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ORIGINAL RESEARCH ARTICLE



PTK2 regulates tau-induced neurotoxicity via phosphorylation of p62 at Ser403

Shinrye Lee^{a*}, Myungjin Jo^{a*}, Younghwi Kwon^{a,b}, Yu-Mi Jeon^a, Seyeon Kim^{a,b}, Kea Joo Lee^c  and Hyung-Jun Kim^{a,b} 

^aDementia Research Group, Korea Brain Research Institute (KBRI), Daegu, South Korea; ^bDepartment of Brain & Cognitive Sciences, DGIST, Daegu, South Korea; ^cNeural Circuits Research Group, Korea Brain Research Institute (KBRI), Daegu, South Korea

ABSTRACT

Tau is a microtubule-associated protein that forms insoluble filaments that accumulate as neurofibrillary tangles in neurodegenerative diseases such as Alzheimer's disease and other related tauopathies. A relationship between abnormal Tau accumulation and ubiquitin-proteasome system impairment has been reported. However, the molecular mechanism linking Tau accumulation and ubiquitin proteasome system (UPS) dysfunction remains unclear. Here, we show that overexpression of wild-type or mutant (P301L) Tau increases the abundance of polyubiquitinated proteins and activates the autophagy-lysosome pathway in mammalian neuronal cells. Previous studies found that PTK2 inhibition mitigates toxicity induced by UPS impairment. Thus, we investigated whether PTK2 inhibition can attenuate Tau-induced UPS impairment and cell toxicity. We found that PTK2 inhibition significantly reduces Tau-induced death in mammalian neuronal cells. Moreover, overexpression of WT or mutant Tau increased the phosphorylation levels of PTK2 and p62. We also confirmed that PTK2 inhibition suppresses Tau-induced phosphorylation of PTK2 and p62. Furthermore, PTK2 inhibition significantly attenuated the climbing defect and shortened the lifespan in the *Drosophila* model of tauopathy. In addition, we observed that phosphorylation of p62 is markedly increased in Alzheimer's disease patients with tauopathies. Taken together, our results indicate that the UPS dysfunction induced by Tau accumulation might contribute directly to neurodegeneration in tauopathies and that PTK2 could be a promising therapeutic target for tauopathies.

Abbreviations: MAPT/Tau: Microtubule-associated protein tau; AD: Alzheimer's disease; FTD: frontotemporal dementia; PHFs: paired helical filaments; NFTs: neurofibrillary tangles; FTDP-17: FTD with parkinsonism linked to chromosome-17; UPS: ubiquitin-proteasome system; ALS: amyotrophic lateral sclerosis; HD: Huntington's disease; PTK2: protein tyrosine kinase 2; N2a: Neuro-2a; PI: propidium iodide

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MAPT/Tau; PTK2; p62; Alzheimer's disease; ubiquitin-proteasome system

Introduction

Microtubule-associated protein tau (MAPT/Tau) is a neuronal protein that mediates axonal transport of cellular components along microtubules by binding to microtubules and regulating their assembly and stabilization (Cuchillo-Ibanez *et al.*, 2008; Gustke, Trinczek, Biernat, Mandelkow, & Mandelkow, 1994). Tauopathies are one of the most common proteinopathies associated with neurodegenerative diseases, including Alzheimer's disease (AD) and frontotemporal dementia (FTD). The presence of paired helical filaments (PHFs) and neurofibrillary tangles (NFTs) inside neurons or glial cells is the pathological hallmark of AD. In addition, FTD with parkinsonism linked to chromosome-17 (FTDP-17) is characterized by filamentous inclusions that are composed of hyperphosphorylated Tau (Ballatore, Lee, & Trojanowski, 2007; Lee, Goedert, & Trojanowski, 2001; Williams, 2006). Under pathological conditions, Tau undergoes various posttranslational modifications and detaches from microtubules to form insoluble aggregates (Gendron & Petrucelli, 2009; Iqbal, Liu, & Gong, 2016; Krüger & Mandelkow, 2016).

The ubiquitin-proteasome system (UPS) is a major intracellular protein degradation systems (Nalepa, Rolfe, & Harper, 2006). Ubiquitination-dependent degradation is involved in several cellular processes, such as protein quality control, transcription, DNA repair, the cellular stress response, and apoptosis. Previous studies have revealed that dysfunction of the UPS is associated with many neurological disorders, including AD, amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) (Durcan *et al.*, 2011; Liu, Fallon, Lashuel, Liu, & Lansbury, 2002; Zhang *et al.*, 2012). The UPS impairment in these diseases may be related to defective clearance of misfolded and aggregated proteins, leading to cell death. A recent study also found that the tauopathy in AD is associated with impaired proteolysis mediated by the UPS (Myeku *et al.*, 2016; Tai *et al.*, 2012). However, studies about the molecular mechanisms linking UPS impairment and tauopathies are insufficient.

PTK2 (protein tyrosine kinase 2) plays an essential role in regulating cell proliferation, survival, and migration (Armendáriz, Masdeu, Soriano, Ureña, & Burgaya, 2014). The tyrosine phosphorylation sites in PTK2 are Y397, 407,

576, 577, 861, and 925. Initiation of PTK2 activation requires autophosphorylation at Y397 and subsequent phosphorylation of other residues in PTK2 by SRC protein family members to activate the PTK2 downstream signaling pathway (Hanks, Ryzhova, Shin, & Brábek, 2003; Rasmussen, Müller, Jørgensen, Pedersen, & Hoffmann, 2015). Previous studies have indicated that PTK2 plays a critical role in the deposition of ubiquitinated aggregates and in neurotoxicity induced by UPS impairment through regulation of the TBK1-p62 pathway in TDP-43 proteinopathies (Lee, Jeon, *et al.*, 2020).

In the present study, we found that PTK2 inhibition mitigates UPS impairment and neurotoxicity in cell and fly models of tauopathy through the regulation of p62 phosphorylation. Moreover, the phosphorylation level of p62 is increased in the hippocampal region of AD patients with tauopathies. These results indicate that PTK2 could be a promising therapeutic target for tauopathies.

Materials and methods

Cell line

The Neuro-2a (N2a) mouse neuroblastoma cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, 11995-065) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, 16000-044) and 50 µg/ml penicillin-streptomycin (Gibco, 15140-122).

Reagents

The following reagents were purchased as indicated: dimethyl sulfoxide (DMSO; Sigma, D8418), mifepristone (RU-486; Sigma, M8046), MG132 (Calbiochem/Merck-Millipore, 474791), and a PTK2 inhibitor (PF573228; APEX BIO, B1523).

Antibodies

Antibodies against the following proteins were used for immunoblotting: p-PTK2 (Y397) (Cell Signaling Technology, 3283), PTK2 (Cell Signaling Technology, 3285), HRP-conjugated alpha-tubulin (Cell Signaling Technology, 9099), polyubiquitin (FK1, Enzo Life Science, BML-PW8805), JL-8 (GFP, Clontech, 632380), p62 (Sigma, P0067), p-p62 (S403) (GeneTex, GTX128171), LC3 (MBL, PM036), Tau (Invitrogen, 13-6400), p-Tau (S396) (Abcam, ab109390), and beta-actin (Abcam, ab16039). HRP-conjugated anti-mouse IgM (Abcam, ab97230), HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, sc-2004), and HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology, sc-2005) were used as secondary antibodies.

Transfection

N2a cells were plated in 6-well plates and transfected with 5 µg of EGFP (pRK5-EGFP), EGFP-human Tau WT (pRK5-EGFP-hTau (WT), Addgene, #46908), or EGFP-human Tau

(P301L) (pRK5-EGFP-hTau (P301L), Addgene, #46908) cDNA by using Lipofectamine 3000 reagent (Invitrogen, L3000-015) according to the manufacturer's instructions.

Western blot analysis

For total protein extraction, either cells or the heads of 20 adult flies were homogenized in Cell Lysis Buffer (Cell Signaling Technology, 9803) supplemented with protease and phosphatase inhibitor cocktails. The protein concentrations in the cell lysates were determined by a BCA protein assay (Thermo Fisher Scientific, 23225). Next, the protein extracts were mixed with 4x Bolt LDS Sample Buffer (Invitrogen) and 10x Bolt Sample Reducing Agent Buffer (Invitrogen) and then boiled at 95 °C for 5 min. An equal amount of protein from each sample was separated on Bolt 4–12% Bis-Tris Gels (Invitrogen, NW04120BOX) or NuPAGE 3–8% Tris-Acetate Gels (Invitrogen, EA0378BOX) and was then transferred to polyvinylidene difluoride (PVDF, Invitrogen, LC2005) membranes. After blocking with 5% skim milk (BD Difco, 232100) in TBS with 0.025% Tween 20 (Sigma, P2287), the membranes were probed with antibodies as indicated, and immunoreactions were detected with an ECL Prime Kit (GE Healthcare, RPN2232). Samples from three independent experiments were used, and the relative expression levels were determined using a Fusion-FX imaging system (Viber Lourmat).

Preparation of soluble and insoluble cell extracts

Cells were homogenized in Cell Lysis Buffer (Cell Signaling Technology, 9803) supplemented with protease and phosphatase inhibitor cocktails (Roche, 11836153001 and 04906837001). Soluble and insoluble fractions were obtained by centrifugation at 100,000 × g for 30 min at 4 °C. The supernatants containing the soluble fractions were harvested, and the pellets constituting the insoluble fractions were solubilized with a detergent (2% SDS). After sonication, the cell lysates were mixed with 4x Bolt LDS Sample Buffer (Invitrogen, B0007) and 10x Bolt Sample Reducing Agent Buffer (Invitrogen, B0009) and then boiled at 95 °C for 5 min.

Flow cytometric analysis

N2a cells were detached with trypsin-EDTA and washed twice with cold PBS. The cells were then resuspended in 250 µl of binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂; pH 7.4). After adding 20 µg/ml propidium iodide, flow cytometric analysis was performed within 1 h using a MoFlo Astrios cell sorter (Beckman Coulter).

Fly strains

Drosophila stocks were maintained on standard cornmeal agar medium at 24 °C unless otherwise noted. The wild-type elavGS-Gal4 (pan-neuronal GeneSwitch driver), UAS-Atg8a-GFP (autophagy reporter), UAS-CL1-GFP (UPS reporter), and UAS-TAU-V337M flies were described previously

Table 1. Human brain samples.

Case	Gender	Age at death (years)	Braak stages	
			(Tau pathology)	(Amyloid beta)
CNL (<i>n</i> = 4)				
CNL1	M	89	2	0
CNL2	F	93	2	0
CNL3	F	82	1	A
CNL4	F	72	1	A
AD (<i>n</i> = 6)				
AD1	F	85	6	C
AD2	M	84	6	C
AD3	F	91	6	C
AD4	M	81	6	C
AD5	F	78	6	C
AD6	F	90	6	C

CNL: indicates control; AD: Alzheimer disease; M: male; F: female.

0–6, Braak stages of AD stages depending on the distribution and amount of neurofibrillary tangles.

A–C, Braak stages of AD stages depending on amyloid plaques.

(Couthouis *et al.*, 2011; Lee, Jeon, *et al.*, 2020; Lee *et al.*, 2021). All other stocks were obtained from the Bloomington *Drosophila* Stock Center.

Climbing and lifespan assays

Adult male flies (0 to 1 d old) were separated and transferred into experimental vials containing fly medium mixed with or without RU-486 (in ethanol, 40 µg/ml) at a density of 25 flies per vial ($n > 100$ flies). The number of dead flies was determined daily, and flies were transferred to fresh medium every other day. Adult locomotor function was assessed by a previously described method (Feany & Bender, 2000; Lee, Kwon, *et al.*, 2020) in 100 flies per genotype per time point in all experiments. The experiments were repeated twice to ensure consistent results.

Human subjects

Postmortem tissue samples were obtained from the Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam. This study was approved by the committee of KBRI (201610-BR-004-01). Brain tissues from patients with AD ($n = 6$) and normal control individuals ($n = 4$) were used (Table 1).

Statistical analyses

Data were analyzed by unpaired Student's *t* test (Vassar Stats, www.vassarstats.net) or one-way ANOVA depending on the comparison variable with *post hoc* analysis as indicated (GraphPad Prism Software). Differences were considered significant when $p < 0.05$, and significance is indicated as follows: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$; n.s., not significant.

Results

Tau-induced UPS impairment and neuronal toxicity are suppressed by PTK2 inhibition in mammalian neuronal cells

Previous studies showed that accumulation of pathologic tau protein causes UPS impairment in a mouse model of

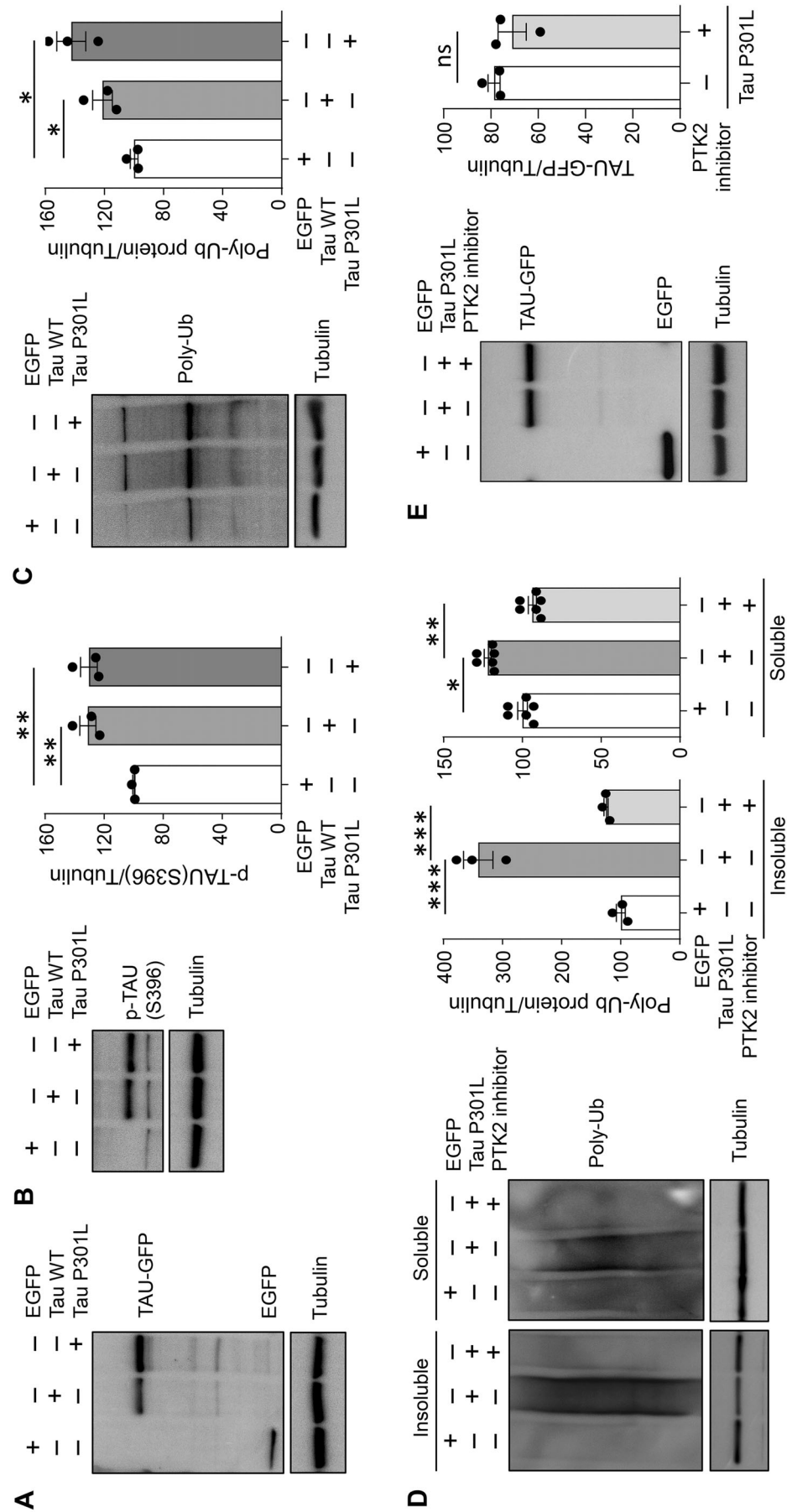
tauopathy and in AD patients (Liu *et al.*, 2021; Mori, Kondo, & Ihara, 1987; Myeku *et al.*, 2016; Tai *et al.*, 2012). We assessed the levels of TAU and phosphorylated TAU in N2a cells transfected with EGFP-tagged Tau or Tau P301L. Tau P301L is the disease-associated mutation commonly found in patients with FTDP-17 (Hutton *et al.*, 1998; Ramsden *et al.*, 2005). Moreover, overexpression of Tau P301L induces neurofibrillary pathology, neuronal loss, and behavioral impairments in mice (Santacruz *et al.*, 2005). We found that the levels of TAU and TAU phosphorylated at serine 396 (S396; a marker for pathologic Tau) were significantly increased in Tau- or Tau P301L-transfected N2a cells (Figure 1(A,B)).

Previous studies have shown that PTK2 inhibition suppresses TDP-43-induced UPS impairment and toxicity in mammalian cells and *Drosophila* (Lee, Jeon, *et al.*, 2020; Lee, Kwon, *et al.*, 2020). Moreover, overexpression of Tau P301L induces neurofibrillary pathology, neuronal loss, and behavioral impairments (Santacruz *et al.*, 2005). We observed that overexpression of Tau or Tau P301L increased the level of total polyubiquitinated protein (Figure 1(C)). In addition, Tau P301L overexpression markedly increased the level of polyubiquitinated proteins in the insoluble fractions, whereas this level was only slightly increased in the soluble fractions (Figure 1(D)). Strikingly, PTK2 inhibition significantly reduced the Tau P301L-induced accumulation of insoluble and soluble polyubiquitinated proteins (Figure 1(D)). We also found that PTK2 inhibition did not affect the Tau protein level in tau P301L-transfected N2a neuronal cells (Figure 1(E)). Accumulation of polyubiquitinated proteins is a marker for UPS impairment and can induce cytotoxicity (Lee, Jeon, *et al.*, 2020; Lee, Kwon, *et al.*, 2020; Li, Arnaud, Rockwell, & Figueiredo-Pereira, 2004; McKinnon *et al.*, 2020).

We further assessed whether PTK2 regulates tau-induced neurotoxicity using flow cytometric analysis. We used propidium iodide (PI) as a marker for cell death and apoptosis. As expected, the number of PI-positive cells was greatly increased among Tau- and Tau P301L-transfected N2a neuronal cells compared to EGFP-transfected cells (Figure 2(A,B)). Inhibition of PTK2 significantly attenuated Tau- or Tau P301L-induced cell death (Figure 2(A,B)). These findings indicate the possibility that PTK2 inhibition attenuates Tau-induced UPS impairment and neurotoxicity in a mammalian model of tauopathy.

PTK2 inhibition suppresses ser 403 phosphorylation of p62 in tau-overexpressing cells

Previous studies have reported that UPS impairment induces ALP activation as a compensatory mechanism (Wang & Wang, 2015). To determine whether overexpression of Tau or Tau P301L induces autophagy, we examined the levels of LC3-I and II. The LC3-I/II ratio is a key indicator of autophagy activation (Tanida, Ueno, & Kominami, 2008). Compared to overexpression of EGFP, overexpression of Tau or Tau P301L significantly increased the levels of LC3-I and II (Figure 3(B)), supporting the previous finding that



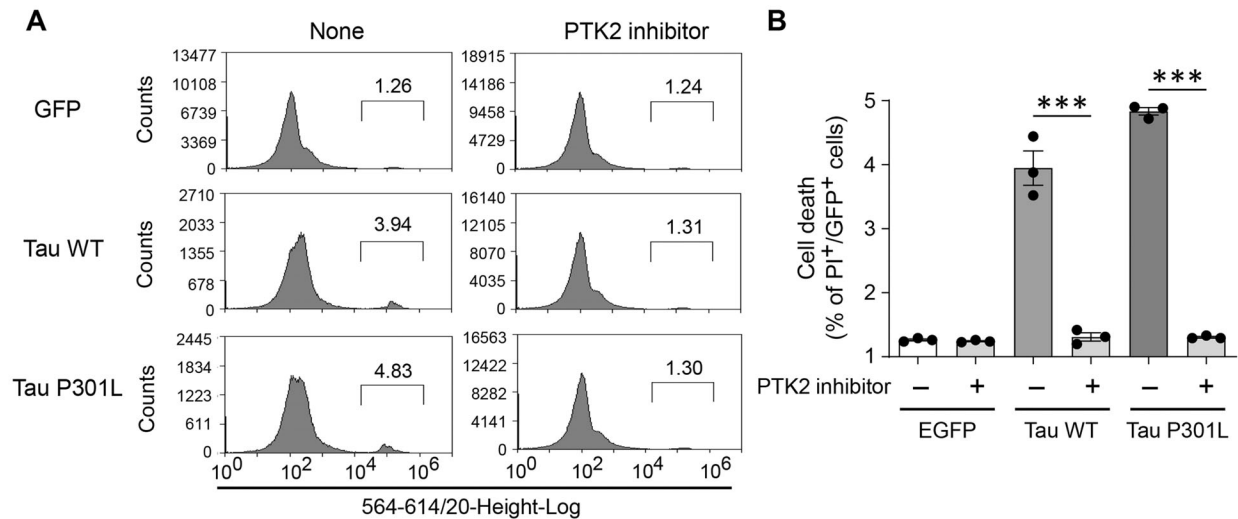


Figure 2. PTK2 inhibition mitigates Tau-induced cell death. (A,B) N2a cells were transfected with the GFP, Tau WT-GFP or Tau P301L-GFP expression construct for 24 h and subsequently treated with a PTK2 inhibitor (1 μ M) for 24 h. (A) FACS analysis was then performed (10,000 cells per condition). Tau WT-GFP- or Tau P301L-GFP-induced neuronal toxicity was significantly reduced by treatment with the PTK2 inhibitor. (B) The cell death rate determined by flow cytometric analysis is shown. The data are presented as the mean $\pm$ SD of 3 independent experiments. *** p < 0.001 (one-way ANOVA with Tukey's multiple comparison test).

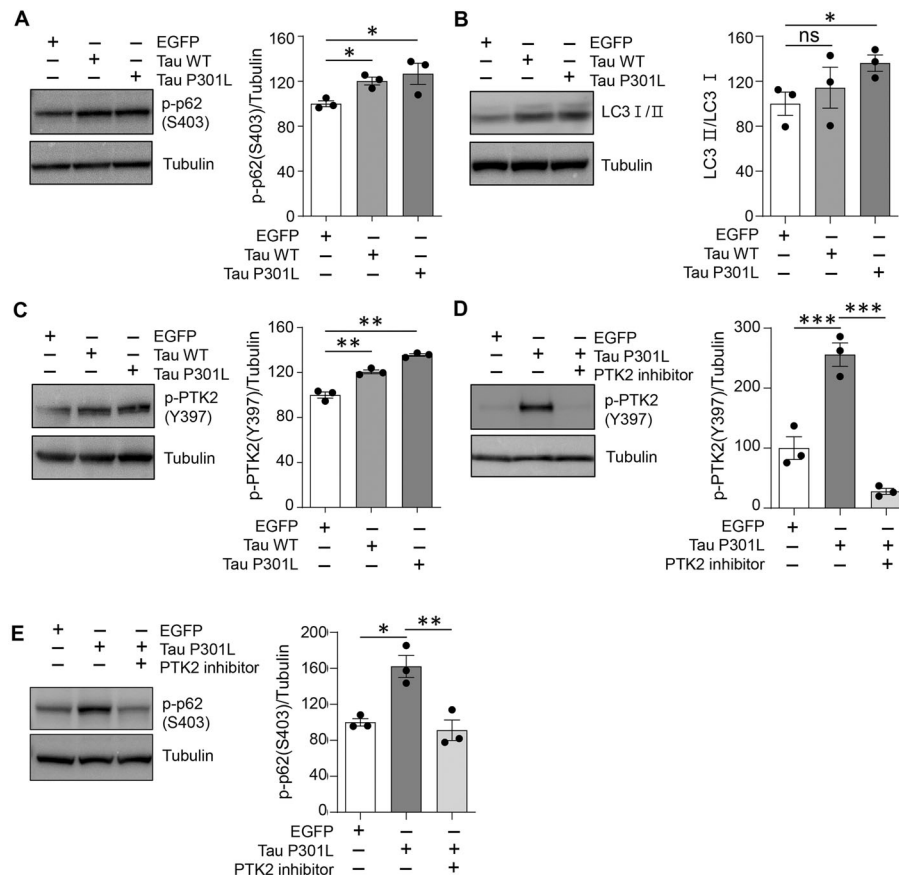


Figure 3. PTK2 inhibition suppresses Tau-induced phosphorylation of PTK2 and p62. (A–C) N2a cells were transfected with the EGFP, Tau WT-EGFP or Tau P301L-EGFP expression construct for 24 h, and the cells were then harvested for protein extraction. (A,B) Western blot analysis was performed to determine the p-p62 (S403) and LC3-II levels. The p-p62 (S403) and LC3-II levels were significantly increased in cells expressing Tau WT-EGFP or Tau P301L-EGFP, which indicates ALP activation. Quantification of expression was performed with immunoblots from 3 independent experiments, and protein levels were normalized to that of tubulin. The data are presented as the means $\pm$ SDs. * p < 0.05 and *n.s.*, not significant (one-way ANOVA with Tukey's multiple comparison test). (C) Western blot analysis was performed to determine the p-PTK2 (Y397) level. The p-PTK2 (Y397) level was significantly increased by expression of Tau WT-EGFP or Tau P301L-EGFP. Quantification of expression was performed with immunoblots from 3 independent experiments, and protein levels were normalized to that of tubulin. The data are presented as the means $\pm$ SDs. ** p < 0.005 (one-way ANOVA with Tukey's multiple comparison test). (D,E) N2a cells were transfected with the EGFP or Tau P301L-EGFP expression construct for 24 h and subsequently treated with a PTK2 inhibitor (1 μ M) for 24 h. (D) Western blot analysis was performed to determine the p-PTK2 (Y397) level. The Tau P301L-EGFP-induced increase in the p-PTK2 (Y397) level was significantly reduced by treatment with the PTK2 inhibitor. Quantification of expression was performed with immunoblots from 3 independent experiments, and protein levels were normalized to that of tubulin. The data are presented as the means $\pm$ SDs. *** p < 0.001 (one-way ANOVA with Tukey's multiple comparison test). (E) Western blot analysis was performed to determine the p-p62 (S403) level. The Tau P301L-EGFP-induced increase in the p-p62 (S403) level was significantly reduced by treatment with the PTK2 inhibitor. Quantification of expression was performed with immunoblots from 3 independent experiments, and protein levels were normalized to that of tubulin. The data are presented as the means $\pm$ SDs. * p < 0.05, ** p < 0.005 (one-way ANOVA with Tukey's multiple comparison test).

overexpression of Tau or Tau P301L induces ALP activation in N2a cells.

It was previously shown that PTK2 regulates toxicity induced by UPS impairment via phosphorylation of p62 at serine 403 (p-p62 (S403)) (Lee, Jeon, *et al.*, 2020). Moreover, a decrease in p-p62 (S403) facilitates autophagy-mediated degradation of ubiquitinated proteins (Lee, Jeon, *et al.*, 2020). Notably, we found that the levels of p-p62 (S403) and PTK2 phosphorylated at Tyr397 (p-PTK2 (Y397)) were greatly increased in Tau- and Tau P301L-overexpressing cells (Figure 3(A,C)). The Y397 is the autophosphorylation site in PTK2, and autophosphorylation at Y397 activates PTK2 and subsequently activates its downstream pathway (Hanks *et al.*, 2003).

To determine whether PTK2 regulates Tau P301L-induced phosphorylation of p62, we analyzed the level of p-p62 (S403) in PTK2 inhibitor-treated N2a cells expressing Tau P301L by western blotting. We observed that PTK2 inhibition significantly reduced Tau P301L-induced phosphorylation of p-PTK2 (Y397) (Figure 3(D)). Moreover, the level of p-p62 (S403) in Tau P301L-overexpressing cells was significantly decreased by PTK2 inhibition (Figure 3(E)). These results indicate that PTK2 inhibition markedly mitigates Tau P301L-induced neurotoxicity via phosphorylation of p62 at Ser403.

Therapeutic modulation of PTK2 attenuates tau-induced neurotoxicity in drosophila

Given the strong cell-based evidence that PTK2 regulates Tau-induced neurotoxicity, we next examined whether PTK2 inhibition suppresses Tau-induced toxicity *in vivo* using a tauopathy model in *Drosophila* that express the human Tau V337M mutant in the nervous system (*elavGS-GAL4*, pan-neuronal GeneSwitch driver). Tau V337M is a disease-associated mutation identified in FTDP-17. Moreover, human Tau V337M-expressing flies show neuropathological features similar to those of human tauopathy patients (Hutton, 2000; Wittmann *et al.*, 2001). The protein level of TAU in the Tau V337M flies was verified by western blot analysis (Figure 4(A)).

We first determined whether the UPS is impaired in tauopathy model flies. To assess ubiquitin-dependent proteasome activity in flies, we used CL1-GFP reporter flies that express GFP fused to the CL1 degron (a signal peptide for ubiquitin-mediated proteasomal degradation) (Pucher, Jayaprakash, Aftyka, Sigman, & Van Swol, 1995). The protein level of CL1-GFP was dramatically increased in tauopathy model flies compared to control flies (Figure 4(B)). Moreover, the level of polyubiquitinated proteins was significantly increased in the heads of tauopathy model flies

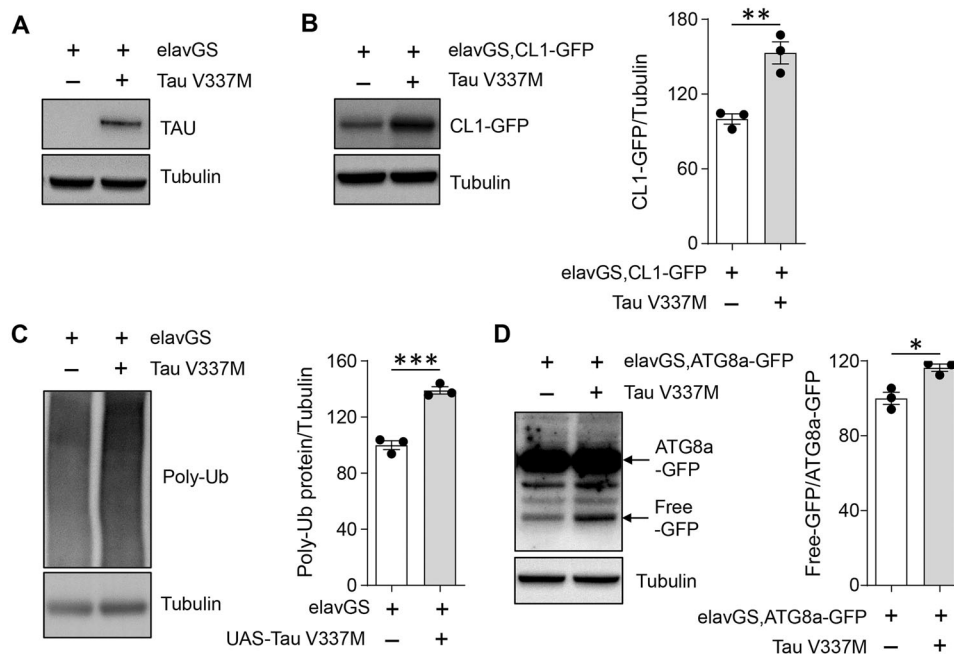


Figure 4. Tau overexpression induces UPS impairment in a *Drosophila* model of tauopathy. (A) Western blot analysis of the Tau protein level in lysates of fly heads. Genes were expressed in the nervous system in a drug-inducible manner with the *elavGS* driver. Quantification of expression was performed with immunoblots from 3 independent experiments. Genotypes: Control is *elavGS/+*. Tau V337M is *elavGS,UAS-Tau V337M/+*. (B) Western blot analysis of the CL1-GFP (a reporter of UPS activity) protein level in lysates of fly heads. The level of CL1-GFP was significantly upregulated in Tau V337M flies. Quantification of expression was performed with immunoblots from 3 independent experiments, and protein levels were normalized to that of tubulin. The data are presented as the means $\pm$ SDs. Genotypes: Control is *elavGS,UAS-CL1-GFP/+*. Tau V337M is *elavGS,UAS-Tau V337M/+;elavGS,UAS-CL1-GFP*. ** $p < 0.005$ (Student's *t* test). (C) Western blot analysis of insoluble polyubiquitinated protein levels in lysates of fly heads. The level of polyubiquitinated proteins was significantly increased in Tau V337M flies. Quantification of expression was performed with immunoblots from 3 independent experiments, and protein levels were normalized to that of tubulin. Quantification of expression was performed with immunoblots from 3 independent experiments, and protein levels were normalized to that of tubulin. The data are presented as the means $\pm$ SDs. *** $p < 0.001$ (Student's *t* test). Genotypes: Control is *elavGS/+*. Tau V337M is *elavGS,UAS-Tau V337M/+*. (D) Western blot analysis of Atg8a-GFP and free GFP levels in lysates of fly heads. The level of free GFP was significantly increased in Tau V337M flies, which indicates increased ALP activity. Quantification of expression was performed with immunoblots from 3 independent experiments, and protein levels were normalized to that of Atg8a-GFP. The data are presented as the means $\pm$ SDs. Genotypes: Control is *elavGS,UAS-Atg8a-GFP/+*. Tau V337M is *elavGS,UAS-Tau V337M/+;elavGS,UAS-Atg8a-GFP*. * $p < 0.05$ (Student's *t* test).

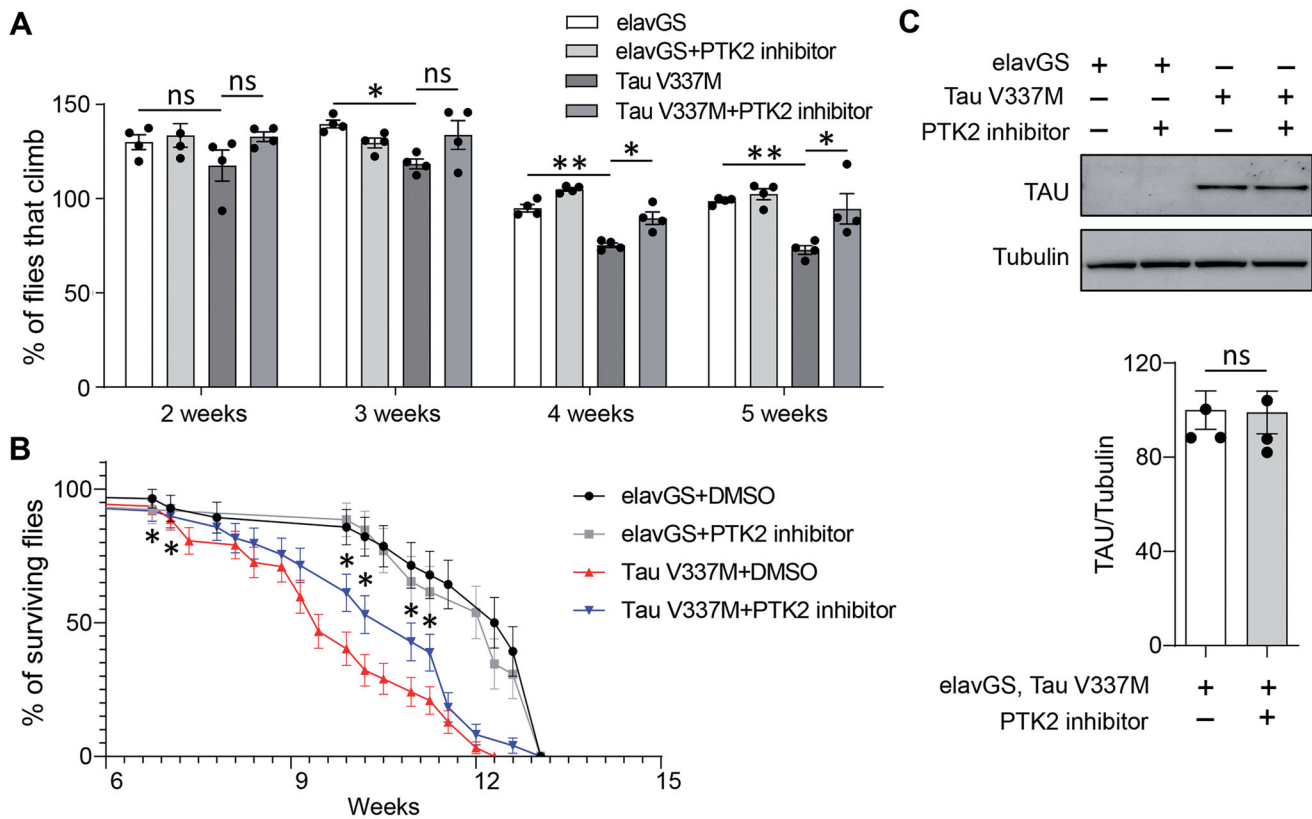


Figure 5. PTK2 inhibition attenuates Tau-induced neurotoxicity in a *Drosophila* model of tauopathy. (A,B) Climbing ability and lifespan of Tau V337M flies treated with a PTK2 inhibitor (10 μ M) at the indicated time points. The Tau V337M-induced climbing deficit and shortened lifespan were weakly mitigated by treatment with the PTK2 inhibitor. The percentages of flies that climbed or survived were quantified. The data are presented as the means $\pm$ SEMs ($n = 4$ replicates per condition, each with $n = 25$ flies per sample). * $p < 0.05$; ** $p < 0.005$; n.s., not significant (one-way ANOVA with the Bonferroni correction for multiple comparisons). (C) Western blot analysis of the TAU protein level in Tau V337M flies treated with the PTK2 inhibitor (10 μ M). Inhibition of PTK2 did not affect the TAU protein level. Quantification of expression was performed with immunoblots from 3 independent experiments, and protein levels were normalized to that of tubulin. The data are presented as the means $\pm$ SDs. n.s., not significant (Student's t test). Genotypes: Control is *elavGS/+*. Tau V337M is *elavGS,UAS-Tau V337M/+*.

(Figure 4(C)), suggesting that the UPS is impaired in the nervous system of tauopathy model flies.

To confirm the *in vitro* results, we assessed ALP activity using the Atg8a-GFP reporter. According to a previous study, the ratio of free GFP to Atg8a-GFP correlates with the level of ALP activation because globular GFP is much more resistant to acidic lysosomal conditions than is Atg8a-GFP (Klionsky *et al.*, 2012). By adopting this approach, we examined the free GFP/Atg8a-GFP ratio in our system and found that the ratio was significantly higher in Tau V337M-expressing flies than in control flies (Figure 4(D)). Notably, a previous study showed that ALP activation is a mechanism compensating for UPS impairment (Wang & Wang, 2015). Thus, it seems likely that the UPS impairment caused by Tau overexpression leads to ALP activation. Consistent with this hypothesis, we found that PTK2 inhibitor treatment significantly ameliorated the Tau V337M-induced climbing deficit and the shortened lifespan (Figure 5(A,B)). In addition, the TAU level was not significantly affected by the PTK2 inhibitor in Tau V337M flies (Figure 5(C)). Taken together, these results suggest that the PTK2 inhibitor attenuates Tau V337M-induced toxicity in a *Drosophila* model of tauopathy.

Serine 403 phosphorylation of p62 is significantly increased in Alzheimer's disease patients with tauopathy

A recent study reported that p-p62 (S403)-positive inclusions are found in the affected neurons of Alzheimer's disease and ALS patients (Kurosawa *et al.*, 2016). Furthermore, Tau pathology is one of the most common features of AD (Grundke-Iqbal *et al.*, 1986; Iqbal *et al.*, 1989; Nelson *et al.*, 2012). To investigate the relationship between p-p62 (S403) and tauopathies in human patients, we used postmortem hippocampal tissues from patients with AD and age-matched control subjects. We assessed the level of p62 phosphorylation at S403 in the hippocampal regions of patients with AD ($n = 6$) and control subjects ($n = 4$) using western blotting (Table 1). The levels of p-TAU (S396) (the marker for Tau pathology) were increased in the brains of AD patients (Figure 6(A), $611 \pm 136\%$ compared to normal controls). In addition, the levels of p-p62 (S403) were significantly increased in the brains of AD patients compared with the brains of control subjects (Figure 6(B), $152 \pm 16\%$). This result indicates that accumulation of p-p62 (S403) is associated with the progression of AD with tauopathy. Taken together, all of the evidence suggests that PTK2 inhibition

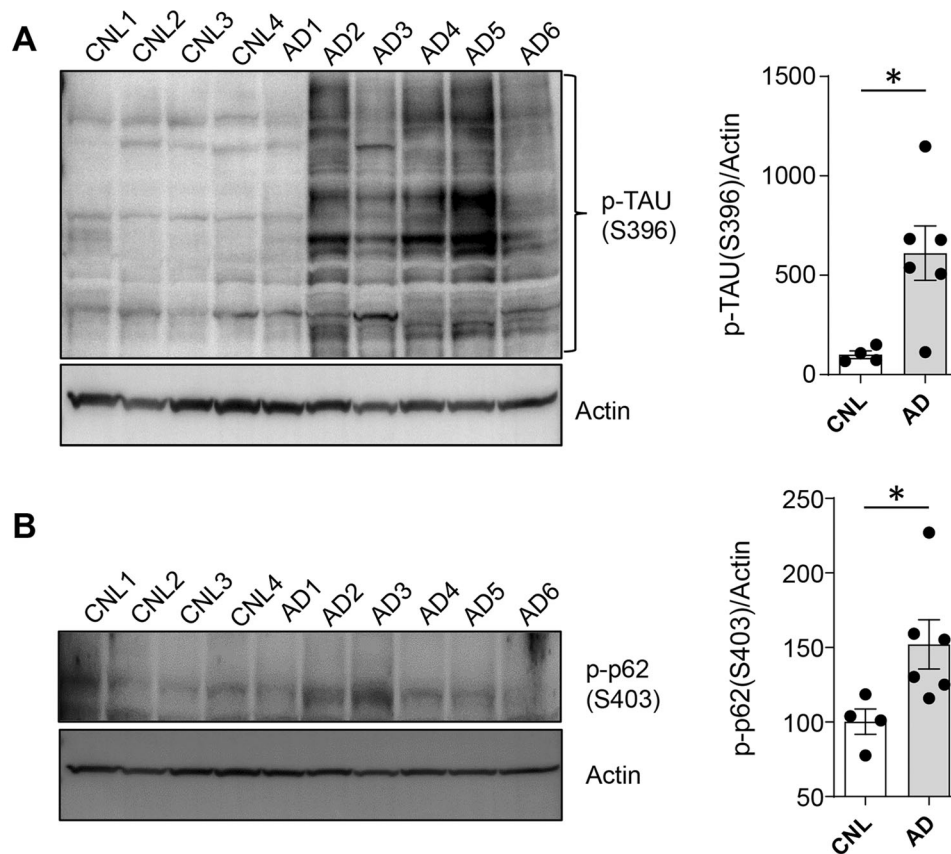


Figure 6. p-p62 (Ser403) is significantly increased in Alzheimer's disease patients with tauopathy. (A) Western blot analysis of phosphorylated TAU (S396) protein levels in brain lysates from control subjects (Braak stage 0–3, $n = 4$) or AD patients (Braak stage 6, $n = 6$). Brain lysates from control subjects and AD patients were prepared from postmortem human hippocampal tissue. Quantification of expression was performed with immunoblots from 3–5 independent experiments, and protein levels were normalized to that of actin. The data are presented as the means $\pm$ SDs. * $p < 0.05$ (Student's t test). (B) Western blot analysis of phosphorylated p62 (S403) protein levels in brain lysates from control subjects (Braak stage 0–3, $n = 4$) and AD patients without TDP-43 proteinopathy (Braak stage 6, $n = 6$). The brains of AD patients showed increased p-p62 (S403) protein levels compared with those in the brains of control subjects. Quantification of expression was performed with immunoblots from 3–5 independent experiments, and protein levels were normalized to that of actin. The data are presented as the means $\pm$ SDs. * $p < 0.05$ (Student's t test).

may be a promising therapeutic strategy for neurodegenerative diseases featuring tauopathies.

Discussion

Tau is the major component of NFTs, and abnormal accumulation of Tau proteins in AD and FTD patients contributes to AD/FTD pathology (Bodea, Eckert, Ittner, Piguet, & Götz, 2016; Cash *et al.*, 2003). Notably, Tau-induced UPS impairment plays a major role in the pathogenesis of tauopathies (Liu *et al.*, 2021; Myeku *et al.*, 2016; Tai *et al.*, 2012). In this study, we demonstrated that the UPS is impaired by Tau overexpression in cell and *Drosophila* models of tauopathy. We observed that expression of the FTDP-17-associated mutant Tau P301L markedly reduced UPS activity in mammalian neuronal cells and that expression of another FTDP-17-associated mutant, Tau V337M, impaired UPS function in *Drosophila*. Our findings suggest that UPS impairment could be a critical factor for the progression of tauopathies. Interestingly, UPS impairment is also associated with other neurodegenerative diseases, such as TDP-43 proteinopathies. Recent studies have demonstrated that TDP-43 overexpression impairs the UPS in cellular and *Drosophila* models of TDP-43 proteinopathies (Lee, Jeon, *et al.*, 2020). Moreover, ubiquitin–proteasome activity is reduced in

the motor neurons of patients with sporadic ALS (Bendotti *et al.*, 2012). Indeed, mutant TDP-43 associated with ALS interacts with the proteasome complex proteins PSMG2 and PSD13. Yin *et al.* also showed that the physical interaction between these proteins can suppress UPS activity (Yin *et al.*, 2021). These findings suggest that UPS dysfunction may be a major pathological mechanism in many neurodegenerative diseases.

Moreover, previous studies demonstrated that PTK2 regulates toxicity induced by UPS impairment. In addition, PTK2 inhibition mitigates TDP-43-induced accumulation of ubiquitinated aggregates and neurotoxicity in mammalian neuronal cells and flies (Lee, Jeon, *et al.*, 2020). These studies revealed that PTK2 inhibition regulates the phosphorylation of p62 at Ser403 to facilitate the clearance of TDP-43-induced ubiquitin aggregates via ALP. These data indicate that PTK2 inhibition mitigates TDP-43-induced neurotoxicity. Thus, we analyzed the effect of PTK2 on Tau-induced UPS impairment and neurotoxicity. We found that Tau P301L-induced PTK2 (Y397) and p62 (S403) phosphorylation was diminished by PTK2 inhibition. We also showed that PTK2 inhibition significantly reduced Tau P301L-induced UPS impairment and neurotoxicity. These results suggest that PTK2 may be a promising therapeutic target for tauopathies as well as TDP-43 proteinopathies.

ALP is involved in the degradation of polyubiquitinated proteins, thereby attenuating proteotoxic stress in neurodegenerative diseases associated with proteinopathy (Martini-Stoica, Xu, Ballabio, & Zheng, 2016; Nah, Yuan, & Jung, 2015; Pan, Kondo, Le, & Jankovic, 2008). In this study, we observed that Tau overexpression causes ALP activation in mammalian neuronal cells and *Drosophila*. Previous results suggested that p62 transports polyubiquitinated proteins into the autophagosome and that the p-p62 (S403) level is significantly increased by UPS impairment (Lee, Jeon, *et al.*, 2020). We also found that the p-p62 (S403) level is increased in Tau-overexpressing N2a cells. In addition, we found that the hippocampal level of p-p62 (S403) is increased in AD patients. Consistent with these findings, p-p62 (S403)-positive inclusions have been observed in the brains of AD patients (Kurosawa *et al.*, 2016). These results suggest that increased p62 phosphorylation may be implicated in the neuropathological mechanism of tauopathies. Furthermore, we showed that the Tau P301L-induced increase in the p-p62 (S403) level was significantly reduced by PTK2 inhibition. Thus, we concluded that PTK2 regulates Tau-mediated UPS impairment and the neuronal toxicity of Tau through phosphorylation of p62 at Ser403.

Current research suggests that UPS impairment is a major contributor to the pathogenesis of tauopathies. Our results also suggest that UPS dysfunction in tauopathies increases the phosphorylation of p62 at Ser403 and that the reduction in p-p62(S403) mediated by PTK2 inhibition attenuates Tau-induced neurotoxicity. Therefore, determining the molecular mechanisms and key molecules, including PTK2, responsible for regulating UPS activity may help identify suitable therapeutic targets for tauopathies.

Ethics approval

Human tissue experiments were performed in accordance with the guidelines of the Institutional Review Board (IRB) at the Korea Brain Research Institute. Postmortem tissue samples were obtained from the Netherlands brain bank (NBB), Netherlands Institute for Neuroscience, Amsterdam. This study was approved by the IRB at Korea Brain Research Institute (#201610-BR-004-01).

Author contributions

S.L., M.J., and Y.K., and planned and performed the experiments. Y.-M.J., S.K., and K.J.L. provided ideas for the project and participated in writing the paper. S.L., M.J., and H.-J.K. wrote the paper. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Kea Joo Lee  <http://orcid.org/0000-0002-1366-4709>
Hyung-Jun Kim  <http://orcid.org/0000-0002-3385-5510>

References

- Armendáriz, B.G., Masdeu, M. d M., Soriano, E., Ureña, J.M., & Burgaya, F. (2014). The diverse roles and multiple forms of focal adhesion kinase in brain. *The European Journal of Neuroscience*, 40(11), 3573–3590. doi:10.1111/ejn.12737
- Ballatore, C., Lee, V.M.-Y., & Trojanowski, J.Q. (2007). Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nature Reviews. Neuroscience*, 8(9), 663–672. doi:10.1038/nrn2194
- Bendotti, C., Marino, M., Cheroni, C., Fontana, E., Crippa, V., Poletti, A., & De Biasi, S. (2012). Dysfunction of constitutive and inducible ubiquitin-proteasome system in amyotrophic lateral sclerosis: implication for protein aggregation and immune response. *Progress in Neurobiology*, 97(2), 101–126. doi:10.1016/j.pneurobio.2011.10.001
- Bodea, L.-G., Eckert, A., Ittner, L.M., Piguet, O., & Götz, J. (2016). Tau physiology and pathomechanisms in frontotemporal lobar degeneration. *Journal of Neurochemistry*, 138(Suppl 1), 71–94. doi:10.1111/jnc.13600
- Cash, A.D., Aliev, G., Siedlak, S.L., Nunomura, A., Fujioka, H., Zhu, X., ... Perry, G. (2003). Microtubule reduction in Alzheimer's disease and aging is independent of tau filament formation. *The American Journal of Pathology*, 162(5), 1623–1627. doi:10.1016/s0002-9440(10)64296-4
- Couthouis, H., Shorter, D.-H., Erion, O., Liu, R., Jethava, H., Epstein, C., ... Rademakers, M., & G. (2011). A yeast functional screen predicts new candidate ALS disease genes. *Proceedings of the National Academy of Sciences of the United States of America*, 108(52), 20881–20890. doi:10.1073/pnas.1109434108
- Cuchillo-Ibanez, I., Seereeram, A., Byers, H.L., Leung, K.-Y., Ward, M.A., Anderton, B.H., & Hanger, D.P. (2008). Phosphorylation of tau regulates its axonal transport by controlling its binding to kinesin. *FASEB Journal : official Publication of the Federation of American Societies for Experimental Biology*, 22(9), 3186–3195. doi:10.1096/fj.08-109181
- Durcan, T.M., Kontogiannia, M., Thorarinsdottir, T., Fallon, L., Williams, A.J., Djarmati, A., ... Fon, E.A. (2011). The Machado-Joseph disease-associated mutant form of ataxin-3 regulates parkin ubiquitination and stability. *Human Molecular Genetics*, 20(1), 141–154. doi:10.1093/hmg/ddq452
- Feany, M.B., & Bender, W.W. (2000). A *Drosophila* model of Parkinson's disease. *Nature*, 404(6776), 394–398. doi:10.1038/35006074
- Gendron, T.F., & Petrucelli, L. (2009). The role of tau in neurodegeneration. *Molecular Neurodegeneration*, 4, 13. doi:10.1186/1750-1326-4-13
- Grundke-Iqbal, I., Iqbal, K., Tung, Y.C., Quinlan, M., Wisniewski, H.M., & Binder, L.I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences of the United States of America*, 83(13), 4913–4917. doi:10.1073/pnas.83.13.4913
- Gustke, N., Trinczek, B., Biernat, J., Mandelkow, E.M., & Mandelkow, E. (1994). Domains of tau protein and interactions with microtubules. *Biochemistry*, 33(32), 9511–9522. doi:10.1021/bi00198a017
- Hanks, S.K., Ryzhova, L., Shin, N.-Y., & Brábek, J. (2003). Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. *Frontiers in Bioscience : a Journal and Virtual Library*, 8, d982–996. doi:10.2741/1114

- Hutton. (2000). Molecular genetics of chromosome 17 tauopathies. *Annals of the New York Academy of Sciences*, 920, 63–73. doi:10.1111/j.1749-6632.2000.tb06906.x
- Hutton, L., Rizzu, B., Froelich, H., Pickering-Brown, C., Isaacs, G., Hackett, A., ... Mann, L. & H. (1998). Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature*, 393(6686), 702–705. doi:10.1038/31508
- Iqbal, K., Grundke-Iqbal, I., Smith, A.J., George, L., Tung, Y.C., & Zaidi, T. (1989). Identification and localization of a tau peptide to paired helical filaments of Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America*, 86(14), 5646–5650. doi:10.1073/pnas.86.14.5646
- Iqbal, K., Liu, F., & Gong, C.-X. (2016). Tau and neurodegenerative disease: the story so far. *Nature Reviews. Neurology*, 12(1), 15–27. doi:10.1038/nrneurol.2015.225
- Klionsky, A., Abeliovich, A., Acevedo-Arozena, A., Agholme, A., Agostinis, A.-G., Ahn, A.-M. ... Zuckerbraun B, (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy*, 8(4), 445–544. doi:10.4161/auto.19496
- Krüger, L., & Mandelkow, E.M. (2016). Tau neurotoxicity and rescue in animal models of human Tauopathies. *Current Opinion in Neurobiology*, 36, 52–58. doi:10.1016/j.conb.2015.09.004
- Kurosawa, M., Matsumoto, G., Sumikura, H., Hatsuta, H., Murayama, S., Sakurai, T., ... Nukina, N. (2016). Serine 403-phosphorylated p62/SQSTM1 immunoreactivity in inclusions of neurodegenerative diseases. *Neuroscience Research*, 103, 64–70. doi:10.1016/j.neures.2015.08.002
- Lee, V.M., Goedert, M., & Trojanowski, J.Q. (2001). Neurodegenerative tauopathies. *Annual Review of Neuroscience*, 24, 1121–1159. doi:10.1146/annurev.neuro.24.1.1121
- Lee, S., Jeon, Y.-M., Cha, S.J., Kim, S., Kwon, Y., Jo, M., ... Kim, H.-J. (2020). PTK2/FAK regulates UPS impairment via SQSTM1/p62 phosphorylation in TARDBP/TDP-43 proteinopathies. *Autophagy*, 16(8), 1396–1412. doi:10.1080/15548627.2019.1686729
- Lee, S., Jo, M., Lee, H.E., Jeon, Y.-M., Kim, S., Kwon, Y., ... Kim, H.-J. (2021). HEXA-018, a Novel Inducer of Autophagy, Rescues TDP-43 Toxicity in Neuronal Cells. *Frontiers in Pharmacology*, 12, 747975. doi:10.3389/fphar.2021.747975
- Lee, S., Kwon, Y., Kim, S., Jo, M., Jeon, Y.-M., Cheon, M., ... Kim, H.-J. (2020). The Role of HDAC6 in TDP-43-Induced Neurotoxicity and UPS Impairment. *Frontiers in Cell and Developmental Biology*, 8, 581942. doi:10.3389/fcell.2020.581942
- Li, Z., Arnaud, L., Rockwell, P., & Figueiredo-Pereira, M.E. (2004). A single amino acid substitution in a proteasome subunit triggers aggregation of ubiquitinated proteins in stressed neuronal cells. *Journal of Neurochemistry*, 90(1), 19–28. doi:10.1111/j.1471-4159.2004.02456.x
- Liu, Y., Ding, R., Xu, Z., Xue, Y., Zhang, D., Zhang, Y., ... Li, X. (2021). Roles and Mechanisms of the Protein Quality Control System in Alzheimer's Disease. *International Journal of Molecular Sciences*, 23(1), 345. doi:10.3390/ijms23010345
- Liu, Y., Fallon, L., Lashuel, H.A., Liu, Z., & Lansbury, P.T. (2002). The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell*, 111(2), 209–218. doi:10.1016/s0092-8674(02)01012-7
- Martini-Stoica, H., Xu, Y., Ballabio, A., & Zheng, H. (2016). The Autophagy-Lysosomal Pathway in Neurodegeneration: A TFEB Perspective. *Trends in Neurosciences*, 39(4), 221–234. doi:10.1016/j.tins.2016.02.002
- McKinnon, C., De Snoo, M.L., Gondard, E., Neudorfer, C., Chau, H., Ngana, S.G., ... Kalia, S.K. (2020). Early-onset impairment of the ubiquitin-proteasome system in dopaminergic neurons caused by alpha-synuclein. *Acta Neuropathologica Communications*, 8(1), 17. doi:10.1186/s40478-020-0894-0
- Mori, H., Kondo, J., & Ihara, Y. (1987). Ubiquitin is a component of paired helical filaments in Alzheimer's disease. *Science (New York, N.Y.)*, 235(4796), 1641–1644. doi:10.1126/science.3029875
- Myeku, N., Clelland, C.L., Emrani, S., Kukushkin, N.V., Yu, W.H., Goldberg, A.L., & Duff, K.E. (2016). Tau-driven 26S proteasome impairment and cognitive dysfunction can be prevented early in disease by activating cAMP-PKA signaling. *Nature Medicine*, 22(1), 46–53. doi:10.1038/nm.4011
- Nah, J., Yuan, J., & Jung, Y.-K. (2015). Autophagy in neurodegenerative diseases: from mechanism to therapeutic approach. *Molecules and Cells*, 38(5), 381–389. doi:10.14348/molcells.2015.0034
- Nalepa, G., Rolfe, M., & Harper, J.W. (2006). Drug discovery in the ubiquitin-proteasome system. *Nature Reviews. Drug Discovery*, 5(7), 596–613. doi:10.1038/nrd2056
- Nelson, A., Bigio, B., Braak, C., Castellani, C., Davies, D.T., Duyckaerts, F., ... Woltjer, B. (2012). Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature. *Journal of Neuropathology and Experimental Neurology*, 71(5), 362–381. doi:10.1097/NEN.0b013e31825018f7
- Pan, T., Kondo, S., Le, W., & Jankovic, J. (2008). The role of autophagy-lysosome pathway in neurodegeneration associated with Parkinson's disease. *Brain : a Journal of Neurology*, 131(Pt 8), 1969–1978. doi:10.1093/brain/awm318
- Pucher, J., Jayaprakash, P., Aftyka, T., Sigman, L., & Van Swol, R. (1995). Clinical evaluation of a new flossing device. *Quintessence International (Berlin, Germany : 1985)*, 26(4), 273–278. <https://www.ncbi.nlm.nih.gov/pubmed/7568747>
- Ramsden, M., Kotilinek, L., Forster, C., Paulson, J., McGowan, E., SantaCruz, K., ... Ashe, K.H. (2005). Age-dependent neurofibrillary tangle formation, neuron loss, and memory impairment in a mouse model of human tauopathy (P301L). *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 25(46), 10637–10647. doi:10.1523/JNEUROSCI.3279-05.2005
- Rasmussen, L.J.H., Müller, H.S.H., Jørgensen, B., Pedersen, S.F., & Hoffmann, E.K. (2015). Osmotic shrinkage elicits FAK- and Src phosphorylation and Src-dependent NKCC1 activation in NIH3T3 cells. *American Journal of Physiology. Cell Physiology*, 308(2), C101–110. doi:10.1152/ajpcell.00070.2014
- Santacruz, L., Spire, P., Kotilinek, I., Guimaraes, D., Ramsden, M., Forster, Y., ... Hyman, H., & A. (2005). Tau suppression in a neurodegenerative mouse model improves memory function. *Science*, 309(5733), 476–481. doi:10.1126/science.1113694
- Tai, H.-C., Serrano-Pozo, A., Hashimoto, T., Frosch, M.P., Spire-Jones, T.L., & Hyman, B.T. (2012). The synaptic accumulation of hyperphosphorylated tau oligomers in Alzheimer disease is associated with dysfunction of the ubiquitin-proteasome system. *The American Journal of Pathology*, 181(4), 1426–1435. doi:10.1016/j.ajpath.2012.06.033
- Tanida, I., Ueno, T., & Kominami, E. (2008). LC3 and Autophagy. *Methods in Molecular Biology*, 445, 77–88. doi:10.1007/978-1-59745-157-4_4
- Wang, C., & Wang, X. (2015). The interplay between autophagy and the ubiquitin-proteasome system in cardiac proteotoxicity. *Biochimica et Biophysica Acta*, 1852(2), 188–194. doi:10.1016/j.bba-dis.2014.07.028
- Williams. (2006). Tauopathies: classification and clinical update on neurodegenerative diseases associated with microtubule-associated protein tau. *Internal Medicine Journal*, 36(10), 652–660. doi:10.1111/j.1445-5994.2006.01153.x
- Wittmann, C.W., Wszolek, M.F., Shulman, J.M., Salvaterra, P.M., Lewis, J., Hutton, M., & Feany, M.B. (2001). Tauopathy in *Drosophila*: neurodegeneration without neurofibrillary tangles. *Science*, 293(5530), 711–714. doi:10.1126/science.1062382
- Yin, P., Bai, D., Zhu, L., Deng, F., Guo, X., Li, B., ... Li, X.-J. (2021). Cytoplasmic TDP-43 impairs the activity of the ubiquitin-proteasome system. *Experimental Neurology*, 345, 113833. doi:10.1016/j.expneurol.2021.113833
- Zhang, M., Deng, Y., Luo, Y., Zhang, S., Zou, H., Cai, F., ... Song, W. (2012). Control of BACE1 degradation and APP processing by ubiquitin carboxyl-terminal hydrolase L1. *Journal of Neurochemistry*, 120(6), 1129–1138. doi:10.1111/j.1471-4159.2011.07644.x