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Neurotrophin receptor p75 mediates amyloid β-induced tau pathology

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ABSTRACT

Neurofibrillary tangles of hyperphosphorylated tau protein (p-tau) are a key pathological feature of Alzheimer's disease (AD). Tau phosphorylation is suggested to be secondary to amyloid-beta (Aβ) accumulation. However, the mechanism by which Aβ induces tau phosphorylation in neurons remains unclear. Neurotrophin receptor p75 (p75^{NTR}) is a receptor for Aβ and mediates Aβ neurotoxicity, implying that p75^{NTR} may mediate Aβ-induced tau phosphorylation in AD. Here, we showed that Aβ-induced tau hyperphosphorylation and neurodegeneration, including tau phosphorylation, synaptic disorder and neuronal loss, in the brains of both male wild-type (Wt) mice and male P301L transgenic mice (a mouse model of human tauopathy) were alleviated by genetic knockout of p75^{NTR} in the both mouse models. We further confirmed that the activation or inhibition of cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase-3β (GSK3β) significantly changed Aβ/p75^{NTR}-mediated p-tau levels in neurons. Treatment of male P301L mice with soluble p75^{NTR} extracellular domain (p75ECD-Fc), which antagonizes the binding of Aβ to p75^{NTR}, suppressed tau hyperphosphorylation. Taken together, our findings suggest that p75^{NTR} meditates Aβ-induced tau pathology and is a potential druggable target for AD and other tauopathies.

1. Introduction

Alzheimer's disease (AD) is pathologically characterized by intraneuronal neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau and extracellular senile plaques containing fibrillary amyloid-beta peptide (Aβ) in the brain. Tau hyperphosphorylation is suggested to be a key pathological process of synapse damage and neuronal loss (Hoover et al., 2010; Noble et al., 2013; Thies and Mandelkow, 2007) and is correlated with the severity of AD (Arriagada et al., 1992; Gomez-Isla et al., 1997). The knowledge about the mechanism underlying the onset and progression of tau pathology in AD is limited. Abundant evidence suggests that tau phosphorylation is downstream of Aβ (Bolmont et al., 2007; Busciglio et al., 1995; Geula et al., 1998; Gotz et al., 2001b; Hoerndli et al., 2007) and that tau mediates Aβ toxicity in AD (Haass and Mandelkow, 2010; Ittner et al., 2010; Rapoport et al., 2002). However, how Aβ induces tau pathology remains unclear.

The p75 neurotrophin receptor (p75^{NTR}) is the pan-receptor for nerve growth factor (NGF) and other neurotrophins including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4/5 (NT4/5) and mediates neuronal growth, apoptosis and synapse plasticity (Barker, 2004; Chao and Bothwell, 2002). In addition to neurotrophins, Aβ has also been shown to be a ligand of p75^{NTR}, and binding of Aβ to p75^{NTR} induces Aβ production, neurite degeneration and neuronal death (Coulson et al., 2009; Knowles et al., 2009; Murphy et al., 2015; Perini et al., 2002; Sotthibundhu et al., 2008). Importantly, expression of p75^{NTR} is associated with tau hyperphosphorylation in hippocampal neurons of AD patients (Hu et al., 2002). Whether p75^{NTR}

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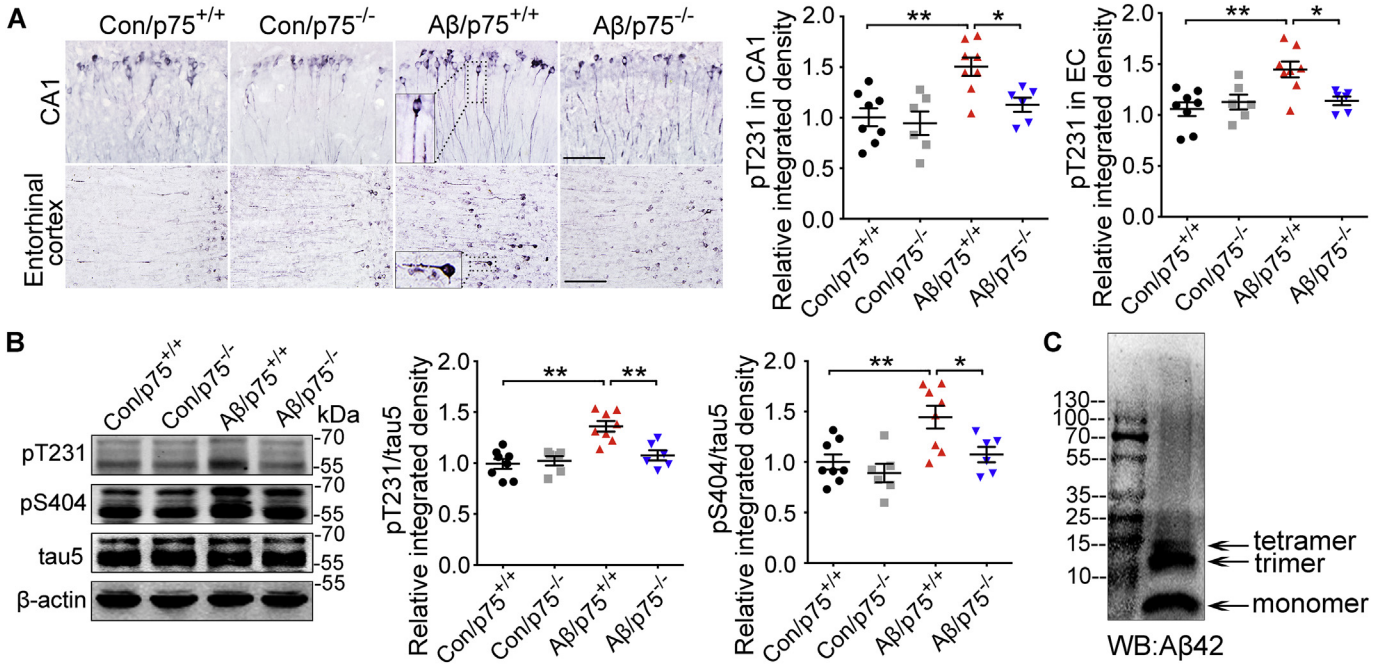


Fig. 1. p75^{NTR} mediates Aβ-induced tau hyperphosphorylation in P301L mice. Male P301L mice aged 2.5 months were subjected to intracerebroventricular injection with Aβ or saline as a control (Con) for 2 weeks. (A) Tau immunostaining (identified by pT231) analysis of the hippocampal CA1 region (one-way ANOVA, F[3,24] = 7.781, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.002, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.044) and entorhinal cortex (one-way ANOVA, F[3,24] = 6.912, p = 0.002; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.002, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.024) in Con/p75$^{+/+}$, Con/p75$^{-/-}$, Aβ/p75$^{+/+}$ and Aβ/p75$^{-/-}$ sections. The pT231 tau relative integrated density values were normalized to those in the Con/p75$^{+/+}$ mice. Scale bar: 100 μm. (B) Immunoblot analysis of pT231 (pT231/tau5: one-way ANOVA, F[3,24] = 12.771, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.001, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.003) and pS404 (pS404/tau5: one-way ANOVA, F[3,24] = 7.166, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.007, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.045) and total tau (tau5) in Con/p75$^{+/+}$, Con/p75$^{-/-}$, Aβ/p75$^{+/+}$ and Aβ/p75$^{-/-}$ mice. The ratios of pT231/tau5 and pS404/tau5 were normalized to those in the Con/p75$^{+/+}$ mice. For each experiment, n = 8 for Con/p75$^{+/+}$ and Aβ/p75$^{+/+}$, and n = 6 for Con/p75$^{-/-}$ and Aβ/p75$^{-/-}$. (C) Characterization of Aβ42 preparations used in the in vivo and in vitro experiments with the monoclonal antibody 6E10. Data are represented as the mean ± s.e.m. Statistical comparisons were performed using one-way ANOVA and Tukey's test. Statistical significance: *$P < 0.05$, **$P < 0.01$.

is the link between Aβ and tau phosphorylation remains elusive. In the present study, we found that p75^{NTR} mediates Aβ-induced neuronal tau hyperphosphorylation and neurodegeneration in AD.

2. Materials and methods

2.1. Animals

The breeding pairs of p75^{NTR} knockout mice (p75^{NTR}/ExonIII$^{-/-}$, p75$^{-/-}$) on a 129/sv background were from Jackson Laboratory (USA), and P301L transgenic mice (pR5) on a C57BL/6 background were provided by Professor Jürgen Götz from the University of Queensland, Australia. All the animals used in this study were raised and bred in the Daping Hospital Animal House. The pR5 mice, which mimic human tauopathy, express the longest human tau isoform (2N4R) with the P301L mutation driven by the neuron-specific mThy1.2 promoter (Deters et al., 2008; Gotz et al., 2001a). To generate transgenic mice with genetic knockout of p75^{NTR}, we crossed p75^{NTR} knockout (p75$^{-/-}$) mice with wild-type (Wt) mice on 129sv background to generate p75^{NTR} heterozygous (p75$^{+/-}$) mice. p75$^{+/-}$ mice were crossed with P301L mice to generate P301L/p75$^{+/-}$ mice followed by backcrossing with p75$^{+/-}$ mice on a C57BL/129sv mixed background to obtain the following mouse lines used in the study: P301L/p75$^{+/+}$ (P301L); P301L/p75$^{-/-}$; wild-type (Wt, p75$^{+/+}$); p75$^{-/-}$. The animals were assigned randomly to the respective groups based on the genotype in all experiments.

Mice were housed in a 12-h light/dark cycle with a room temperature of 22 °C and access to a standard chow diet. All animal experiments were performed in accordance with China Ministry of Science and Technology guidelines and were approved by the Third Military Medical University Animal Welfare Committee.

2.2. General experimental design

To investigate the in vivo role of p75^{NTR} in Aβ-induced tau hyperphosphorylation, several animal models were used, including: (1) P301L/p75$^{+/+}$ (P301L), (2) P301L/p75$^{-/-}$, (3) p75$^{+/+}$ (Wt); (4) p75$^{-/-}$. Mice were subjected to experiments at the age of 2.5 months when obvious NFTs and neurodegeneration have not been formed in the brain of P301L mice (Deters et al., 2008). Male mice (n = 8 for p75$^{+/+}$ and P301L/p75$^{+/+}$, n = 6 for p75$^{-/-}$ and P301L/p75$^{-/-}$) were subjected to a single intracerebroventricular (ICV) injection of 5 μl saline or Aβ42 (82 pmol/μl, a dose inducing memory impairment in mice (Ji et al., 2014)). The mice were euthanized for analysis two weeks after injection.

To investigate the signalling pathway of Aβ/p75^{NTR} in tau hyperphosphorylation, hippocampal neurons obtained from neonatal mice (n = 3 per group) were cultured and treated with signalling pathway modulators. The tau phospho-epitopes including ps199, pT231 and pS404, which are common in tauopathies, were selected for assessment.

To investigate the effects of p75^{NTR} modulation on Aβ-induced tau pathology, primary hippocampal neurons were cultured and treated with recombinant extracellular domain of p75^{NTR} fused with the human IgG Fc fragment (p75ECD-Fc) or human IgG. In addition, male P301L mice aged 2.5 months (n = 6) were intracerebroventricularly injected with Aβ and pre-treated the mice with p75ECD-Fc.

2.3. Intracerebroventricular injection

Mice were stereotactically injected with saline or Aβ as previously

described (Dahlgren et al., 2002; Yao et al., 2015). Under anaesthesia by intraperitoneal injection of 6% chloral hydrate (0.15 ml/g body weight), mice were placed in a stereotactic frame (Stoelting, Wood Dale, USA) for right lateral ventricle injection. Bregma was clearly marked, and a small burr hole was formed in the skull over the stereotaxic coordinates: anteroposterior, −0.6 mm to bregma; lateral, 1.2 mm to the sagittal suture; and ventral, 2.2 mm to the skull surface. ICV injections were performed using a 10-μl Hamilton microsyringe fitted with a 26-gauge needle. Pre-treatment of p75ECD-Fc were performed by injecting p75ECD-Fc (3 μl p75ECD-Fc, 1.5 μg/μl) into the left lateral ventricle 15 min before Aβ injection. The injection was performed by hand at a speed of 2.5 μl/min.

2.4. Oligomeric Aβ42 preparation

Synthetic Aβ42 (American Peptide, Sunnyvale, CA, USA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma) at 1 mg/ml and then aliquoted in Eppendorf tubes (100 μg/tube). HFIP was removed by evaporation in the fume hood, and the remaining peptide was dried under vacuum overnight. The dried pellet was kept at −20 °C until use. For oligomeric Aβ preparation, Aβ42 peptides were resuspended in DMEM to a final concentration of 100 μM and then incubated at 4 °C for 24 h following previous protocols (Dahlgren et al., 2002; De Felice et al., 2008; Yao et al., 2015). For examination of the species of the Aβ preparation, the Aβ solution (200 ng) were separated on a 10–20% Tris-tricine gel and identified with the monoclonal antibody 6E10. As showed in Fig. 1C, the preparations of Aβ were consisted of monomer, trimer and tetramer with entirely free of fibrils. (See Fig. 2.)

2.5. Tissue sampling

Mice were anaesthetized with 6% chloral hydrate (6 ml/kg). Following anaesthesia, mice were perfused transcardially with normal saline containing 0.1% $NaNO_2$ that can dilate blood vessels, thereby enhancing perfusion. The left brain hemisphere was pulverized in liquid nitrogen and then frozen at −80 °C until biochemical analysis. The right brain hemisphere was post-fixed in 4% paraformaldehyde for 24 h and cryoprotected in 30% sucrose for 48 h for histological analysis. Then, 35-μm-thick coronal sections were cut with a cryosectioning microtome and stored in freezing buffer (PBS containing 30% sucrose and 30% ethylene glycol) at −20 °C.

2.6. Protein extraction and sample preparation

For semi-quantitative analysis of protein, brain powder was suspended in lysis buffer supplemented with protease inhibitor and phosphatase inhibitor. Protein lysate was centrifuged at 12,000*g* for 10 min at 4 °C, and the supernatants were collected. Total protein content was determined using a BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Western blotting was performed as previously described. Samples (15–30 μg) were subjected to SDS-PAGE (8–12% acrylamide) gels in running buffer. The blots were probed with antibodies including those against pS199 tau (1:2000, monoclonal, Signalway), pT231 tau

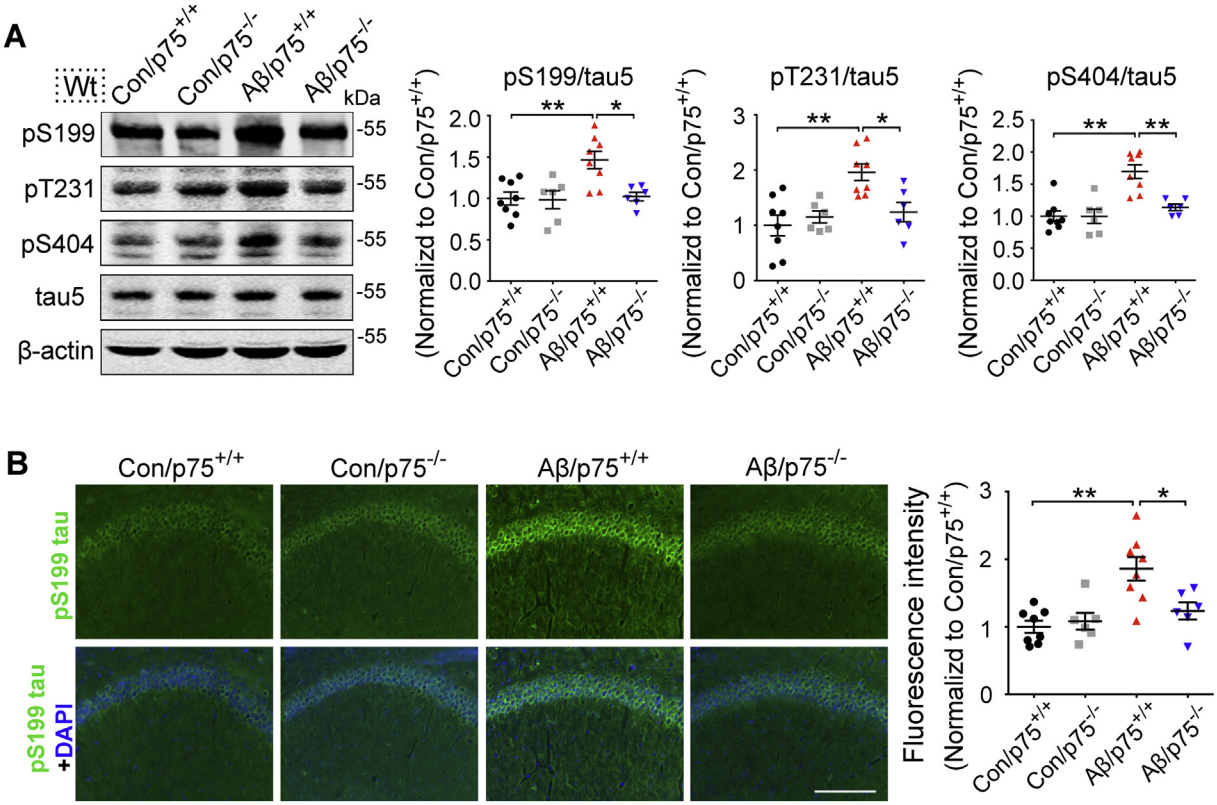


Fig. 2. p75^{NTR} mediates Aβ-induced tau hyperphosphorylation in wild-type (Wt) mice. Male Wt mice aged 2.5 months were subjected to intracerebroventricular injection of Aβ or saline as a control (Con) for 2 weeks. (A) Immunoblot analysis of pS199 (one-way ANOVA, F[3,24] = 7.085, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.004, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.012), pT231 (one-way ANOVA, F[3,24] = 7.703, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.001, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.023) and pS404 (one-way ANOVA, F[3,24] = 13.643, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.001, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.002) tau and total tau (tau5) in Con/p75$^{+/+}$, Con/p75$^{-/-}$, Aβ/p75$^{+/+}$ and Aβ/p75$^{-/-}$ mice. (B) pS199 tau immunostaining analysis of the hippocampal CA1 region in Con/p75$^{+/+}$, Con/p75$^{-/-}$, Aβ/p75$^{+/+}$ and Aβ/p75$^{-/-}$ sections (one-way ANOVA, F[3,24] = 9.196, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.001, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.017). The p-tau relative integrated density values were normalized to those in the Con/p75$^{+/+}$ mice. Scale bar, 200 μm. For each experiment, n = 8 for Con/p75$^{+/+}$ and Aβ/p75$^{+/+}$, and n = 6 for Con/p75$^{-/-}$ and Aβ/p75$^{-/-}$. Data are represented as the mean ± s.e.m. Statistical comparisons were performed using one-way ANOVA, Tukey's test. Statistical significance: *P < 0.05, **P < 0.01.

(1:1000, monoclonal, Signalway), pS404 tau (1:2000, monoclonal, Signalway), tau5 (1:1000, monoclonal, Abcam), glycogen synthase kinase 3 beta (GSK3β) (1:1000, monoclonal, Abcam), pS9-GSK3β (1:1000, monoclonal, Abcam), CDK5 (1:1000, monoclonal, Abcam), p35 (1:1000, polyclonal, Abcam), calpain1 (1:1000, polyclonal, Cell Signaling), spectrin αII (1:1000, monoclonal, Santa Cruz Biotech), AKT (1:1000, polyclonal, Cell Signaling), pS473-AKT (1:1000, polyclonal, Cell Signaling), synapsin-1 (1:1000, monoclonal, Syn-1, Millipore), VAMP1 (1:1000, monoclonal, Epitomics), PSD93 (1:1000, monoclonal, Abcam), PSD95 (1:1000, monoclonal, Millipore), synaptosomal-associated protein 25 (SNAP-25, 1:1000, monoclonal, Millipore), NeuN (1:1000, monoclonal, Abcam), microtubule-associated protein 2 (1:1000, MAP-2, monoclonal, Millipore) and β-actin (1:1000, monoclonal, Sigma-Aldrich). Protein was scanned using the Odyssey scanner software (Li-COR Bioscience) and quantified using Quantity One 6.0. The band density was normalized to that of β-actin. Phosphorylated proteins were normalized to the total amount of the respective protein.

2.7. Immunostaining

The consecutive sections including the hippocampal CA1, hippocampal CA3 or entorhinal cortex were chosen based on the mouse brain atlas (Fig. S1). For immunohistochemistry, sections were stained with primary antibodies overnight at 4 °C, followed by the secondary antibody for 1 h. The antibody (identified pT231-tau) was used at a 1:200 dilution. Slices were visualized using a DAB substrate kit for peroxidase (DAB kit, DAKO). For immunofluorescence, sections were stained overnight at 4 °C with primary antibodies and then with the secondary antibody, followed by counterstaining with DAPI. Images were obtained with a confocal laser scanning microscope (Leica DMI 6000, Wechslar, Germany). Positive staining in the hippocampal CA1, hippocampal CA3 or entorhinal cortex, defined based on the mouse brain atlas. The photos were taken at the same areas of the hippocampal CA1, hippocampal CA3 or entorhinal cortex for each animal. The integrated density for immunohistochemistry and the fluorescence intensity for immunofluorescence were quantified in a blinded manner with ImageJ 6.0.

2.8. Cell culture

Hippocampal neurons were isolated from one-day-old P301L or P301L/p75$^{-/-}$ mouse brains and cultured on poly-D-lysine precoated coverslips. To reduce number of neonatal mice used in our experiments, the primary neurons of the male and female were mixed first and were equally divided into different treatment groups. The cultures were maintained for 7 days before being harvested for further analysis. In some experiments, inhibitors or activators for kinases and receptors were added as indicated 30 min before the administration of Aβ. An antibody to the extracellular domain of p75^{NTR} (1.5 μl/ml, ab1554, Millipore) was added with Aβ to block the binding of Aβ and p75^{NTR}. All drugs were dissolved in DMSO and then diluted using cell culture medium without bovine serum with the final concentration of DMSO < 0.05%. Primary hippocampal neuron cultures were collected and solubilized in RIPA buffer containing protease inhibitors. The cell lysates were sonicated and then centrifuged at 12,000*g* for 10 min at 4 °C. The total protein concentration of supernatants was determined using a by BCA Protein Assay Kit, and 30 μg of total protein was subjected to Western blot analysis.

2.9. Statistical analyses

For each statistical analysis, appropriate tests were selected based on whether the data were normally distributed. Statistical comparisons were made by using unpaired Student's *t*-test (two-tailed, 95% confidence interval) for two groups or one-way ANOVA followed by Tukey's test in cases of multiple comparisons. Differences were considered statistically significant when $P < 0.05$. All statistical analyses were performed with SPSS software (version 13.0) in a blinded manner.

3. Results

3.1. Aβ/p75^{NTR} mediates tau hyperphosphorylation in mouse models

We observed that injection of Aβ oligomers (Fig. 1C) induced an increase in tau phosphorylation, as demonstrated by pT231 tau staining in the hippocampal CA1 and entorhinal cortex of P301L mice compared to that in those injected with saline (Fig. 1A). In these areas, the tau pathology revealed by pT231 tau staining was lower in the p75$^{-/-}$ mice than in the p75$^{+/+}$ mice after ICV injection of Aβ (Fig. 1A). Immunoblotting results showed that Aβ increased pT231 and pS404, which are characteristically hyperphosphorylated in AD, but not total tau (tau5 staining) in the brains of p75$^{+/+}$ mice, and these effects were also rescued in p75$^{-/-}$ mice (Fig. 1B). In addition, we found that the expression of p75^{NTR} and the precursor of nerve growth factor (proNGF), which can induce tau phosphorylation via p75^{NTR} (Shen et al., 2018), was not changed in P301L mice compared with Wt mice at age of 3 months (Fig. S2), suggesting that the increased tau phosphorylation observed in above studies was mainly due to Aβ injection. These data suggest that Aβ-induced tau hyperphosphorylation is dependent on p75NTR.

3.2. Aβ-induced CDK5 and GSK3β activation are p75^{NTR}-dependent

We next investigated the signalling pathway underlying p75^{NTR}-mediated tau hyperphosphorylation. Previous studies reported that tau is phosphorylated by kinases such as glycogen synthase kinase 3β (GSK3β) (Hooper et al., 2008; Ma, 2014), cyclin-dependent kinase 5 (CDK5) (Cruz et al., 2003; Noble et al., 2003) and mitogen-activated protein kinase (MAPK) (Fontaine et al., 2015; Munoz and Ammit, 2010). Thus, we measured the levels of CDK5 and GSK3β, as well as the levels of p38 and ERK1/2 of the MAPK pathway, in vivo. We found that injection of Aβ led to an increase in the p25-CDK5/p35-CDK5 (activated form of CDK5) ratio (Fig. 3A) and a decrease in p-Ser9-GSK3β (pS9-GSK3β, inactivated form of GSK3β) (Fig. 3B) in the brains of P301L mice. These changes were ameliorated by genetic deletion of p75^{NTR}, whereas the expression of p-p38 or p-ERK was unaffected by p75^{NTR} deletion (Fig. 3C and D). These results suggested that Aβ-induced activation of CDK5 and GSK3β were p75^{NTR}-dependent.

3.3. Roles of calpain/CDK5 and AKT/GSK3β signalling in Aβ/p75^{NTR}-mediated tau hyperphosphorylation in P301L hippocampal neurons

We then used primary hippocampal neurons isolated from new-born P301L/p75$^{+/+}$ and P301L/p75$^{-/-}$ mice to further investigate the role of the Aβ/p75^{NTR} signalling pathway in tau hyperphosphorylation. Administration of Aβ increased pT231 and pS404 tau levels and the ratio of p25-CDK5/p35-CDK5 (activated form), while decreasing pS9-GSK3β (inactivated form) expression in p75$^{+/+}$ hippocampal neurons. These changes were not present in p75$^{-/-}$ neurons (Fig. 4A and B). In addition, Aβ activated calpain1, a critical upstream regulator of CDK5 (Kamei et al., 2007), as reflected by an increase in calpain1 expression and the 150KD/220KD α-spectrin ratio, which is mediated by calpain1 activation (Czogalla and Sikorski, 2005). Aβ also decreased the level of AKT phosphorylated at Ser473 (pS473-AKT, activated form), a critical upstream regulator of GSK3β (Cross et al., 1995; Hooper et al., 2008), in p75$^{+/+}$ neurons. These effects of Aβ were alleviated in p75$^{-/-}$ neurons (Fig. 4C). The effects of Aβ on tau phosphorylation (pT231 and pS404 tau) were blocked by the inhibitors of GSK3β (SB216763), CDK5 (roscovitine) and calpain (MDL28170), as well as an activator of AKT (SC79) (Fig. 4D). In addition, the anti-p75^{NTR} antibody was found to reduce Aβ-induced tau hyperphosphorylation (Fig. 4E). Ionomycin (a

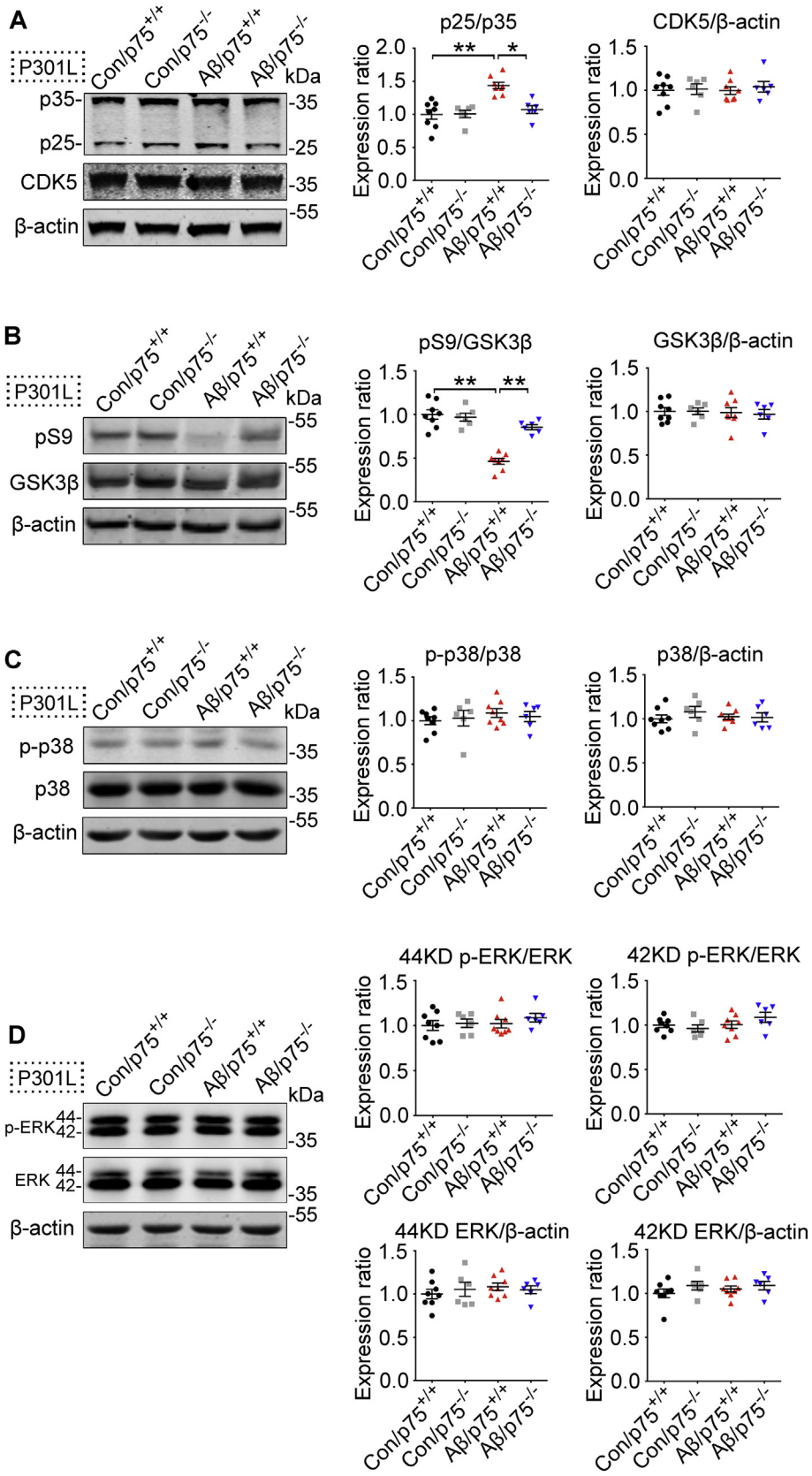
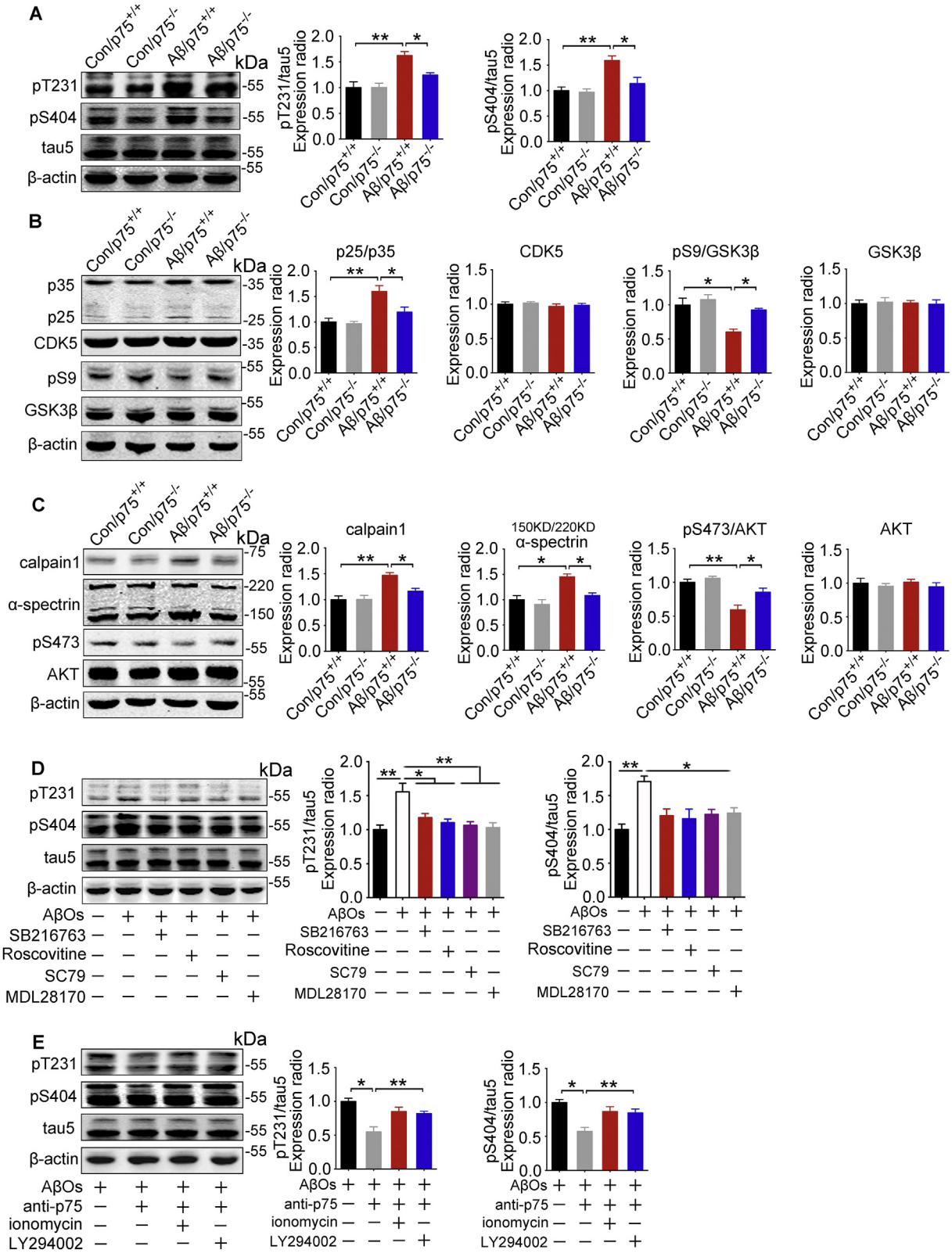


Fig. 3. Genetic removal of p75^{NTR} alleviates GSK3β and CDK5 kinase activation induced by Aβ in P301L mice. (A) Immunoblot analyses of the ratio of p25-CDK5/p35-CDK5 and the levels of total CDK5 (p25-CDK5/p35-CDK5, one-way ANOVA, F [3,24] = 12.653, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.001, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.002; CDK5, one-way ANOVA, F [3,24] = 0.128, p = 0.942). (B) Immunoblot analyses of pS9-GSK3β and total GSK3β levels (pS9/GSK3β, one-way ANOVA, F[3,24] = 36.215, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$ p = 0.001, Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.001; GSK3β, one-way ANOVA, F[3,24] = 0.084, p = 0.968). (C) Immunoblot analyses of p-p38 and total p38 levels (p-p38/p38, one-way ANOVA, F [3,24] = 0.442, p = 0.725; p38, one-way ANOVA, F [3,24] = 0.510, p = 0.679). (D) Immunoblot analyses of p-ERK and total ERK levels (p-ERK/ERK 44KD, one-way ANOVA, F[3,24] = 0.527, p = 0.668; ERK 44KD, one-way ANOVA, F [3,24] = 0.446, p = 0.722; p-ERK/ERK 42KD, one-way ANOVA, F[3,24] = 1.374, p = 0.275; ERK 42KD, one-way ANOVA, F[3,24] = 0.897, p = 0.457). For each experiment, n = 8 for Con/p75$^{+/+}$ and Aβ/p75$^{+/+}$, and n = 6 for Con/p75$^{-/-}$ and Aβ/p75$^{-/-}$. Data are represented as the mean ± s.e.m. Statistical comparisons were performed using one-way ANOVA and Tukey's test. Statistical significance: *$P < 0.05$, **$P < 0.01$.

specific activator of the calpain/CDK5 pathway) and LY294002 (a specific inhibitor of the PI3K, which has been shown to block the AKT/GSK3β pathway (Shen et al., 2018; Wang et al., 2016) were found to reverse the anti-hyperphosphorylation effect induced by the anti-p75^{NTR} antibody (Fig. 4E). These results suggested that activation of the calpain/CDK5 pathway and inactivation of the AKT/GSK3β pathway are involved in Aβ/p75^{NTR}-mediated tau hyperphosphorylation. Moreover, MDL28170 (an inhibitor of calpain) alleviated the effects of



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Aβ on increased levels of pT231 and pS404 tau (Fig. 5A) and on reduced levels of pS473-AKT and pS9-GSK3β (Fig. 5B) in primary hippocampal neurons. Interestingly, the decrease in pS404 tau by MDL28170 was absent when hippocampal neurons were pre-treated with LY294002 (Fig. 5). These results are indicative of crosstalk between the calpain/CDK5 and AKT/GSK3β pathways in the regulation of Aβ/p75^{NTR}-mediated tau hyperphosphorylation.

Fig. 4. Aβ/p75^{NTR} mediates tau hyperphosphorylation through the calpain/CDK5 and AKT/GSK3β pathways in vitro. Cultured hippocampal neurons were isolated from P301L mice with (p75$^{-/-}$) or without (p75$^{+/+}$) gene knockout of p75^{NTR} and were treated with Aβ (3 μM) or saline as a control (Con) for 6 h. (A) Immunoblot analyses of pT231 and pS404 tau and total tau (tau5) in cultured hippocampal neurons (pT231/tau5: one-way ANOVA, F[3,8] = 13.049, p = 0.002; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.003; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.045; pS404/tau5: one-way ANOVA, F[3,8] = 10.327, p = 0.004; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.007; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.030). (B) Immunoblot analyses of pS9-GSK3β and total GSK3β levels (pS9/GSK3β: one-way ANOVA, F[3,8] = 9.923, p = 0.005; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.012; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.035; GSK3β: one-way ANOVA, F [3,8] = 0.060, p = 0.980), as well as the ratio of p25-CDK5/p35-CDK5 and total CDK5 levels in cultured hippocampal neurons (p25-CDK5/p35-CDK5: one-way ANOVA, F[3,8] = 11.445, p = 0.003; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.005; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.042; CDK5: One-way ANOVA, F [3,8] = 0.472, p = 0.710). (C) Immunoblot analyses of calpain1 levels (One-way ANOVA, F[3,8] = 11.649, p = 0.003; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.004; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = .039), ratio of 150 KD/220 KD α-spectrin (one-way ANOVA, F[3,8] = 10.543, p = 0.004; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.011; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.030), and pS473-AKT and total AKT levels (pS473/AKT: one-way ANOVA, F[3,8] = 15.299, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.003; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.034; AKT: one-way ANOVA, F[3,8] = 0.369, p = 0.778) in cultured hippocampal neurons. (D) Immunoblot analyses of pT231 and pS404 tau and total tau (tau5) in hippocampal neurons treated with Aβ (3 μM) for 6 h with roscovitine pre-treatment (10 μM, 1 h) (pT231/tau5: one-way ANOVA, F[5,12] = 7.325, p = 0.002; Tukey's test, Aβ vs Con, p = 0.002; Aβ vs Aβ + SB216763, p = 0.038; Aβ vs Aβ + roscovitine, p = 0.012; Aβ vs Aβ + SC79, p = 0.006; Aβ vs Aβ + MDL28170, p = 0.004; pS404/tau5: one-way ANOVA, F[5,12] = 6.295, p = 0.004; Tukey's test, Aβ vs Con, p = 0.002; Aβ vs Aβ + SB216763, p = 0.026; Aβ vs Aβ + roscovitine, p = 0.015; Aβ vs Aβ + SC79, p = 0.033; Aβ vs Aβ + MDL28170, p = 0.040). (E) Immunoblot analyses of pT231, pS404 tau and total tau (tau5) in hippocampal neurons treated with Aβ (3 μM) and incubated with the anti-p75^{NTR} antibody for 24 h with LY294002 pre-treatment (10 μM, 1 h) (pT231/tau5: one-way ANOVA, F[3,8] = 10.873, p = 0.003; Tukey's test, Aβ vs Con, p = 0.002; Aβ vs Aβ + ionomycin, p = 0.023; Aβ vs Aβ + LY294002, p = 0.040; pS404/tau5: one-way ANOVA, F[3,8] = 9.836, p = 0.005; Tukey's test, Aβ vs Con, p = 0.003; Aβ vs Aβ + ionomycin, p = 0.027; Aβ vs Aβ + LY294002, p = 0.038). For each experiment, n = 3 per group. Data are represented as mean ± s.e.m. Statistical comparisons were performed using one-way ANOVA, Tukey's test. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$.

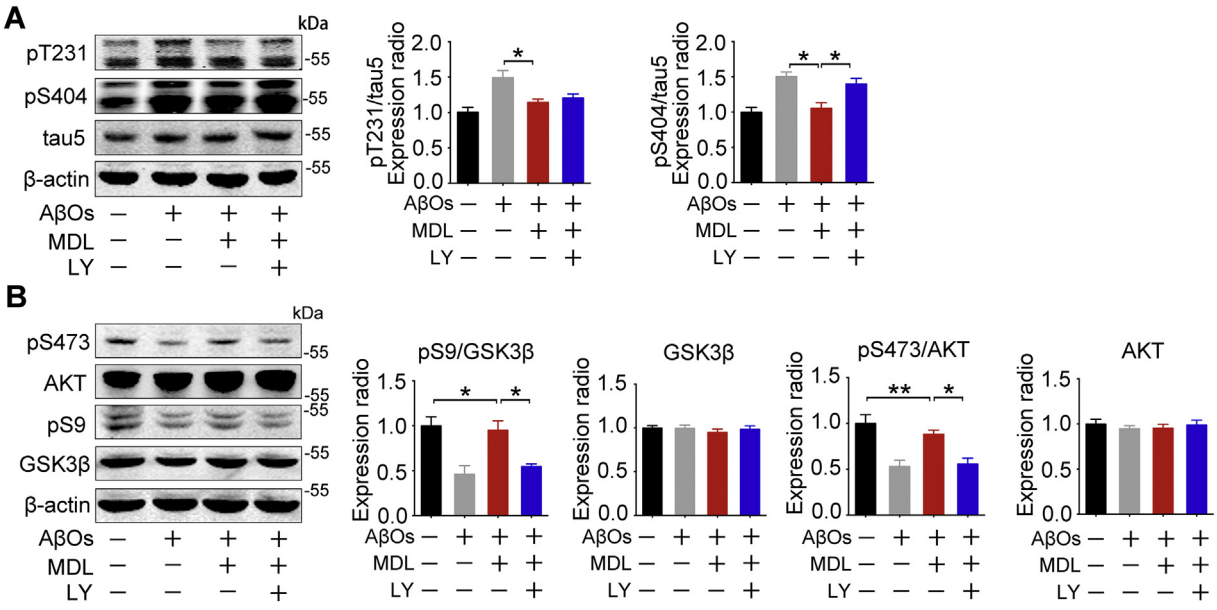

Fig. 5. Activation of the AKT/GSK3β pathway contributes to the anti-hyperphosphorylation role of calpain inhibition. (A) Immunoblots of pT231, pS404 tau and total tau (tau5) in cultured hippocampal neurons treated with Aβ (3 μM, 6 h) and incubated with MDL28170 (MDL, 10 nM, 1 h) with or without LY294002 (LY, 10 μM, 1 h) pre-treatment (pT231/tau5: one-way ANOVA, F[3,8] = 8.271, p = 0.008; pS404/tau5: one-way ANOVA, F[3,8] = 8.333, p = 0.008). (B) Immunoblot analyses of pS9-GSK3β, total GSK3β, pS473-AKT and total AKT in cultured hippocampal neurons treated with Aβ and incubated with MDL28170 (MDL, 10 nM, 1 h) with or without LY294002 (LY, 10 μM, 1 h) pre-treatment (pS9/GSK3β: One-way ANOVA, F[3,8] = 9.768, p = 0.005; GSK3β: One-way ANOVA, F[3,8] = 0.431, p = 0.737; pS473/AKT: one-way ANOVA, F[3,8] = 10.937, p = 0.003; GSK3β: one-way ANOVA, F[3,8] = 0.315, p = 0.815). For each experiment, n = 3 per group. Data are represented as the mean ± s.e.m. Statistical comparisons were performed using One-way ANOVA and Tukey's test. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$.

3.4. Genetic knockout of p75^{NTR} alleviates Aβ-induced tau pathology

To examine the role of p75^{NTR} in the regulation of Aβ-induced tau phosphorylation in 2.5-month-old mice with the MAPT P301L transgene, we intraventricularly injected Aβ42 into animals with or without the genetic deletion of p75^{NTR}. Compared with saline-treated P301L controls, the Aβ-treated mice displayed synaptic disorders reflected by lower levels of pre-synaptic proteins including synapsin-1, VAMP-1 and SNAP-25 and post-synaptic proteins including PSD93 and PSD95 in p75$^{+/+}$ mice (Fig. 6A and B). Genetic knockout of p75^{NTR} alleviated the synaptic disorder caused by Aβ (Fig. 6A and B). Immunofluorescence analysis of brain sections demonstrated that the genetic knockout of p75NTR increased VAMP-1 and PSD95 expression in the CA1 of Aβ-treated p75$^{-/-}$ mice, compared with Aβ-treated p75$^{+/+}$ mice (Fig. 6C–F). Moreover, genetic knockout of p75NTR alleviated the neuronal loss induced by Aβ in the CA1 of p75$^{-/-}$ mice, as reflected by the greater expression of NeuN and MAP-2 in Aβ-treated p75$^{-/-}$ mice than in Aβ-treated p75$^{+/+}$ mice (Fig. 7A and B). These results suggest that deletion of p75^{NTR} alleviates Aβ-induced tau pathology in P301L mice.

3.5. p75ECD-Fc treatment attenuates Aβ-induced tau hyperphosphorylation

The above findings and other evidence suggest that modulation of p75^{NTR} may be a valid therapeutic approach to alleviate Aβ-induced tau hyperphosphorylation in AD (Nguyen et al., 2014; Simmons et al., 2014; Yang et al., 2008). Therefore, we investigated whether treatment with p75ECD-Fc, which blocks the binding of p75^{NTR} with Aβ (Yao

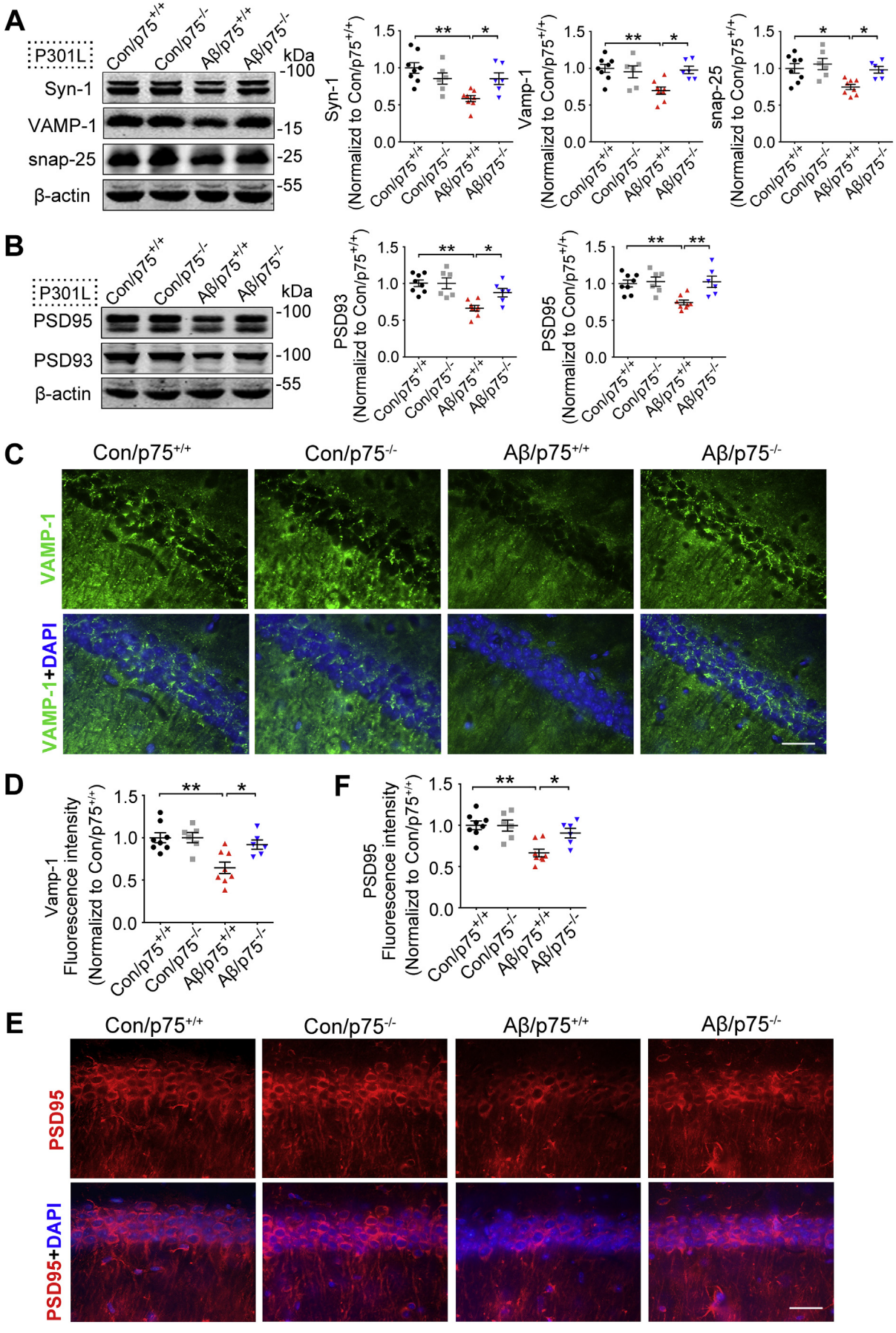

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Fig. 6. Genetic removal of p75^{NTR} alleviates Aβ-induced synaptic disorder in P301L mice. (A) Immunoblot analyses of synapsin-1 (Syn-1) (one-way ANOVA, F [3,24] = 7.651, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.001; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$ p = 0.044), VAMP-1 (one-way ANOVA, F [3,24] = 6.310, p = 0.003; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.004; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.014), SNAP-25 (one-way ANOVA, F [3,24] = 6.126, p = 0.003; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.004,; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.014) in brain homogenates. (B) Immunoblot analyses of PSD93 (one-way ANOVA, F[3,24] = 7.083, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.007; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.006) and PSD95 (one-way ANOVA, F[3,24] = 10.110, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.007; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.044) in brain homogenates. Immunofluorescence labelling (C and E) and quantification of fluorescence intensity for (D) VAMP-1 (one-way ANOVA, F[3,24] = 8.021, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.001; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = .025) and (F) PSD95 (one-way ANOVA, F[3,24] = 9.000, p = .001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = .001; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = .026) expression in the hippocampal CA1 region in Con/p75$^{+/+}$, Con/p75$^{-/-}$, Aβ/p75$^{+/+}$ and Aβ/p75$^{-/-}$ sections. Scale bar, 50 μm. For each experiment, n = 8 for Con/p75$^{+/+}$ and Aβ/p75$^{+/+}$, and n = 6 for Con/p75$^{-/-}$ and Aβ/p75$^{-/-}$. Data are represented as the mean ± s.e.m. Statistical comparisons were performed using one-way ANOVA and Tukey's test. Statistical significance: *$P < 0.05$, **$P < 0.01$.

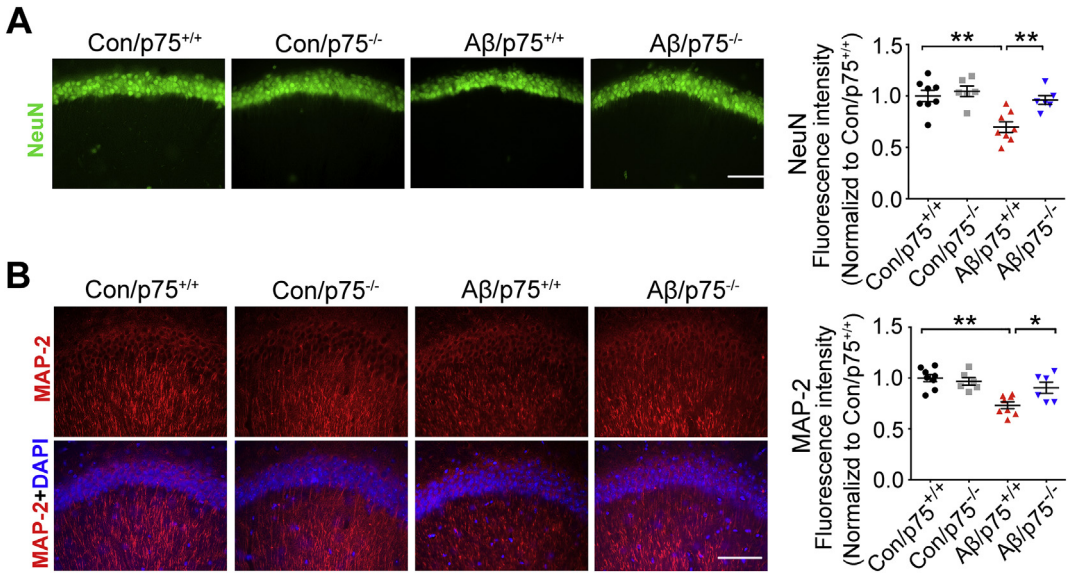

Fig. 7. Genetic removal of p75^{NTR} alleviates Aβ-induced neuronal loss in P301L mice. Immunofluorescence labelling and quantification of fluorescence intensity for (A) NeuN (one-way ANOVA, F[3,24] = 9.728, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.001; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.008) and (B) MAP-2 (one-way ANOVA, F[3,24] = 9.866, p = 0.001; Tukey's test, Con/p75$^{+/+}$ vs Aβ/p75$^{+/+}$, p = 0.001; Aβ/p75$^{+/+}$ vs Aβ/p75$^{-/-}$, p = 0.028) expression in the hippocampal CA1 region in Con/p75$^{+/+}$, Con/p75$^{-/-}$, Aβ/p75$^{+/+}$ and Aβ/p75$^{-/-}$ sections. Scale bar, 100 μm. For each experiment, n = 8 for Con/p75$^{+/+}$ and Aβ/p75$^{+/+}$, and n = 6 for Con/p75$^{-/-}$ and Aβ/p75$^{-/-}$. Data are represented as the mean ± s.e.m. Statistical comparisons were performed using One-way ANOVA and Tukey's test. Statistical significance: *$P < 0.05$, **$P < 0.01$.

et al., 2015), could rescue Aβ-induced tau pathology in P301L mice. The in vitro studies showed that Aβ-induced tau phosphorylation in primary P301L hippocampal neurons was reduced by treatment with p75ECD-Fc rather than human IgG (Fig. 8A). Moreover, the in vivo experiments found that treatment with p75ECD-Fc by ICV injection effectively alleviated tau hyperphosphorylation (such as pT231 and pS404 tau) in the hippocampus of P301L mice (Fig. 8B and C). The data above support that modulation of p75^{NTR} is a potential therapeutic approach to reduce tau pathology in AD.

4. Discussion

In this study, we found that p75^{NTR} mediates Aβ-induced neuronal tau hyperphosphorylation. The calpain/CDK5 and AKT/GSK3β pathways are involved in Aβ/p75^{NTR}-induced tau hyperphosphorylation. Genetic knockout of p75^{NTR} or blockage of p75^{NTR} with p75ECD-Fc alleviated the tau pathology in P301L mice, supporting the view that modulation of p75^{NTR} is a potential therapeutic approach to rescue tau pathology in AD (Nguyen et al., 2014; Simmons et al., 2014; Yang et al., 2008; Zeng et al., 2011).

In the present study, we provided in vivo and in vitro evidence that Aβ/p75^{NTR}-mediated tau hyperphosphorylation is linked to the calpain/CDK5 and AKT/GSK3β pathways. The cytoplasmic juxtamembrane region of p75^{NTR} has been reported to be necessary and sufficient to induce calpain-mediated cell death in a variety of neural and non-neural cell types (Coulson et al., 2000). Evidence suggests that calpain negatively regulates PI3K/AKT pathway activity in NIH 3T3 cells (Beltran et al., 2011). Calpain has been shown to truncate and activate GSK3β in AD (Jin et al., 2015). The CDK5 activator protein p25 preferentially binds with and activates GSK3β (Chow et al., 2014). Here, we show that MDL28170 (an inhibitor of calpain) activated AKT/GSK3β and reduced the pS404 tau level, and these changes were reversed by LY294002, indicating that the AKT/GSK3β pathway might underlie the anti-hyperphosphorylation effect of MDL28170. The in vitro findings implicate a crosstalk between the calpain/CDK5 and AKT/GSK3β pathways downstream of Aβ/p75^{NTR} signalling in the regulation of p-tau levels in AD. Further studies are needed to dissect the exact contribution of calpain/CDK5 and AKT/GSK3β to tau hyperphosphorylation.

Aging is the primary risk factor for AD and other neurodegenerative disorders. How aging promotes the occurrence of AD remains largely unknown. We previously found that p75^{NTR} expression is elevated with the aging process (Wang et al., 2011) and can also be up-regulated by other risk factors for AD, such as central nervous system injury (Ibanez and Simi, 2012), diabetes (Barcelona et al., 2016) and vascular dysfunction (Obata et al., 2006; Taniuchi et al., 1986). In addition, the accumulation of Aβ in the brain activates p75^{NTR} expression (Wang et al., 2011; Yao et al., 2015). In turn, Aβ/p75^{NTR} signalling further increases Aβ production by regulating the convergence and endocytosis of APP and BACE1 in endosomes (Saadipour et al., 2018) and induces

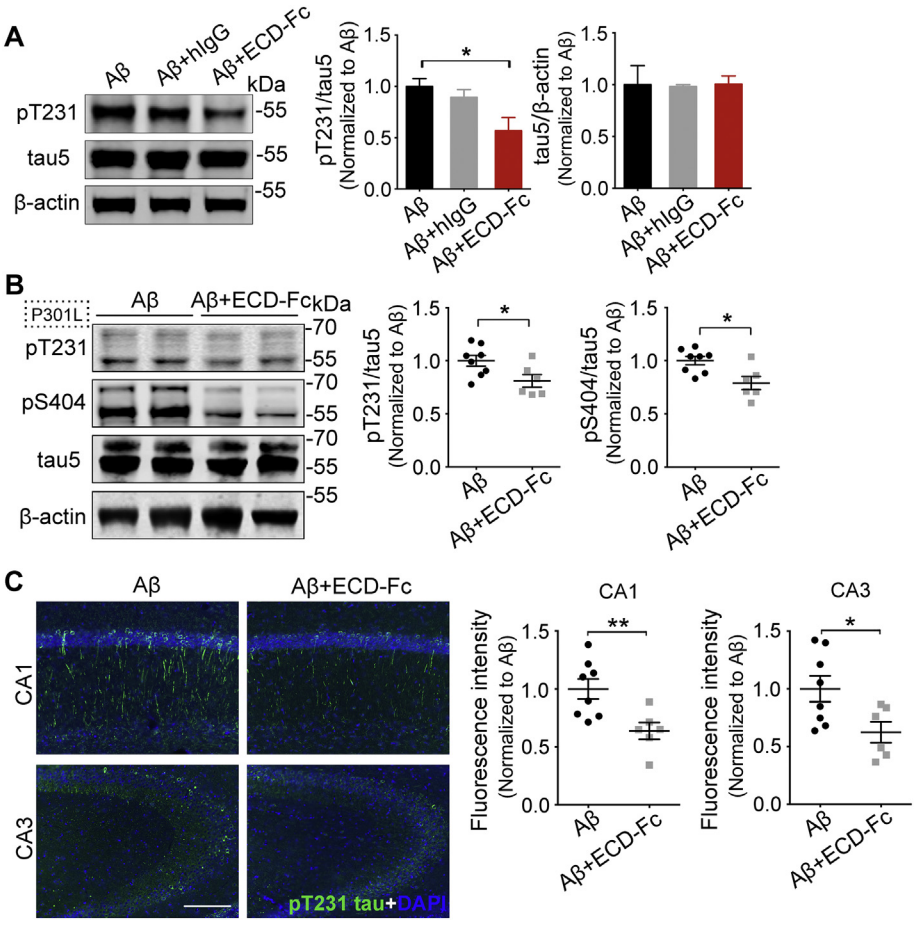

Fig. 8. P75ECD-Fc attenuates the tau hyperphosphorylation induced by Aβ in cultured hippocampal neurons and P301L mice. (A-B) Immunoblot analysis of pT231 and total tau (tau5) in cultured P301L hippocampal neurons treated with Aβ (3 μM, 6 h) and incubated with p75ECD-Fc (0.15 μg/μl, 30 min) or human IgG (h IgG, 0.15 μg/μl, 30 min) pre-treatment (pT231/tau5: one-way ANOVA, $F[2,6] = 5.416$, $p = 0.045$; tau5/β-actin: one-way ANOVA, $F[2,6] = 0.11$, $p = 0.989$). For each in vitro experiment, n = 3 per group. Data are represented as the mean ± s.e.m. Statistical comparisons were performed using one-way ANOVA and Tukey's test. Statistical significance: $^*P < 0.05$. (B) Immunoblot analysis of pT231 tau, pS404 tau and total tau (tau5) in 2.5-month-old P301L mice injected with Aβ with or without p75ECD-Fc pre-treatment (pT231/tau5, $p = 0.032$, $t = 2.42$, $df = 12$; pS404/tau5, $p = 0.011$, $t = 3.014$, $df = 12$). (C) Immunofluorescence labelling and quantification of fluorescence intensity for pT231 tau in the hippocampal CA1 and CA3 regions of 2.5-month-old P301L mice injected with Aβ with or without p75ECD-Fc pre-treatment. The relative fluorescence intensity values were normalized to those in the mice injected with Aβ (CA1, $p = 0.010$, $t = 3.059$, $df = 12$; CA3, $p = 0.030$, $t = 2.469$, $df = 12$). Scale bar, 100 μm. For each in vivo experiment, n = 8 for Aβ, and n = 6 for Aβ + p75ECD. Data are represented as the mean ± s.e.m. Statistical comparisons were performed using an unpaired two-tailed *t*-test. Statistical significance: $^*P < 0.05$.

neuronal death and neurite degeneration (Zeng et al., 2011) Furthermore, $p75^{NTR}$ promotes Aβ production in cortical neurons of APP/PS1 transgenic mice by increasing the phosphorylation of APP and BACE1 in a $p75^{NTR}$-dependent manner (Saadipour et al., 2018). Production of NGF by Aβ-exposed astrocytes promotes excess tau phosphorylation through $p75^{NTR}$ (Saez et al., 2006). Moreover, we found that $p75^{NTR}$ signalling meditates Aβ-induced neurotoxicity, especially tau hyperphosphorylation in P301L mice. In this sense, $p75^{NTR}$ and Aβ might form a vicious cycle that drives the development of AD hallmarks including Aβ production, tau phosphorylation and neuronal degeneration in the pathogenesis of AD (Yao et al., 2015). Dysregulation of p75NTR/TrkA signalling has been regarded as a pathogenic mechanism associated with NFT formation in AD (Tiernan et al., 2018). Other neurodegenerative substances, such as proNGF and Nogo, also exert detrimental effects via p75NTR signalling, including neuronal death, neurite degeneration and tau hyperphosphorylation (Masliah et al., 2010; Shen et al., 2018), though P301L mice aged 3 months had similar levels of $p75^{NTR}$ and proNGF compared with age-matched wild-type mice (Fig. S2). These studies suggest that p75NTR is a critical player in the neurodegeneration of AD.

Two animal models were used here trying to illustrate the effect of $p75^{NTR}$ on Aβ-mediated tau hyperphosphorylation. The purpose that we performed this study using P301L mice was to see whether Aβ/p75NTR pathway regulate human tau phosphorylation. The wild-type mice were used to study the effect of Aβ on endogenous tau phosphorylation of mice. Based on the intra-group analysis, the magnitude of the effects seen in wild-type mice was similar to that seen in P301L mice. However, we did not directly compare the increase of tau phosphorylation levels between wild-type and P301L mice as experiments were carried out on the two groups of mice separately. Genetic background has potential impact on the tau pathology. In our study, due to the availability of animal models, the $p75^{-/-}$/p301L mouse was made with a mixed background of 129/sv and C57BL/6. It is necessary to examine whether our findings based on this mixed genetic background can be produced in animals with other genetic background in the future.

Diverse therapeutic approaches targeting tau have been proposed, including reducing tau expression, inhibiting the phosphorylation and assembly of tau, disrupting tau aggregates, stabilizing microtubules, and inhibiting the spread of misfolded tau (Holtzman et al., 2016; Li and Gotz, 2017; Sun et al., 2018). Considering that Aβ and tau both have important pathological roles, effective medicine needs to target both Aβ and tau (Wang, 2014). Our results suggest that blocking the binding between $p75^{NTR}$ and Aβ is a promising therapeutic approach to target the Aβ-induced phosphorylation of tau. Meanwhile, the discovery of multiple roles of $p75^{NTR}$ in the pathogenesis of AD also suggests that $p75^{NTR}$ is a potential target for multi-target treatment for AD.

In conclusion, the present study reveals an important role of $p75^{NTR}$ in Aβ-induced tau pathology and provides proof-of-concept that genetic or pharmacological (e.g., p75ECD) modulation of $p75^{NTR}$ may be a valid and multi-target therapeutic approach for the treatment of AD and perhaps other tauopathies.

Author contributions

Y.J.W. and X.F.Z. conceived and designed the project; L.L.S., W.W.L., Y.L.X., S.H.G., X.L.B., Y.H.L., J.W., M.Y.X. and F.Z. performed animal and in vitro experiments: J.Z., C.Y.G., X.Q.Y., F.Z, X.F.Z. and Y.J.W. analyzed the data. L.L.S. and Y.J.W. wrote the manuscript.

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Availability of data and material

The datasets collected and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All animal experiments were approved by the Third Military Medical University Animal Welfare Committee (China).

Declaration of Competing Interest

Xin-Fu Zhou and Yan-Jiang Wang are the co-inventors of the patents on the p75ECD as a therapeutic approach for AD, which were transferred to Fujian Tiantai Medical Technology Ltd. by University of South Australia Venture.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.104567.

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