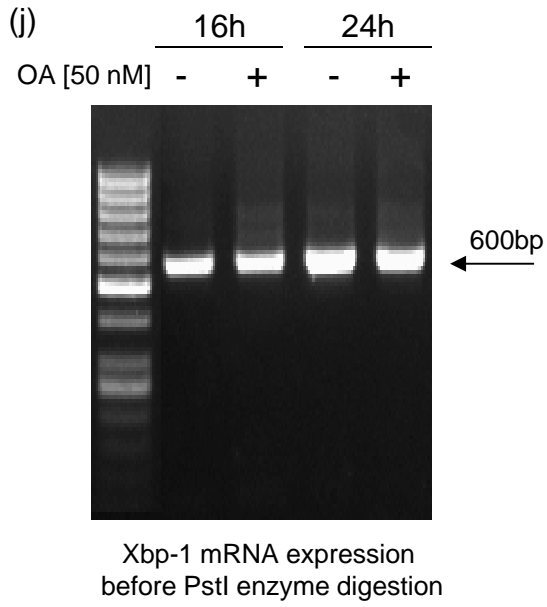


Fig. 2

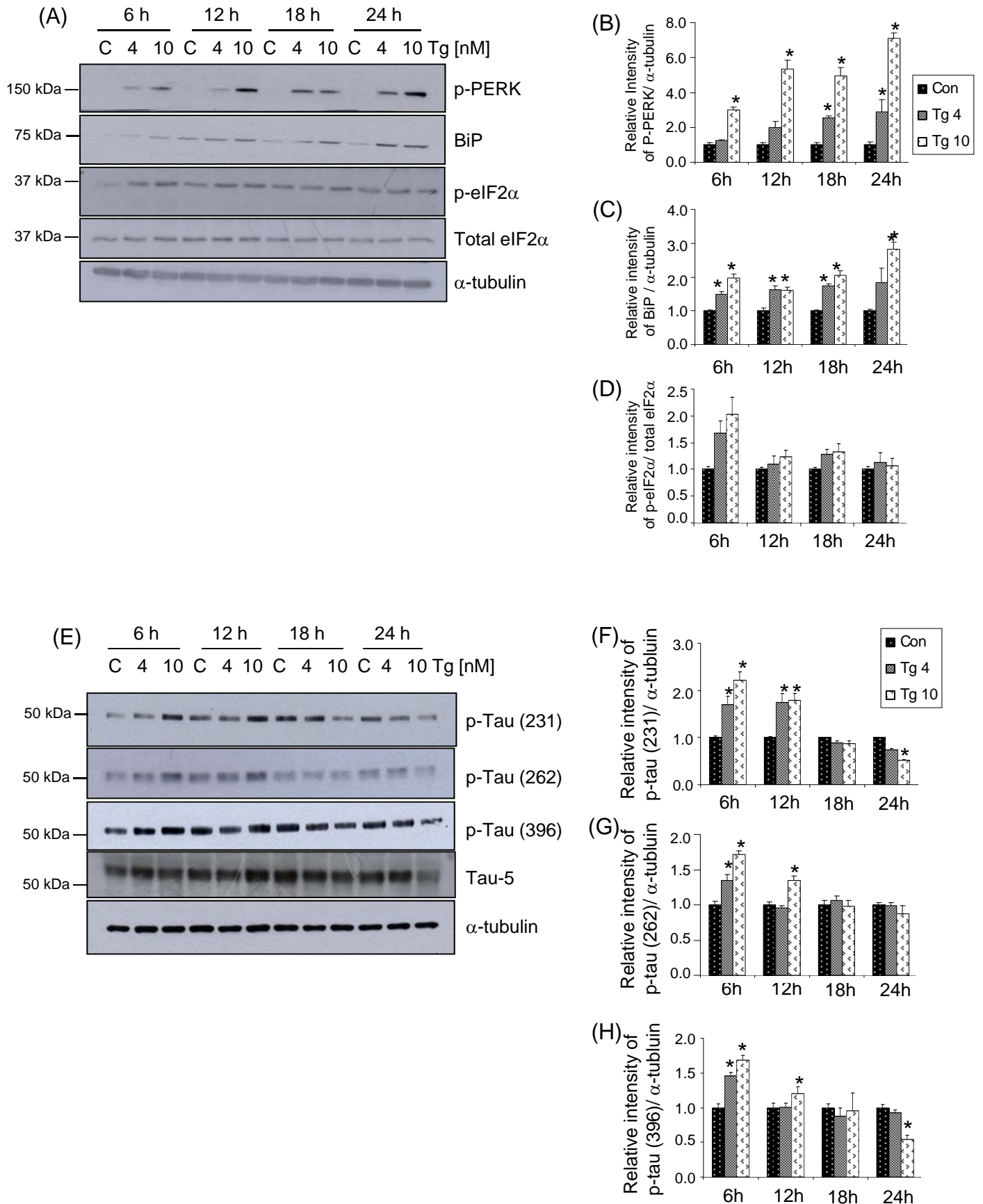


Fig. 3

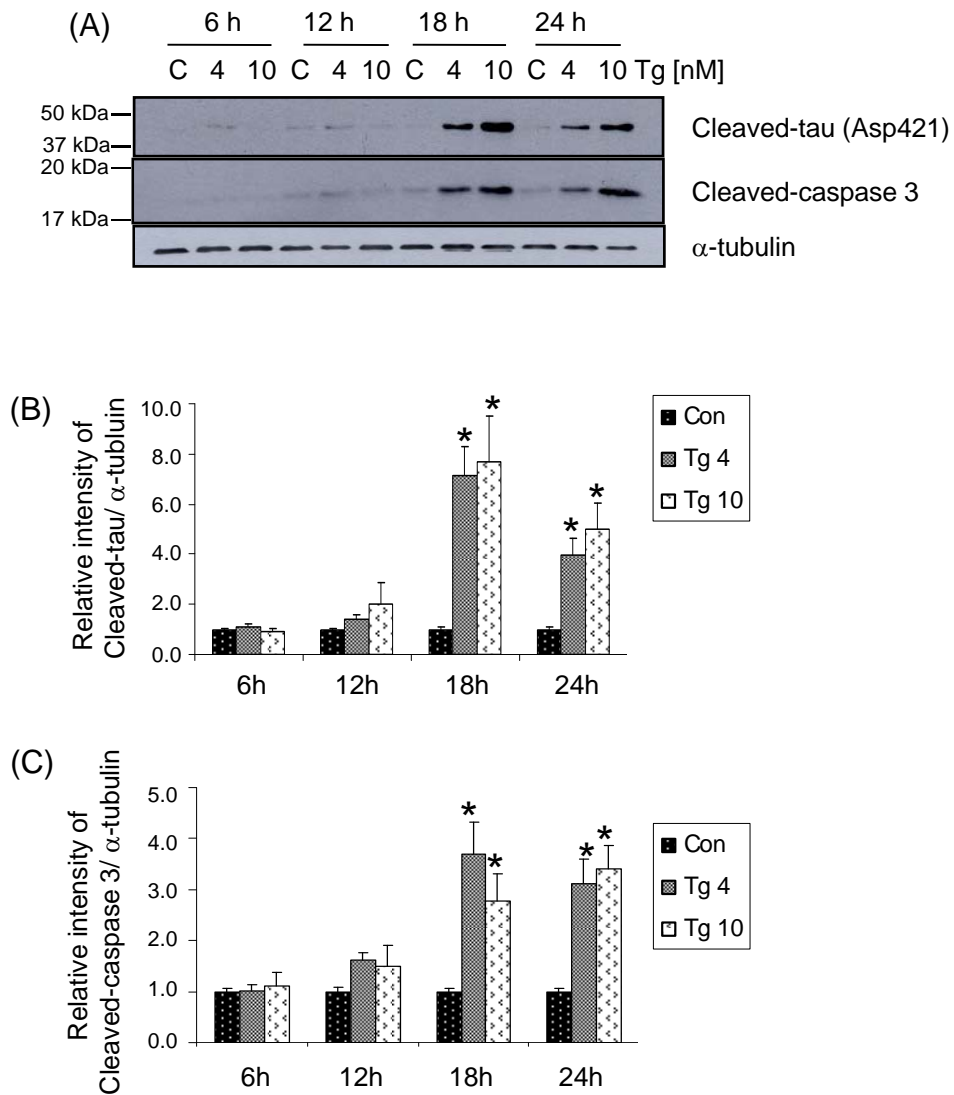


Fig. 4

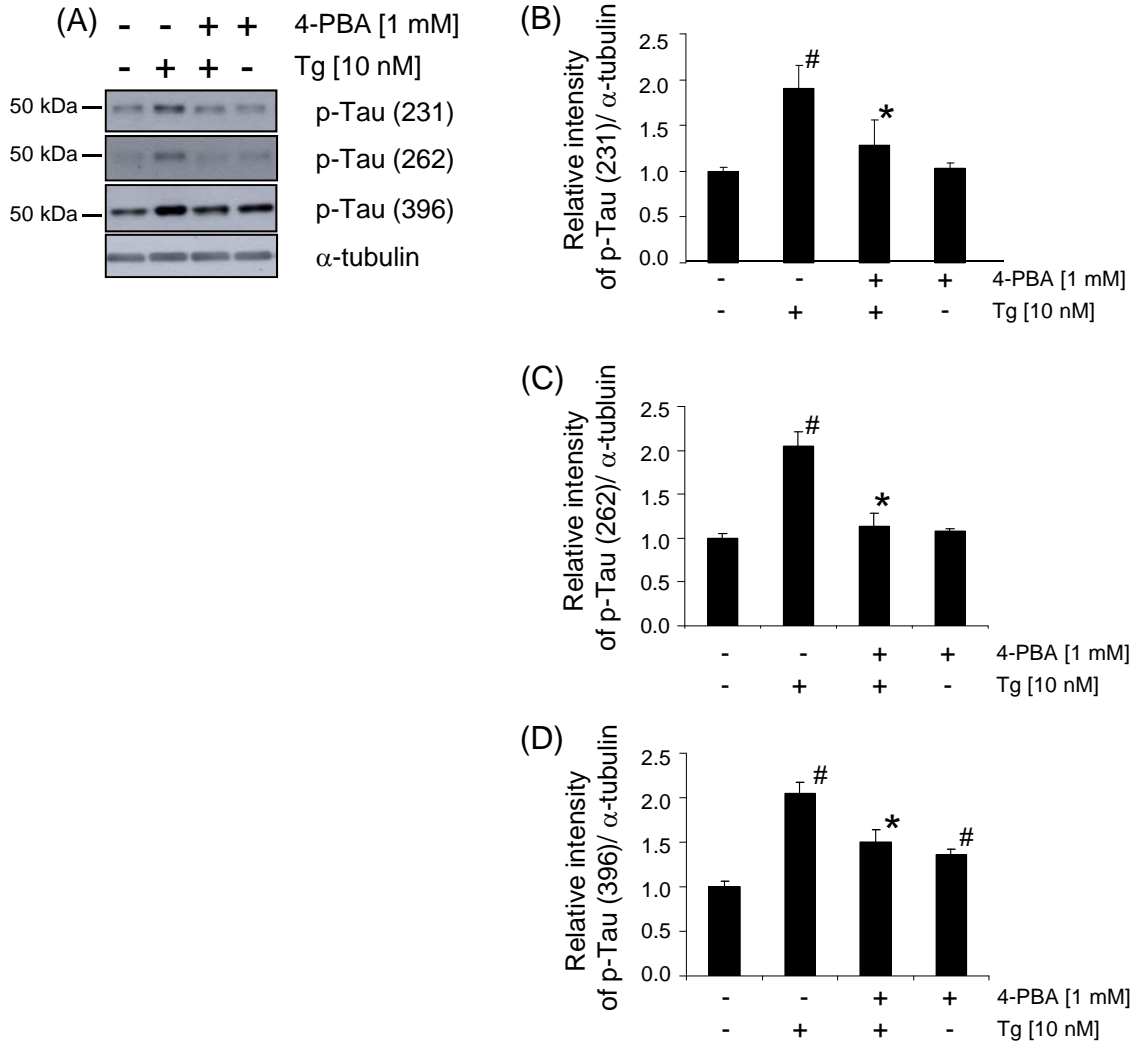


Fig. 5

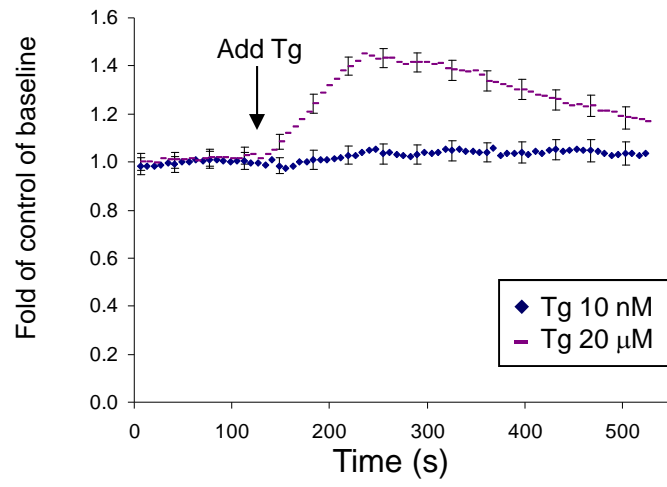


Fig. 6

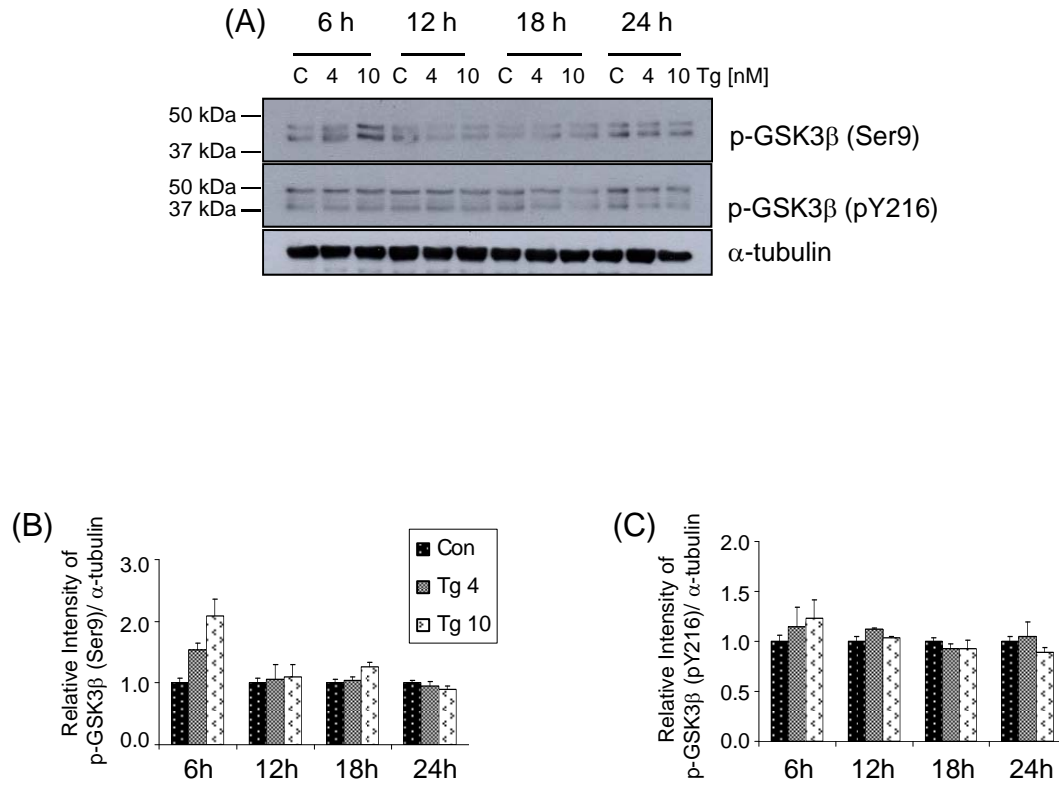
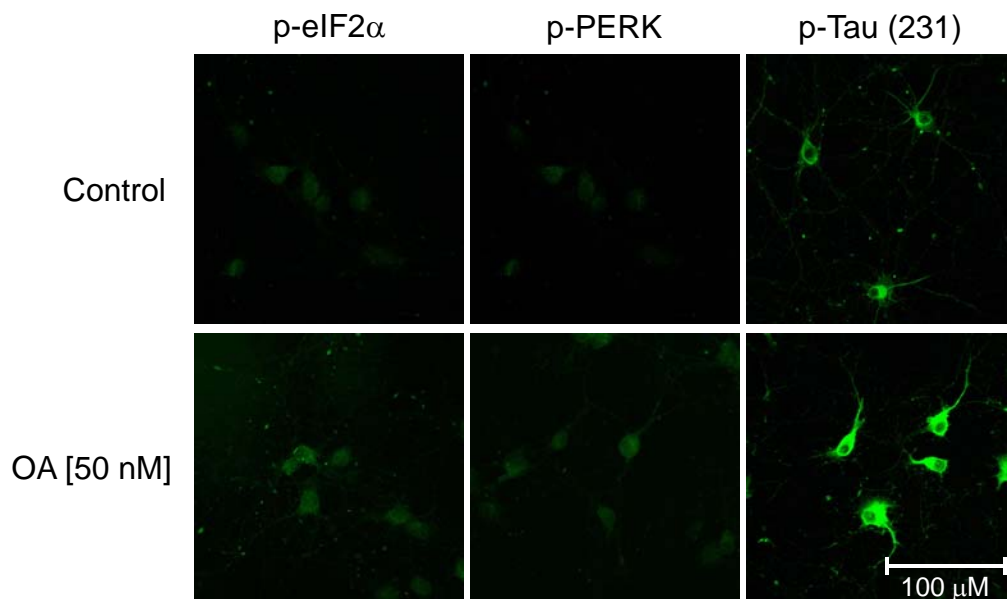
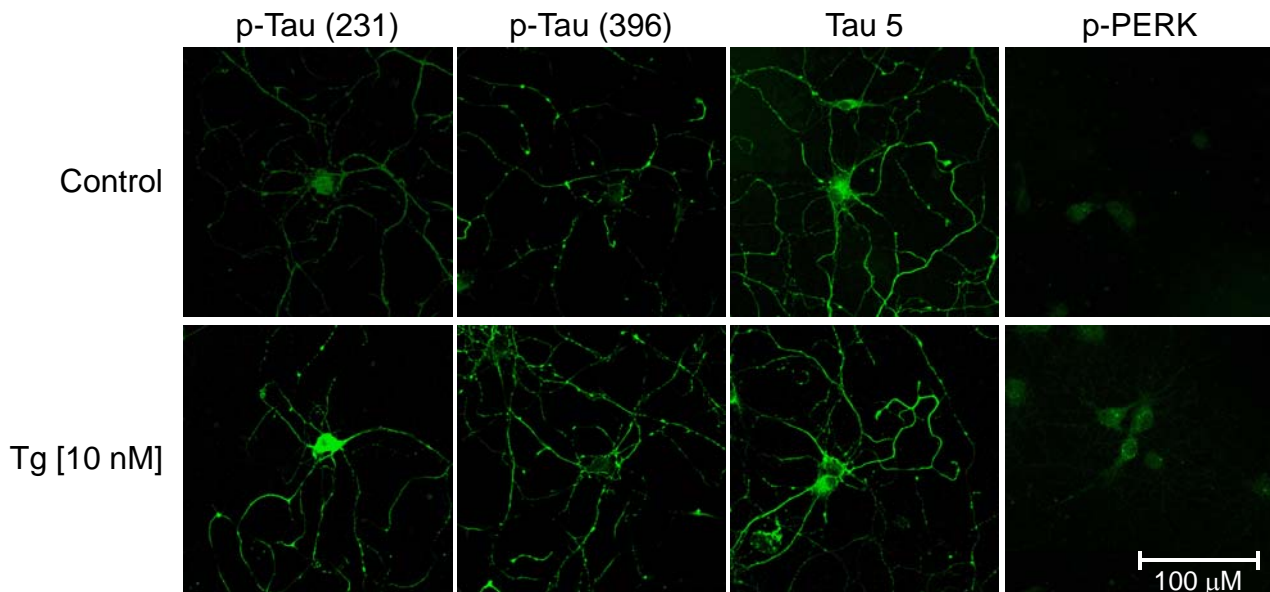


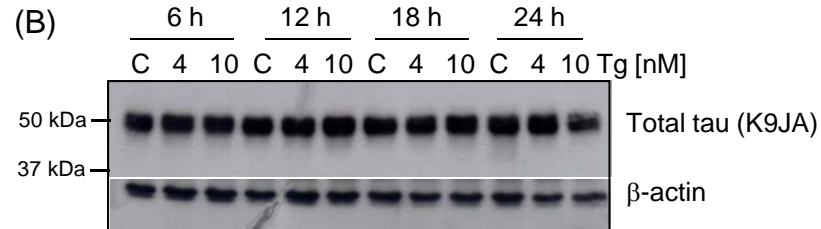
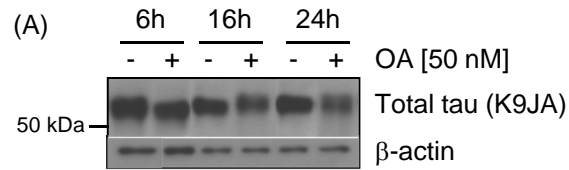
Fig. 7



Supplementary Fig.1 OA-induced hyperphosphorylation of tau led to ER-stress in primary cultures of hippocampal neurons. OA [50 nM] was added to neuronal cultures and incubated for 6h to induce tau hyperphosphorylation. The neurons were fixed with 4% paraformaldehyde and stained with specific antibodies against p-Tau (231), p-Tau (396), Tau 5 and p-PERK. Representative photos of each group are shown, magnification = 40X. The level of p-Tau (231) was markedly increased in the OA-treated group. In the same time, the levels of p-eIF2 α A and p-PERK were also increased.



Supplementary Fig.2 Tg induced UPR and phosphorylation of tau in primary cultures of hippocampal neurons. Tg [10 nM] was added to neuronal cultures and incubated for 6h to induce ER-stress. The neurons were fixed with 4% paraformaldehyde and stained with specific antibodies against p-Tau (231), p-Tau (396), Tau 5 and p-PERK. Representative photos of each group are shown, magnification = 40X. The level of p-PERK, a UPR marker, was elevated in the Tg-treated group. In the same time, the levels of p-Tau (231), p-Tau (396) were also increased. The level of total tau, which detected by the Tau 5 antibody remained unchanged.



Supplementary Fig.3 Levels of total tau were further confirmed by using the Tau-5 antibody. (A) The pattern of total tau detected by Tau-5 antibody in OA-treated cultures was similar to those detected by the K9JA pan-tau antibody in Fig. 2A. (B) The pattern of total tau detected by Tau-5 antibody in Tg-treated cultures was similar to those detected by the K9JA pan-tau antibody in Fig. 3E.