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ORIGINAL ARTICLE

Thymosin beta 4-Induced Autophagy Increases Cholinergic Signaling in PrP (106–126)-Treated HT22 Cells

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Abstract

Prion protein peptide (PrP) has been associated with neurotoxicity in brain cells and progression of prion diseases due to spongiform degeneration and accumulation of the infectious scrapie prion protein (PrP^{Sc}). Autophagy has been shown to provide protective functions for neurodegenerative diseases, including prion disease. Thymosin beta 4 ($T\beta_4$) plays a key role in the nervous system, providing a neuronal growth effect that includes motility, neurite outgrowth, and proliferation. However, the effect of $T\beta_4$ on autophagy in prion disease has not been investigated. In this study, we investigated the neuroprotective effects of $T\beta_4$, an activator of autophagy, in cholinergic signaling activation in PrP (106–126)-treated HT22 cells. We found that $T\beta_4$-induced autophagy markers, LC3A/B and Beclin1, were protective against PrP-induced neurotoxicity. Interestingly, a balance between autophagy markers and autophagy pathway factors (AKT, p-AKT, mTOR, and p-mTOR) was maintained by $T\beta_4$ competitively against each protein factors reacted to PrP (106–126). The cholinergic signaling markers ChTp and AChE, which play an important role in the brain, were maintained by $T\beta_4$ competitively against each protein factors reacted to PrP (106–126). However, these results were reversed by 3-MA, an autophagy inhibitor. Taken together, our results indicate that $T\beta_4$ has cholinergic signaling activities through the induction of autophagy. Thus, $T\beta_4$ may be to a potential therapeutic agent for preventing neurodegenerative diseases.

Keywords Thymosin beta 4 · Autophagy · Cholinergic signaling · Prion protein

Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), belong to a group of neurodegenerative disorders. Prion diseases arise due to the misfolding of the normal cellular prion (PrP^{C}) into the scrapie isoform of the prion protein (PrP^{Sc}) (Prusiner 1982). The PrP fragment (106–126) has demonstrated similar properties to PrP^{sc} and induces neurotoxicity in brain cells due to spongiform degenerative, amyloidogenic, and aggregative properties (Melo et al. 2007). It also results in neurotoxicity through other unknown mechanisms.

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Autophagy is a life-sustaining response to cellular stress conditions (Boya et al. 2013; Hubbi and Semenza 2015). Autophagy plays an important role in adaptation to starvation (Larsen and Sulzer 2002), cancer (Rosenfeldt and Ryan 2011), immunity (Deretic et al. 2013; Zhong et al. 2016), and neuroprotection (Cherra III and Chu 2008). Previous studies have shown that functional impairment of autophagy is observed in neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases as well as in prion diseases (Boellaard et al. 1991; Kiriyama and Nochi 2015; Sikorska et al. 2007). In addition, induction of autophagy had been shown to reduce the amount of PrP^{Sc} (Heiseke et al. 2010; Nakagaki et al. 2013).

Thymosin beta 4 (Tβ4) is a small actin monomer-binding molecule present in all mammalian species (Yu et al. 1993). Previous studies have reported that Tβ4 plays an important role in neuronal survival, anti-apoptotic, anti-inflammatory, and angiogenic functions both in vitro and in vivo (Malinda et al. 1999; Philp et al. 2004; Sosne et al. 2001). However, the effect of Tβ4 on PrP^{Sc}-induced autophagy inhibition is still unknown.

Here, we investigated the neuroprotective effect of Tβ4 in PrP (106–126)-treated hippocampal neuronal cells. In addition, we investigated how Tβ4 changes the expression of autophagy pathway proteins in PrP (106–126)-treated HT22 cells.

Materials and Methods

Chemicals

$T\beta_4$ was purchased from Tocris Bioscience (Bristol, UK). Synthetic PrP (106–126) were synthesized by Peptron (Seoul, Korea) (Na et al. 2014). The PrP peptides were dissolved in sterile dimethyl sulfoxide at a concentration of 10 mM and stored at − 72 °C. Primary antibodies raised against LC3A/B, Beclin1, mammalian target of rapamycin (mTOR), phosphorylated (p) - mTOR, protein kinase B (AKT), and p-AKT were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies raised against β-actin and Acetylcholinesterase (AChE) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), the choline transporter (ChTp) antibody was purchased from Millipore (Temecula, CA, USA). Secondary antibodies (i.e., anti-rabbit, anti-goat and anti-mouse IgG antibody conjugated with horseradish peroxidase) were obtained from Millipore. All other chemicals and reagents were analytical grade.

Cell Culture

Hippocampal neuronal cells (HT22 cells) were maintained in Dulbecco's modified Eagle medium (DMEM) (Hyclone, Logan, UT, USA) containing 10% FBS (Hyclone, Canada) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator with 5% CO_2 at 37 °C. The medium was changed every 2–3 days. The cells were treated with 0.4 μg/mL of Tβ4 and 100 μM PrP (106–126). Then, the autophagy inhibitor 3-Methyladenine (3-MA; 5 mM) was added for 3 h, and the mTOR inhibitor rapamycin (1 μM) was applied for 24 h.

Cell Viability Assay

Cell survival was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma-Aldrich, St. Louis, MO, USA). HT22 cells were plated at 2×10^4cells/well on 48-well plates (Nunc, NY, USA). After a 24 h incubation, Tβ4 (0.1–1 μg/mL) and PrP (25–200 μM) were applied for 24 h. HT22 cells were then incubated with MTT for 2 h at 37 °C in a 5% CO_2 incubator. The absorbance was determined at 540 nm using a microplate reader (PowerWave 2, Bio-Tek Instruments, Winooski, VT, USA).

Western Blotting

Cell pellets were treated with lysis buffer (140 mM NaCl, 25 mM Tris–HCl (pH 7.4), and 1% NP-40) and freshly added protease inhibitor cocktail (BD Biosciences, San Jose, CA). Proteins were subjected to SDS-polyacrylamide gel electrophoresis on a 6–15% gel and electrophoretically transferred to PVDF membranes (BioRad, Hercules, CA). Membranes were blocked in 5% skim milk in PBS and then incubated with each primary antibody, diluted 1:1000 in 1% skim milk in PBS, and membranes were incubated with primary antibodies overnight at 4 °C. Membranes were then incubated with each secondary antibody, diluted to 1:10000 at room temperature for 1 h. Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Rockford, IL, USA) on a chemiImager system (Alpha Innotech, San Leandro, CA, USA).

Lactate Dehydrogenase (LDH) Assay

Level of LDH was measured using an LDH cytotoxicity assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Briefly, cells were plated at 1×10^4cells/well on 96-well plates. Cells were treated with either PrP (106–126) or PrP (106–126) and $T\beta_4$ and then added with 3-MA or rapamycin. Supernatants were used for the determination of LDH concentration according to the manufacturer's instructions.

Statistical Analysis

All the data in this paper were performed in triplicate, at a minimum. And all values were expressed as mean ± standard error (SE). Statistical differences between two groups were determined by one-way ANOVA and Student's *t* test. p values < 0.05 were considered statistically significant.

Results

Tβ4 Protects HT22 Cells against PrP (106–126) -Induced Neurotoxicity

To assess the effects of Tβ4 on the viability of PrP (106–126)-treated HT22 cells, we conducted an MTT assay. Tβ4-treated cells did not exhibit significant cell death (Fig. 1a). Cell viability of PrP (106–126)-treated cells was significantly decreased in a dose-dependent manner compared to cell viability in control cells. Cell viability decreased by 60% with PrP (106–126) 100 μM treatment (Fig. 1b). We also confirmed the effect of Tβ4 on PrP (106–126)-treated HT22 cells. Cell

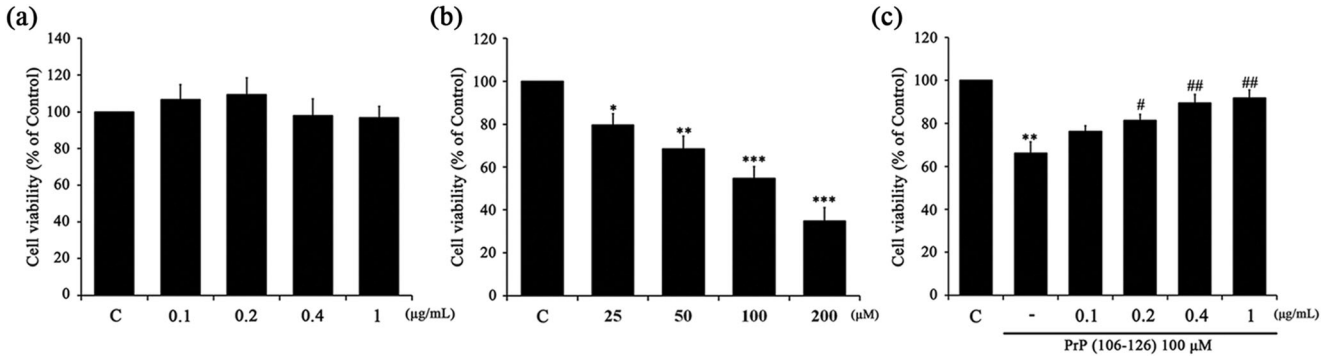

Fig. 1 Treatment with thymosin beta 4 inhibits PrP (106–126)-induced cell death. Cell viability was determined with an MTT assay. **a** HT22 cells were treated with various concentrations of Tβ4 (0.1–1 μg/mL). **b** HT22 cells were treated with various concentrations of PrP (106–126) (25–200 μM). **c** HT22 cells were treated with various concentrations of Tβ4 (0.1–1 μg/mL) and were then exposed to 100 μM PrP (106–126) for 24 h. Data are expressed as the mean ± SEM ($n = 3$). *$p < 0.05$, compared to control. **$p < 0.01$, compared to control. ***$p < 0.001$, compared to control. #$p < 0.05$, compared to the PrP-only treated group. ##$p < 0.01$, compared to the PrP-only treated group

viability of Tβ4-treated cells was significantly increased in a dose-dependent manner in cells treated with PrP (106–126) (Fig. 1c). These results suggested that Tβ4 inhibits neurotoxicity induced by PrP (106–126) in HT22 cells.

Tβ4 Treatment Leads to an Increase in Autophagy Markers

The expression of LC3A/B and Beclin1, which are markers of autophagy, was determined by Western blot analysis of Tβ4 or PrP (106–126)-treated HT22 cells. Tβ4 treatment significantly increased the protein expression of LC3A/B-II and Beclin1. In addition, the greatest expression was shown with 0.4 μg/mL Tβ4 treatment (Fig. 2a). PrP (106–126) significantly decreased the expression of LC3A/B-II and Beclin1 in a dose-dependent manner compared to the control (Fig. 2b). Based on these results, 0.4 μg/mL of Tβ4 and 100 μM of PrP (106–126) were used in subsequent experiments. These results suggest that Tβ4 induces autophagy, whereas PrP (106–126) decreases autophagy.

The PI3K/AKT/mTOR Signaling Pathway Is Involved in Tβ4-Induced Autophagy

mTOR is an important negative regulator of the autophagic process and is regulated by growth factors, starvation, and cellular stressors (Noda and Ohsumi 1998; Schmelzle and Hall 2000). The PI3K/AKT pathway is a critical upstream modulator of mTOR (Hay 2005). Therefore, we confirmed the association of Tβ4 (0.4 μg/mL) and the PI3K/AKT/mTOR pathway using the mTOR inhibitor rapamycin in HT22 cells treated with PrP (106–126) 100 μM (Fig. 3a, b). The expression of p-AKT and p-mTOR decreased with Tβ4 treatment, while p-AKT and p-mTOR expression increased with PrP (106–126) treatment. Cells co-treated with Tβ4 and PrP (106–126) showed significantly decreased expression of p-AKT and p-mTOR compared to cells treated with PrP (106–126) alone. In addition, rapamycin decreased the expression of p-AKT and p-mTOR compared to Tβ4 and PrP (106–126) treatment. Tβ4 significantly increased the expression of LC3A/B-II and Beclin1, whereas PrP (106–126) significantly decreased the expression of LC3A/B-II and Beclin1 compared to the control. Cells co-treated with Tβ4 and PrP (106–126) showed a significant increase in the expression of LC3A/B-II and Beclin1 compared to PrP (106–126) treatment alone. Moreover, rapamycin increased the expression of LC3A/B-II and Beclin1 compared to Tβ4 and PrP (106–126) treatment. These results indicate that Tβ4 induces autophagy by inhibition of the PI3K/AKT/mTOR pathway in PrP (106–126)-treated HT22 cells.

Tβ4 Increases Cholinergic Signaling Through Induction of Autophagy

We confirmed the association of Tβ4-induced autophagy and cholinergic signaling in HT22 cells treated with PrP (106–126) and autophagy inhibitor (3-MA) (Fig. 4). The expression of ChTp increased in Tβ4-treated cells, but decreased in PrP (106–126)-treated cells. Cells co-treated with Tβ4 and PrP (106–126) showed significantly increased ChTp expression compared to PrP (106–126)-treated cells. However, 3-MA reversed the increase in ChTp expression in cells co-treated with Tβ4 and PrP (106–126). The expression of AChE decreased in Tβ4-treated cells, whereas the expression of AChE in PrP (106–126)-treated cells increased compared to the control. Cells co-treated with Tβ4 and PrP (106–126) showed significantly decreased AChE expression compared to cells treated with PrP (106–126). 3-MA significantly reversed the inhibitory activity of Tβ4 on PrP (106–126)-induced AChE expression. This result showed that Tβ4-induced autophagy affects the expression of ChTp and AChE in PrP (106–126)-treated HT22 cells.

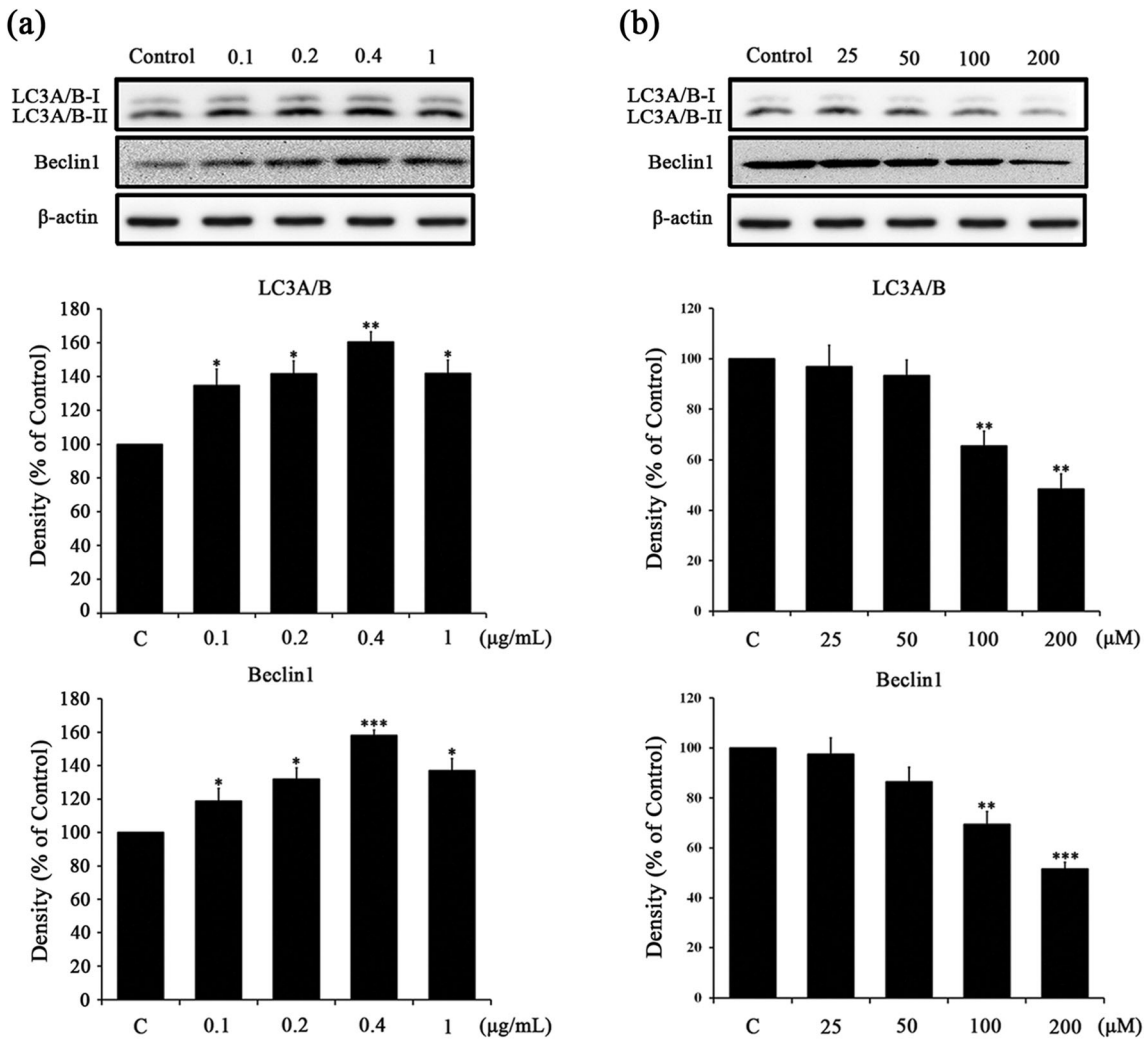

Fig. 2 Thymosin beta 4 induces the production of autophagy markers. **a** HT22 cells were treated with various concentrations of Tβ4 (0.1–1 μg/mL). **b** HT22 cells were treated with various concentrations of PrP (106–126) (25–200 μM). The treated cells were assessed for LC3A/B and Beclin1 production by Western blot analysis. Data are expressed as the mean ± SEM ($n = 3$). *$p < 0.05$, compared to control. **$p < 0.01$, compared to control. ***$p < 0.001$, compared to control

Tβ4-Induced Autophagy Protects Against PrP (106–126)-Induced Cell Death

We evaluated the effects of Tβ4-induced autophagy on the viability of PrP (106–126)-treated HT22 cells using 3-MA or rapamycin (Fig. 5a, b). PrP (106–126) and 3-MA treatment showed a significant decrease compared to PrP (106–126)-only treated group. Moreover, PrP (106–126) and 3-MA treatment induced significant over-production of LDH, which is a marker of toxicity. As expected, Tβ4 treatment significantly increased cell viability and decreased the level of LDH compared to PrP (106–126) and 3-MA treatment. PrP (106–126) and rapamycin treatment showed a significant increase compared to PrP (106–126)-only treated group. Moreover, PrP (106–126) and rapamycin treatment significantly decreased the level of LDH. In addition, Tβ4 treatment significantly increased cell viability and decreased the level of LDH compared to PrP (106–126) and rapamycin treatment. These results suggested that Tβ4-induced autophagy inhibits neurotoxicity induced by PrP (106–126) in HT22 cells.

Discussion

The present study demonstrates for the first time that the association between Tβ4 and cholinergic signaling regulates the autophagy and thus plays a pivotal role in the neuroprotective effects. There is a considerable amount of recent evidence that Tβ4 has survival and angiogenic properties, protects tissue against damage, and promotes tissue regeneration. In addition, Tβ4 plays a key role in anti-apoptosis, neurite formation, cell proliferation, and neuronal survival (Malinda et al. 1999; Pirkkala et al. 2001; Pollard and Borisy 2003; Yang et al. 2008).

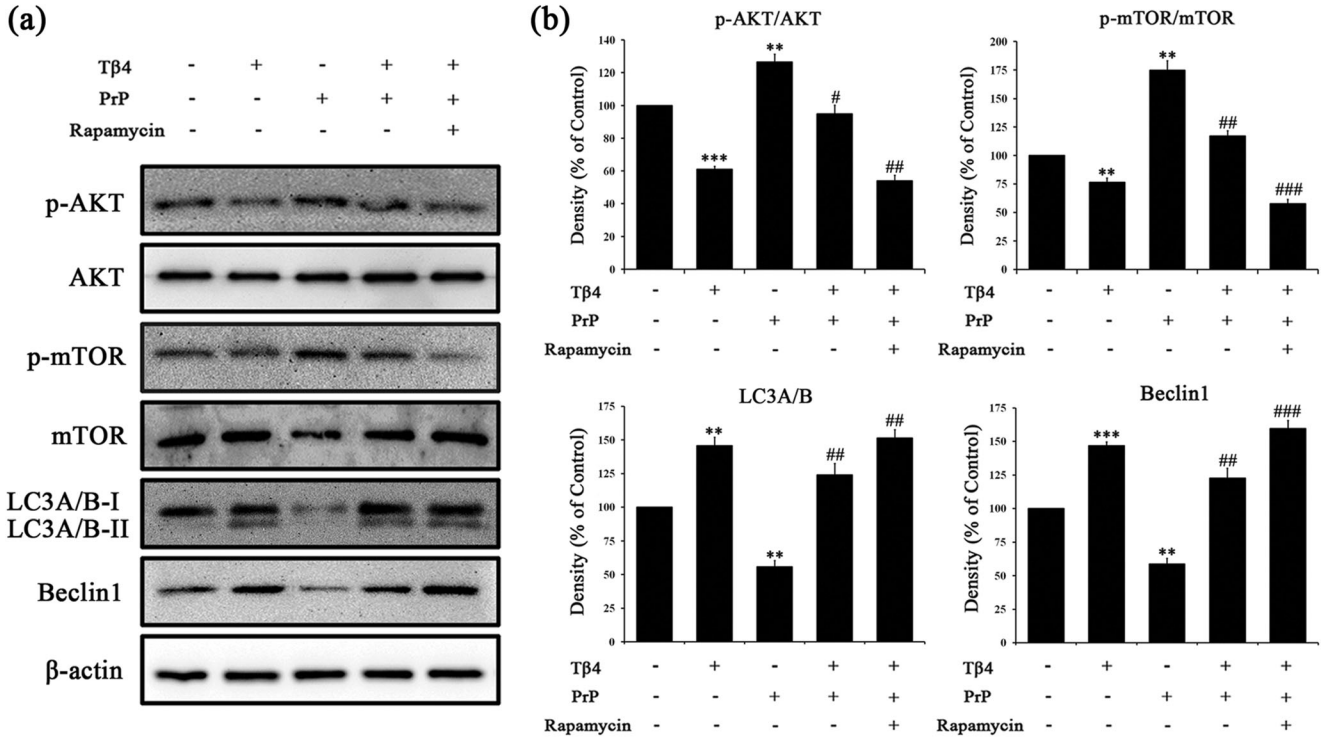

Fig. 3 Thymosin beta 4 induced autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway in PrP (106–126)-treated HT22cells. **a** HT22 cells were stimulated with 100 μM PrP (106–126) for 24 h in the absence or presence of Tβ4 (0.4 μg/mL) or mTOR inhibitor (rapamycin). **b** The bar graphs indicate the average p-mTOR/mTOR, p-AKT/AKT, LC3A/B, and Beclin1 activity. Data are expressed as the mean ± SEM ($n = 3$). $^{**}p < 0.01$, compared to control. $^{***}p < 0.001$, compared to control. $^{\#}p < 0.05$, compared to the PrP-only treated group. $^{\#\#}p < 0.01$, compared to the PrP-only treated group. $^{\#\#\#}p < 0.001$, compared to the PrP-only treated group

The PrP fragment PrP (106–126) possesses the majority of the pathogenic properties associated with the infectious PrP^{Sc}. The accumulation of PrP^{Sc} in the brain of humans and animals has negative effects on the central nervous system. Here, PrP (106–126) decreased cell viability in a dose-dependent manner, whereas Tβ4 increased cell viability in PrP (106–126)-treated HT22 cells in a dose-dependent manner (Fig. 1b, c). These results show that Tβ4 protects against PrP (106–126)-induced neurotoxicity.

Autophagy is an important mechanism in various physiopathological processes, including tumorigenesis, development, cell death, and cell survival (Mizushima et al. 2008; Rubinsztein 2006). Previous studies have reported that autophagy plays a key role in promoting cell survival against apoptosis (Eisenberg-Lerner et al. 2009), and autophagic vacuoles have been observed in prion-infected cultured neuronal cells (Schatzl et al. 1997). The LC3A/B protein is localized to and aggregated on the autophagosome and, therefore, is regarded as a marker for autophagy. Beclin1 is considered an essential protein for the nucleation step of autophagy and is crucial for the autophagy pathway (Meyer et al. 2013). Thus, we investigated autophagy induction by measuring LC3A/B and Beclin1 in Tβ4 or PrP (106–126)-treated HT22 cells. Tβ4 significantly increased expression of LC3A/B-II and Beclin1, whereas PrP (106–126) decreased expression of LC3A/B-II and Beclin1 in a dose-dependent manner (Fig. 2a, b). These results indicate that Tβ4 induces autophagy.

The signaling molecule of the PI3K/AKT/mTOR pathway, mTOR, is closely associated with the inhibition of autophagy (Singh et al. 2012). For these reasons, the total and phosphorylated levels of AKT and mTOR were measured to determine whether the PI3K/AKT/mTOR pathway was involved in Tβ4-induced autophagy. As expected, Tβ4 significantly decreased the phosphorylation expression levels of mTOR and AKT, which had increased in HT22 cells with exposure to PrP (106–126). In addition, phosphorylation levels of mTOR and AKT in cells treated with rapamycin were lower than in cells treated with Tβ4 and PrP (106–126) (Fig. 3a, b). This result suggests that inhibiting the PI3K/AKT/mTOR pathway is important for regulating Tβ4-induced autophagy. In addition, Tβ4 in combination with rapamycin could be useful for therapeutic purposes.

The cholinergic neurotransmitter system requires the co-expression of proteins involved in acetylcholine (ACh) synthesis, storage, and release (Aizawa and Yamamuro 2010). ChTp and AChE play critical roles in the maintenance of ACh level and as cholinergic marker enzymes (Zhao et al. 2015). ChTp is a transmembrane protein localized at the nerve terminals and binds to extracellular acetylcholine with high

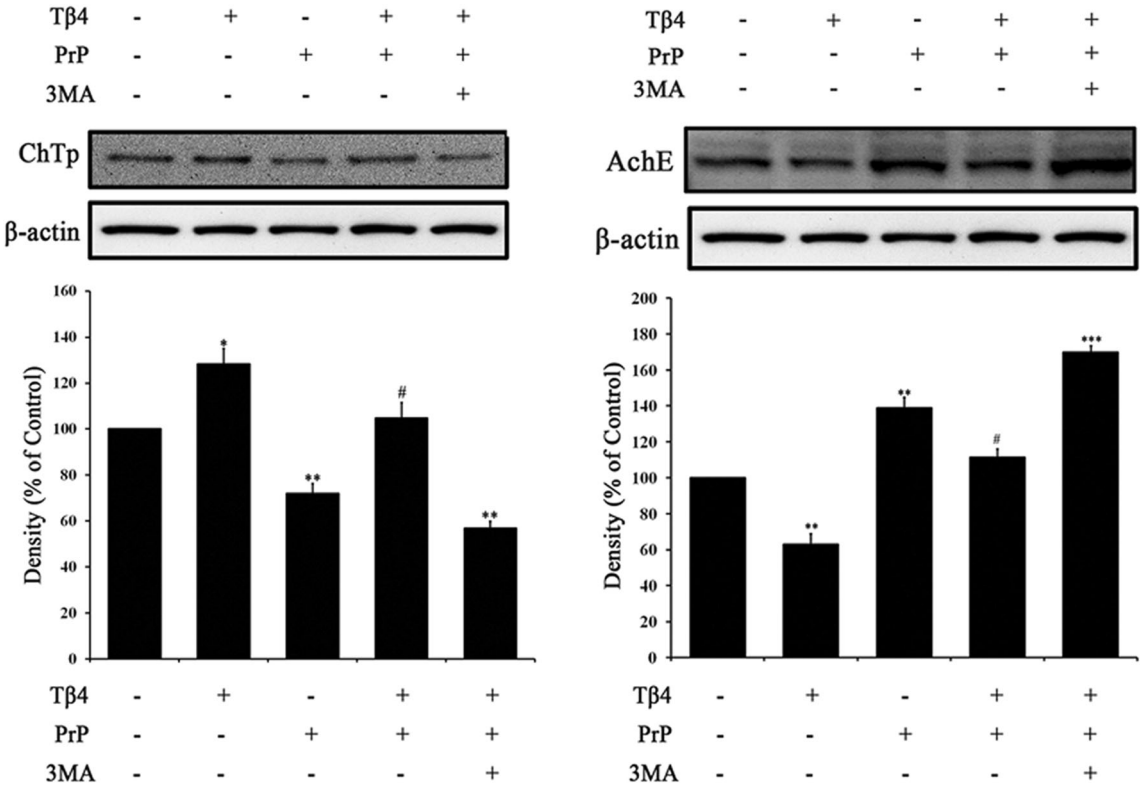

Fig. 4 Expression of cholinergic signaling factors ChTp and AChE in conditions of activated autophagy. HT22 cells were pre-treated for 3 h with the autophagy inhibitor 3-methyladenine (3-MA; 5 mM) and were stimulated with 100 μM PrP (106–126) for 24 h in the absence or presence of Tβ4 (0.4 μg/mL). Data are expressed as the mean ± SEM ($n = 3$). *$p < 0.05$, compared to control. **$p < 0.01$, compared to control. ***$p < 0.001$, compared to control. #$p < 0.05$, compared to the PrP-only treated group

affinity and transports it into the cell (Bales et al. 2006; Perry et al. 1993). AChE hydrolyzes the acetylcholine in neuromuscular junctions and brain cholinergic synapses and terminates signal transmission. A previous study considered

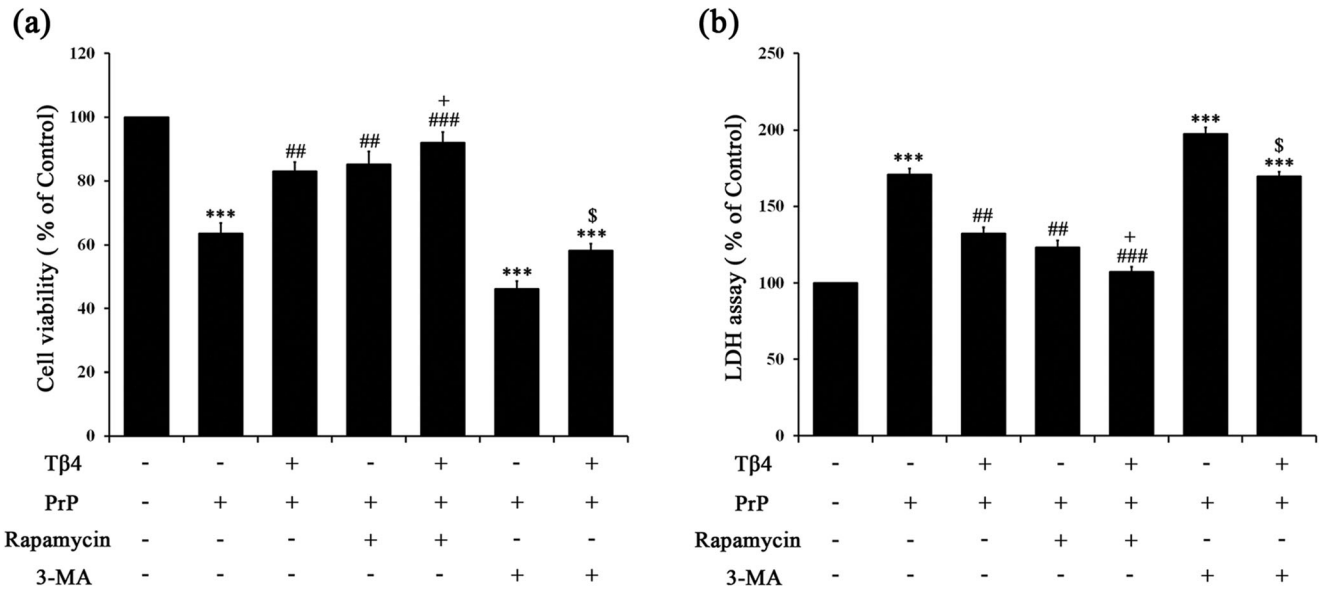

Fig. 5 Tβ4-induced autophagy protects against cell death by PrP (106–126). HT22 cells were stimulated with 100 μM PrP (106–126) for 24 h in the absence or presence of Tβ4 (0.4 μg/mL) or mTOR inhibitor (rapamycin) or autophagy inhibitor (3-MA). Accordingly, cell viability was assessed by **a** MTT assay and **b** LDH release assay. Data are expressed as the mean ± SEM ($n = 3$). ***$p < 0.001$, compared to control. ##$p < 0.01$, compared to the PrP-only treated group. ###$p < 0.001$, compared to the PrP-only treated group. +$p < 0.05$, compared to PrP and rapamycin treated group. \$$p < 0.05$, compared to PrP and 3-MA treated group

the relevance of cholinergic dysfunction in Alzheimer's disease (AD) and related neurodegenerative pathologies (Contestabile et al. 2008). However, the effects of Tβ4 on cholinergic dysfunction induced by PrP^{Sc} are still unknown. Thus, we investigated whether Tβ4 recovered cholinergic dysfunction in PrP (106–126)-treated HT22 cells. In this study, we found that Tβ4 up-regulated ChTp expression in PrP (106–126)-treated HT22 cells, whereas increased levels of ChTp were reversed by 3-MA, an autophagy inhibitor. Tβ4 down-regulated AChE expression in PrP (106–126)-treated HT22 cells, whereas decreased AChE was reversed by 3-MA (Fig. 4). The results of the present study show that Tβ4 increases autophagy and leads to activation of cholinergic signaling through the induction of autophagy in PrP (106–126)-treated HT22 cells. Therefore, this result may highlight one of the key mechanisms underlying the observed neuroprotective effects of Tβ4.

For these reasons, we investigated whether Tβ4-induced autophagy protected PrP (106–126)-induced neurotoxicity in HT22 cells. PrP (106–126) and 3-MA decreased cell viability, increased level of LDH. Whereas, PrP (106–126) and rapamycin increased cell viability, decreased level of LDH. In addition, Tβ4 treatment up-regulated cell viability, down-regulated level of LDH compared to PrP (106–126) with 3-MA or rapamycin treatment. The results of the present study show that autophagy induced by Tβ4 and rapamycin prevents against PrP (106–126)-induced neurotoxicity.

In summary, regulating autophagy could be a therapeutic target for recovering cholinergic function in a cellular prion disease model. Although further studies are needed, autophagy activity from neurons treated with Tβ4 may be an ideal therapeutic target for novel neuroprotective drugs.

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