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Telencephalin protects PAJU cells from amyloid beta protein-induced apoptosis by activating the ezrin/radixin/moesin protein family/phosphatidylinositol-3-kinase/protein kinase B pathway[☆]

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Abstract

Telencephalin is a neural glycoprotein that reduces apoptosis induced by amyloid beta protein in the human neural tumor cell line PAJU. In this study, we examined the role of the ezrin/radixin/moesin protein family/phosphatidylinositol-3-kinase/protein kinase B pathway in this process. Western blot analysis demonstrated that telencephalin, phosphorylated ezrin/radixin/moesin and phosphatidylinositol-3-kinase/protein kinase B were not expressed in PAJU cells transfected with empty plasmid, while they were expressed in PAJU cells transfected with a telencephalin expression plasmid. After treatment with 1.0 nM amyloid beta protein 42, expression of telencephalin and phosphorylated phosphatidylinositol-3-kinase/protein kinase B in the transfected cells gradually diminished, while levels of phosphorylated ezrin/radixin/moesin increased. In addition, the high levels of telencephalin, phosphorylated ezrin/radixin/moesin and phosphatidylinositol-3-kinase/protein kinase B expression in PAJU cells transfected with a telencephalin expression plasmid could be suppressed by the phosphatidylinositol-3-kinase inhibitor LY294002. These findings indicate that telencephalin activates the ezrin/radixin/moesin family/phosphatidylinositol-3-kinase/protein kinase B pathway and protects PAJU cells from amyloid beta protein-induced apoptosis.

Key Words

telencephalin/intercellular adhesion molecule 5; amyloid beta protein; ezrin/radixin/moesin family proteins/phosphatidylinositol-3-kinase/protein kinase B signal transduction; neural regeneration

Research Highlights

Telencephalin (intercellular adhesion molecule-5) activates the ezrin/radixin/moesin protein family/phosphatidylinositol-3-kinase/protein kinase B pathway and protects PAJU cells from amyloid beta protein-induced apoptosis.

Abbreviation

ERM/PI3K/Akt, ezrin/radixin/moesin/phosphoinositide-3-kinase/protein kinase B

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INTRODUCTION

Amyloid beta protein is a major component of the senile plaques characteristic of Alzheimer's disease^[1]. Increasing evidence suggests that as amyloid beta protein accumulates, it initiates a cascade of pathological events that result in not only the formation of senile plaques, but also the accompanying neural damage and death^[2]. It is neurotoxic^[2], and induces axonopathy^[3] and neuronal apoptosis^[4], both in brain tissue^[5] and in neuroblastoma cells *in vitro*^[6].

Telencephalin (intercellular adhesion molecule-5) is a cell adhesion molecule expressed in the somatodendritic membrane of telencephalic neurons in the mammalian brain^[7]. It induces dendritic outgrowth^[6], which participates in memory formation and learning^[7]. Furutani *et al*^[8] demonstrated that the telencephalin cytoplasmic region binds ezrin/radixin/moesin (ERM) family proteins that link membrane proteins to the actin cytoskeleton. Members of the ERM family link integral plasma membrane proteins with the underlying actin cytoskeleton, and participate in signal transduction pathways^[9]. Dendritic filopodia contain telencephalin, PI(4,5)P2, phospho-ERM and F-actin, all of which presumably play important roles in the signal transduction cascade leading to the formation and maintenance of dendritic filopodia^[8]. These functions of telencephalin require its extracellular and cytoplasmic regions, suggesting the involvement of cytoskeleton-associated intracellular binding partners and signal transduction in telencephalin-mediated morphogenesis of dendritic filopodia. The interaction between telencephalin and ERM proteins plays a key role in dendritic morphogenesis^[8], and may be involved in signal transduction. It has been reported that phospho-ERM proteins participate in signal transduction pathways, such as the pro-survival phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway^[10]. Axonopathy is an early marker of amyloid beta protein neurotoxicity, in contrast to apoptosis. We reported previously that telencephalin attenuates axonal disruption induced by amyloid beta protein 35 and amyloid beta protein 42 in the PAJU cell line^[11]. Subsequently, we found that telencephalin may confer resistance to apoptosis^[12]. Because PI3K phosphorylates diverse protein substrates, including precursors in a cascade that results in activation of the survival kinase Akt, we hypothesized that cell survival is promoted by a signaling complex that includes telencephalin, ERM, PI3K and Akt. Therefore, in this study, we examine the end events of neuronal degeneration induced by amyloid beta protein 42 in the differentiated PAJU cell line and the activation of the

PI3K/Akt pathway, focusing on the transduction events between telencephalin and ERM.

RESULTS

Amyloid beta protein 42 inhibited telencephalin expression in PAJU-telencephalin cells

Our previous studies suggest that telencephalin is involved in apoptotic neuronal death triggered by amyloid beta protein peptide^[12-13]. Therefore, we assessed if these peptides affect telencephalin expression. The levels of telencephalin were assessed in PAJU-telencephalin cells (PAJU cells transfected with a telencephalin expression vector) treated with 1.0 nM amyloid beta protein 42 for different time periods (24, 48, and 96 hours). Amyloid beta protein 42 significantly decreased telencephalin expression for all the incubation periods tested compared with control untreated cells ($P < 0.05$; Figure 1), and this effect was time-dependent.

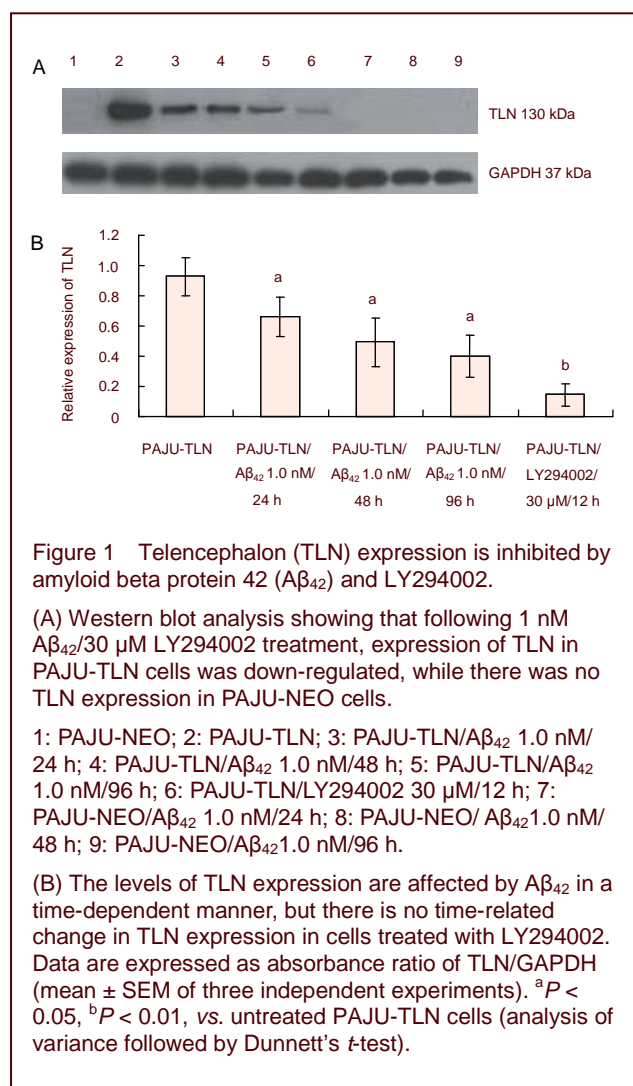


Figure 1 Telencephalin (TLN) expression is inhibited by amyloid beta protein 42 (Aβ₄₂) and LY294002.

(A) Western blot analysis showing that following 1 nM Aβ₄₂/30 μM LY294002 treatment, expression of TLN in PAJU-TLN cells was down-regulated, while there was no TLN expression in PAJU-NEO cells.

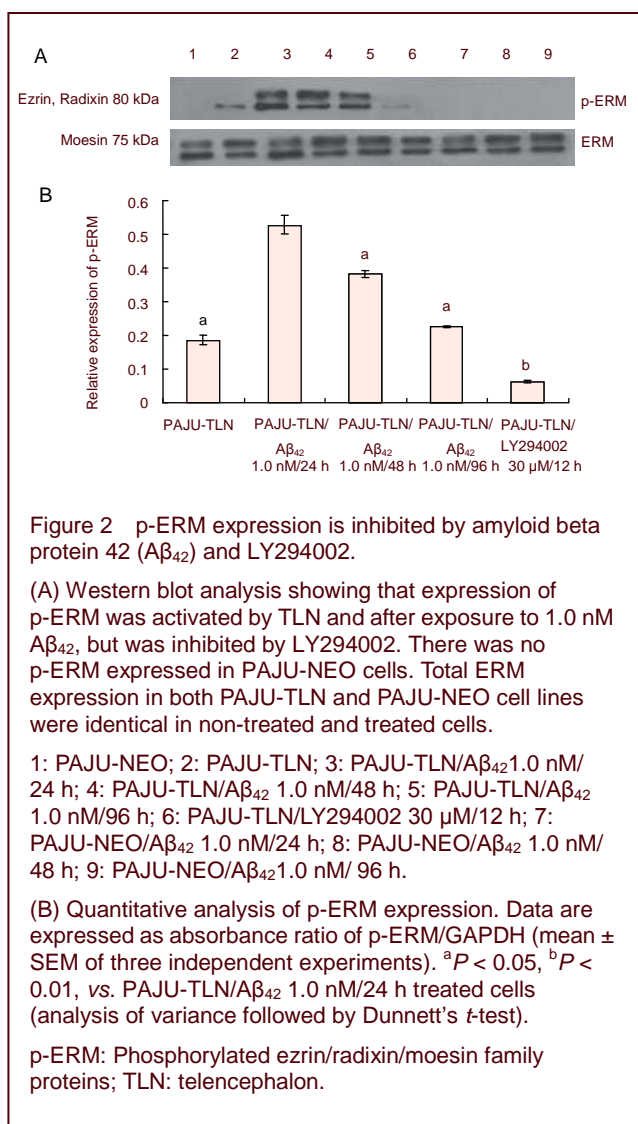
1: PAJU-NEO; 2: PAJU-TLN; 3: PAJU-TLN/Aβ₄₂ 1.0 nM/24 h; 4: PAJU-TLN/Aβ₄₂ 1.0 nM/48 h; 5: PAJU-TLN/Aβ₄₂ 1.0 nM/96 h; 6: PAJU-TLN/LY294002 30 μM/12 h; 7: PAJU-NEO/Aβ₄₂ 1.0 nM/24 h; 8: PAJU-NEO/Aβ₄₂ 1.0 nM/48 h; 9: PAJU-NEO/Aβ₄₂ 1.0 nM/96 h.

(B) The levels of TLN expression are affected by Aβ₄₂ in a time-dependent manner, but there is no time-related change in TLN expression in cells treated with LY294002. Data are expressed as absorbance ratio of TLN/GAPDH (mean ± SEM of three independent experiments). ^a $P < 0.05$, ^b $P < 0.01$, vs. untreated PAJU-TLN cells (analysis of variance followed by Dunnett's *t*-test).

No telencephalin expression was present in PAJU-NEO

cells (PAJU cells transfected with an empty expression vector). Furthermore, we found that telencephalin expression was affected by the PI3K inhibitor LY294002 as in a study from Maruya *et al*^[14]. Telencephalin expression was significantly lower in PAJU-telencephalin cells treated with LY294002 (30 μ M) for 12 hours, compared with untreated cells ($P < 0.05$; Figure 1).

Telencephalin promoted the phosphorylation of ERM in amyloid beta protein 42-treated PAJU- telencephalin cells (Figure 2)



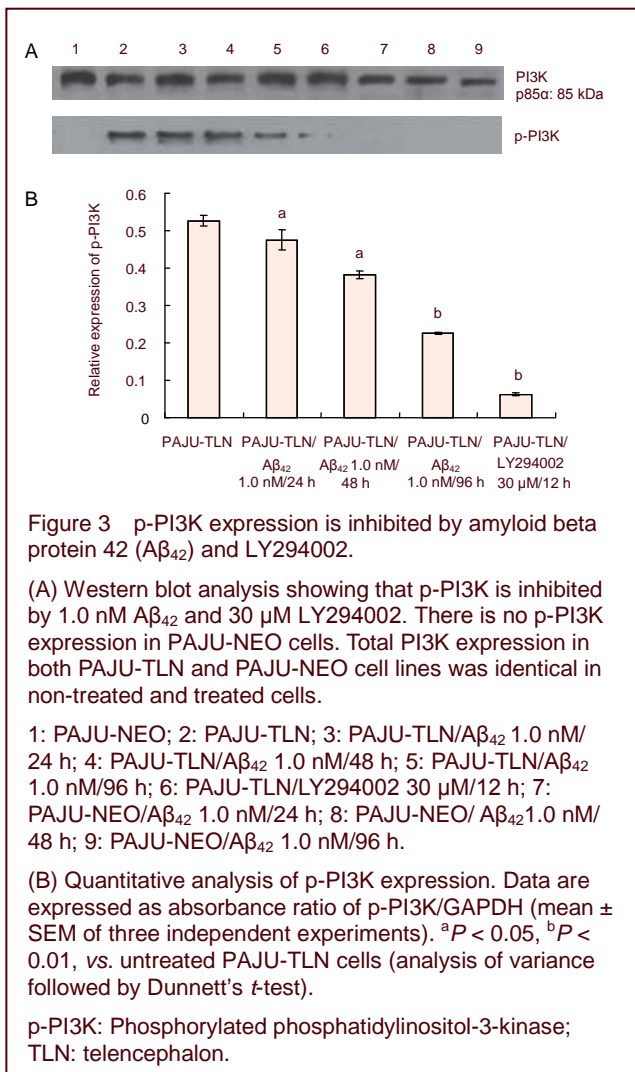
Because the apoptosis of PAJU cells induced by amyloid beta protein 42 was partially inhibited by telencephalin, we sought to clarify the underlying signal transduction events. ERM proteins are specific intracellular binding partners for telencephalin, and the telencephalin cytoplasmic region binds to ERM proteins. In cells expressing telencephalin and constitutively active ezrin, phospho-ERM forms a complex with F-actin and the cytoplasmic region of telencephalin^[8]. Phosphorylation of

ERM proteins at specific domains may be regulated by different kinases and is necessary for signaling, so we investigated whether telencephalin is necessary for ERM phosphorylation. We used the phospho-specific antibody anti-phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558), which recognizes the Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) phosphorylation site of ERM proteins. Western blot analysis showed that in untreated PAJU-telencephalin cells, there was phosphorylation at Moesin (Thr558). In PAJU-telencephalin cells treated with amyloid beta protein 42 for 24 or 48 hours, there was a marked increase in phosphorylation at Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558). In comparison, there was a significant decrease in phosphorylation at Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) in cells treated with amyloid beta protein 42 for 96 hours and in PAJU-telencephalin cells treated with LY294002 for 12 hours. There was no detectable phospho-ERM in amyloid beta protein 42- treated or untreated PAJU-NEO cells. Total ERM was expressed in treated and untreated PAJU-telencephalin and PAJU-NEO cells (Figure 2).

Telencephalin promoted the phosphorylation of PI3K/Akt in amyloid beta protein 42-treated PAJU-telencephalin cells

To determine whether ERM activation is linked to PI3K and Akt activity, cells were stimulated with amyloid beta protein 42 and cell lysates were probed for PI3K and Akt activity with anti-phospho-PI3K and anti-phospho-Akt antibodies. The PI3K-Akt pathway was blocked using the PI3K inhibitor LY294002; PI3K activity is frequently required for Akt activation^[15-17]. First, we evaluated whether telencephalin has an impact on the phosphorylation of PI3K and Akt. We used the phospho-specific antibody anti-phospho-PI3K p85 α and anti-phospho-Akt1/2/3 (Ser-473), which recognizes the PI3K p85 α and Akt1/2/3 (Ser-473) phosphorylation site of PI3K and Akt. Western blot analysis revealed that in untreated PAJU-NEO cells there was no detectable phospho-PI3K or phospho-Akt. In untreated PAJU- telencephalin cells, there was phosphorylation at the PI3-Kinase p85 α and Akt1/2/3 (Ser-473) site (Figures 3, 4).

In amyloid beta protein 42-treated PAJU-telencephalin cells, phospho-PI3K and phospho-Akt were also present. In contrast, there was no detectable phospho-PI3K or phospho-Akt in amyloid beta protein 42-treated PAJU-NEO cells. Because PI3K is a critical component of survival signaling, we next tested the effect of the PI3K inhibitor LY294002 in PAJU cells. In amyloid beta protein 42 and LY294002-treated PAJU-telencephalin cells, we inhibited the upstream Akt regulator PI3K. This reduced Akt phosphorylation to undetectable levels (Figure 4).

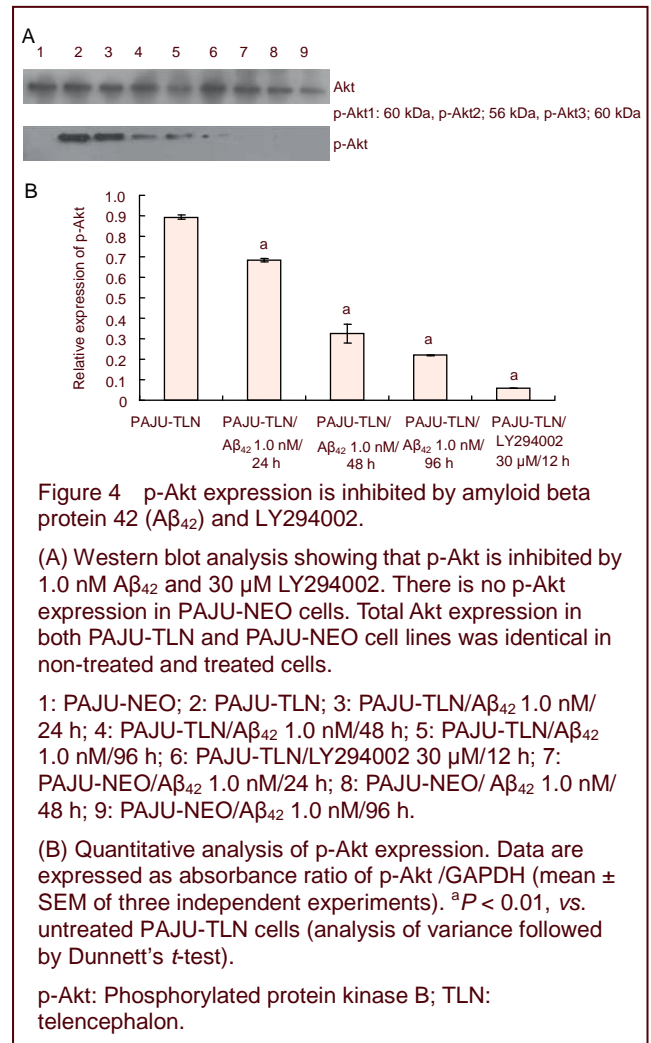


As expected, the levels of telencephalin, which can induce p-PI3K and p-Akt over-activation, were increased in untreated PAJU-telencephalin cells compared with amyloid beta protein 42-treated PAJU-telencephalin cells. There was no phosphorylation at the Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) site, or of PI3-Kinase p85 α or Akt1/2/3 (Ser-473) in untreated or treated PAJU-NEO cells. These results indicate that telencephalin activity directly parallels telencephalin expression levels. Total ERM, PI3K and Akt were expressed in both treated and untreated PAJU-telencephalin and PAJU-NEO cells.

DISCUSSION

Several studies have addressed the mechanisms underlying amyloid beta protein toxicity in different models^[18-20], but no work so far has tried to assess the role of telencephalin in the neurotoxicity caused by amyloid beta protein. Although other studies have shown that telencephalin may interact with amyloid related proteins^[21-22],

this is the first study showing that telencephalin is in part responsible for the neurotoxic effect of amyloid beta protein 42. In this study, we used the human PAJU neuroblastoma cell line to examine the role of telencephalin in amyloid beta protein 42-induced neuronal death as an *in vitro* model of Alzheimer's disease.



Telencephalin expression was inhibited by amyloid beta protein 42 and LY294002

The expression of telencephalin can protect neurons from amyloid beta protein-induced apoptosis^[12-13]. In our experiments, telencephalin expression was down-regulated after long-term exposure to amyloid beta protein 42. Since this reduction was only partial, we speculate that there are additional cell survival pathways, some of which may be inhibited by a specific inhibitor of PI3K, LY294002^[23-27], because the PI3K/Akt pathway is crucial for neuronal survival^[28].

We found that telencephalin expression was significantly reduced in LY294002-treated PAJU-telencephalin cells, compared with untreated PAJU-telencephalin cells. Similar results were obtained by Maruya *et al*^[14], although

not in neurons. These results indicate that telencephalin may be inhibited by long-term exposure to amyloid beta protein 42, as well as by LY294002. It suggests that neuronal apoptosis in Alzheimer's disease may be due to the accumulated amyloid beta protein 42 suppressing telencephalin expression.

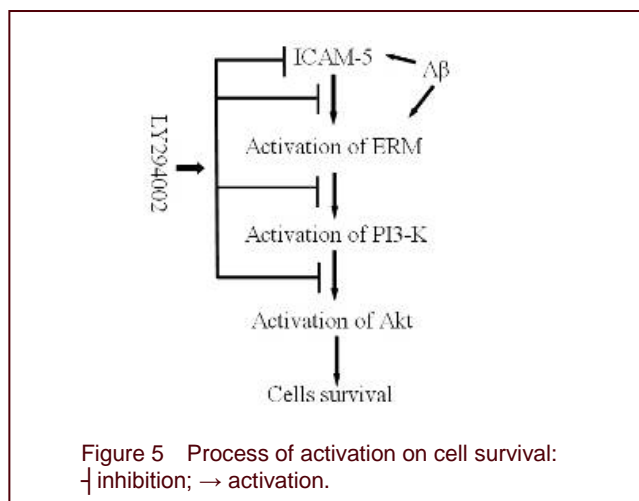
Telencephalin phosphorylated ERM proteins in PAJU cells

ERM family proteins are primarily located in the cytoplasm as inactive forms. The inactive state is maintained by intermolecular interactions between the N and C-terminal domains, which masks a C-terminal F-actin binding site. The functional activation of ERM proteins is triggered by two factors: binding of phosphatidylinositol 4,5-bisphosphate to the N-terminus and phosphorylation of a C-terminal threonine residue^[29], which is conserved in ezrin (T567), radixin (T564) and moesin (T558)^[24, 29-32]. Phosphorylated active forms of ERM proteins (phospho-ERMs) bind to membrane proteins through the N-terminal FERM domain and to the actin cytoskeleton through the C-terminal domain. In cultured hippocampal neurons, phosphorylated active forms of ERM proteins are colocalized with telencephalin in dendritic filopodia, whereas α -actinin, another binding partner of telencephalin, is co-localized with telencephalin at surface membranes of soma and dendritic shafts. Expression of constitutively active ezrin induces dendritic filopodia formation, whereas small interference RNA-mediated knockdown of ERM proteins decreases filopodia density and accelerates spine maturation^[8]. When telencephalin was ectopically expressed in N2a cells, cellular morphology dramatically changed. While control cells were small and round, telencephalin-expressing cells had a spreading morphology and displayed multiple filopodia-like protrusions. The protrusions contained phospho-ERM together with telencephalin and F-actin^[8]. When we examined whether telencephalin affects phosphorylation at the Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) site, we found moderate phosphorylation of moesin at Thr-558 in untreated PAJU-telencephalin cells, while complete phosphorylation of ERM proteins at Thr residues was observed when the cells were treated with amyloid beta protein 42. Furthermore, when telencephalin expression was markedly inhibited by 30 μ M LY294002 in PAJU-telencephalin cells^[14], expression of phospho-ERM proteins was also dramatically down-regulated. No telencephalin or phospho-ERM expression was observed in untreated PAJU-NEO cells or in those treated with amyloid beta protein 42 peptides. These results are in accordance with those of Maruya *et al*^[14] and Furutani *et al*^[8].

Moesin is an ERM family protein that is expressed ubiquitously. The major proposed function of moesin is the linking of the membrane to the actin cytoskeleton^[9]. When moesin is phosphorylated at Thr-558, the intramolecular interaction between the N and C-terminus of moesin is disrupted. Subsequently, the N-terminal FERM domain of moesin is exposed, which is then able to interact with the C-terminal tail of membrane proteins, such as telencephalin, and the actin cytoskeleton. ERM proteins are mostly present in the cytoplasm in an inactive form and only become activated during phosphorylation of a Thr residue near the C-terminus^[33-34]. Based on these observations, we conclude that telencephalin is a regulator of ERM protein phosphorylation in neuronal cells exposed to 1.0 nM amyloid beta protein 42.

Telencephalin phosphorylated ERM proteins to trigger PI3K/Akt activation

Members of the ERM family proteins link integral plasma membrane proteins with the underlying actin cytoskeleton and participate in signal transduction pathways. ERM proteins associate with plasma membrane components upon phosphorylation of a C-terminal threonine residue^[29]. When cells are subjected to apoptotic stress, phosphorylated ERM physically associates with the cytoskeleton-enriched fraction, resulting in activation of the pro-survival kinase, PI3K/Akt^[10, 23-24]. Telencephalin interacts with ERM family proteins and phosphorylates Ezrin (Thr567), Radixin (Thr564) and Moesin (Thr558). Thus, we next investigated phosphorylation of the p85 α -containing PI3K and downstream phosphorylation of Akt1/2/3 (Ser-473) in PAJU-telencephalin and PAJU-NEO cells. The results showed that telencephalin not only coexists with phosphorylated PI3K (Tyr508, p85 α) and its downstream effector Akt (Ser473) in untreated PAJU-telencephalin cells, but also in PAJU-telencephalin cells treated with the amyloid beta protein 42 peptides. There was no detectable phospho-ERM, phospho-PI3K or phospho-Akt proteins in untreated PAJU-NEO cells or in those treated with the amyloid beta protein 42 peptides. We observed that the selective PI3K inhibitor LY294002 (30 μ M) markedly inhibited not only telencephalin, but also phospho-ERM, phospho-PI3K and phospho-Akt in PAJU-telencephalin cells, while the total ERM, PI3K and Akt levels in both PAJU-telencephalin and PAJU-NEO cell lines were identical in untreated and treated cells. These data suggest that phosphorylated ERM proteins recruit PI3K and Akt to regulate cell survival, which is in accordance with the study of Wu *et al*^[24]. These observations establish that telencephalin and ERM proteins modulate Akt activity (Figure 5).



In the inactive state, ERM proteins reside within the cytosol in a closed conformation through head-to-tail interactions between the amino and carboxyl-terminal domains. ERM proteins directly bind to PI3K^[9, 23] and become activated following phosphorylation at conserved carboxyl-terminal Thr residues and interaction with negatively charged phosphatidylinositol 4,5-bisphosphate, which causes protein unfolding and targeting to the plasma membrane^[9, 35]. Multiple stimuli can activate PI3K, which then phosphorylates numerous substrates, thereby regulating cell survival, cytoskeletal rearrangements and transformation^[36]. PI3K catalyzes phosphorylation of phosphatidylinositol 4,5-bisphosphate to PIP3, which serves as a docking site for pleckstrin homology domain proteins, including Akt. Activated Akt facilitates cell growth, cell cycle entry and cell migration, but the best described Akt function is mediation of cell survival, through phosphoregulation of multiple apoptosis-related proteins^[37-38]. Our results indicate that cell survival signaling is initiated by interactions between telencephalin and ERM proteins, but further investigation is needed to understand the detailed mechanisms of this process.

Introduction of exogenous human telencephalin in PAJU cells restores PI3K signaling, suppresses apoptosis and prevents neuronal death. Importantly, these telencephalin effects are blocked by PI3K inhibitors, showing that the effects of telencephalin on the phosphorylation of ERM, PI3K and Akt are due to telencephalin affecting ERM activity. Furthermore, our data suggest that the survival effects of telencephalin are mediated by the ERM/PI3K/Akt cell signaling pathway. Although a contribution of other pathways cannot be excluded, our data show that telencephalin/ERM/PI3K/Akt signaling is both necessary and sufficient to suppress apoptosis and prevent neuronal degeneration. Previous studies have shown that telencephalin is not a γ -secretase sub-

strate^[22], and that PI3K/Akt signaling is independent of γ -secretase activity^[39-40]. Overexpression or microinjection of constitutively active PI3K/Akt promotes neurites outgrowth and neuronal cell survival^[41-43], while the PI3K/Akt signal blocker LY294002 inhibits neurite outgrowth and induces neuronal apoptosis^[44-45]. PI3K activation and PIP3 production are critical for neuronal cell survival and development, neurite formation and elongation, and axon specification^[46-48]. This effect has been demonstrated in several neuronal cell types, including hippocampal neurons^[46, 49-50], cerebellar granule neurons^[50] and PC12 cells^[51]. The inhibition of apoptosis appears to involve suppression of caspase-3^[15]. Mature primary PS1^{-/-} neurons display impaired PI3K/Akt signaling and increased activation of proapoptotic caspase-3 followed by neurodegenerative changes indicated by extensive dendritic retraction and increased pyknotic or fragmented nuclei. These observations support a critical role of PI3K/Akt signaling in suppressing neuronal apoptosis^[52] and dendritic retraction^[53].

In this study, telencephalin-associated Akt activity and cell survival were suppressed by LY294002, an inhibitor of PI3K, an upstream regulator of Akt. The present observations of amyloid beta protein 42-treated PAJU-telencephalin cells raise the possibility that telencephalin-ERM-independent PI3K signaling pathways may upregulate neuronal cell phenotype by promoting neurite and axonal formation, and by reducing apoptosis. This is consistent with other studies^[54-56] showing that telencephalin promotes neurite outgrowth. It has been reported that the C-terminal 17-amino acid motif of telencephalin serves solely as a dendritic targeting signal in neurons^[57], but it may also signal survival in PAJU cells. The transmembrane domain of telencephalin appears to be required for binding to presenilin 1. Since the presenilins are at the crossroads of several important signaling pathways^[58], telencephalin may regulate multiple signaling cascades. Further study is required to clarify the role of telencephalin in other signaling pathways. However, it is clear that the telencephalin/ERM/PI3K/Akt signaling pathway provides novel and potentially important targets for drug development in the fight against Alzheimer's disease.

MATERIALS AND METHODS

Design

A comparative *in vitro* cytology study.

Time and setting

Experiments were performed in the Genetic Laboratory, Xiangya Second Hospital, Central South University,

China, in 2009.

Materials

The neuroblastoma cell line PAJU is a human neural crest-derived cell line^[59] that express no telencephalin. PAJU-telencephalins (Division of Biochemistry, Faculty of Biosciences, University of Helsinki, Finland) are PAJU cells transfected with the complete human telencephalin cDNA. PAJU cells used in our study were obtained by successive passage of PAJU-telencephalin cells without G418. Identity was confirmed by immunofluorescence and western blot (data not shown). The control cells, PAJUNEO, were transfected with the empty pcDNA3.1 vector. Stable transfections were performed with the LipofectamineTM 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA), and expression was analyzed by Colony-forming test.

Methods

Cell culture

The neuroblastoma cell line PAJU was cultured in Dulbecco's modified Eagles medium (Invitrogen) containing 10% fetal calf serum, 1% penicillin-streptomycin, 1% glutamine and 1 mM HEPES. PAJU-telencephalin and PAJU-NEO cell lines were cultured in the above medium containing 0.5 mg/mL G418 (Invitrogen) as instructed by Professor Carl G. Gahmberg.

Peptide preparation and peptide/LY294002 treatment

Amyloid beta protein 42 peptide corresponding to the human amyloid beta protein wild type sequence was reconstituted according to the manufacturers' instructions (Peptide Company, Osaka, Japan), and aged in 0.1% NH₄OH/PBS, in a stock concentration of 1 mM, for 7 days at 37°C^[60]. The cells were grown to confluency at 37°C for 3 days of differentiation in DMEM containing phenol red in 6-well plates at a density of 2 × 10⁶ cells/cm², in a humidified atmosphere containing 5% CO₂. Amyloid beta protein 42 was added into the culture medium, at a final concentration of 1 nM, on day 4 after culture for different time periods, ranging from 24 to 96 hours^[60]. The original supernatant solution was removed from the cells with a pipette, and the new medium (3 mL, 37°C, containing aggregated amyloid beta protein 42 alone or without the peptides) was added rapidly (within 3 seconds) to the wells at 37°C. Sedimented amyloid beta protein clusters remained cell-bound after washing of the cells. As for amyloid beta protein 42 treatment, 30 μM LY294002 (Alexis Company, New York, NY, USA) was added to the cells for 12 hours.

Western blot assay

For the preparation of total cell extracts, untreated and peptide-treated cells were scraped in 400 μL of ice-cold lysis buffer containing (in mM): Tris-HCl (pH 8.0) 50, NaCl 80, CaCl₂ 2^[6], EDTA 1, NaF 20, Na₃VO₄ 1 and β-glycerophosphate 5, supplemented with 100 μM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, protease inhibitor cocktail (containing 1 μg/mL leupeptin, pepstatin A, chymostatin and antipain) and 0.2% NP-40 (all from Sigma, St. Louis, MO, USA). Cell lysates were frozen three times in liquid N₂ and centrifuged at 14 000 × g to remove nuclei and large debris. Protein concentration in the supernatant was measured using the Bio-Rad protein dye assay reagent. 15-μL aliquots of samples were denatured at 95°C for 3 minutes in sample buffer containing 500 mM Tris, 600 mM dithiothreitol, 10% sodium dodecyl sulfate, 30% glycerol and 0.012% bromophenol blue. Equal amounts of each sample were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel and electro-blotted onto nitrocellulose membranes. The identification of proteins of interest was facilitated by the usage of a pre-stained precision protein standard, which was run simultaneously. The proteins in gel were electrophoretically transferred to membranes that were incubated for 1 hour at room temperature in Tris-buffered saline (pH 7.6) containing 5% nonfat dry milk and 3% bovine serum albumin to block nonspecific binding. The components of Tris-buffered saline included 25 mM Tris base, 137 mM NaCl, 3 mM KCl and 0.1% Tween-20 according to Cell Signaling Technology Western Immunoblotting Protocol (Beverly, MA, USA). The membranes were washed three times with Tris-buffered saline. Then the membranes were incubated with the primary antibodies overnight at 4°C in Tris-buffered saline containing 5% nonfat dry milk and 3% bovine serum albumin. The primary antibodies used were: (i) monoclonal mouse anti-human telencephalin (1:500; R&D Systems, Shanghai, China); (ii) goat polyclonal anti-PI3-Kinase p85α (S-20) and antiphospho-PI3-Kinase p85α (Tyr508) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); (iii) rabbit polyclonal anti-Akt1/2/3 (H-136) and antiphospho-Akt1/2/3 (Ser-473) (1:500; Santa Cruz Biotechnology); (iv) rabbit polyclonal anti-Ezrin/Radixin/Moesin and antiphospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (1:500; Cell Signaling Technology); and (v) mouse polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3 000; Santa Cruz Biotechnology). After this incubation, the membranes were washed and incubated in Tris-buffered saline with 5% nonfat dry milk for 2 hours at room temperature, followed by incubation with anti-rabbit, anti-goat or anti-mouse horseradish peroxi-

dase-conjugated secondary antibody (1:2 500 or 1:2 000; Cell Signaling Technology) for 24 hours at 4°C. Immunoreactive bands were detected after incubation of membranes with enhanced chemiluminescence reagent for 1 minute at room temperature as described by the manufacturer. Then, membranes were exposed to film (Kodak, Rochester, New York, USA), developed and fixed. The absorbance of the scanned bands was determined using Image J (National Institutes of Health, Rockville, Maryland, USA). The specific positive value was calculated by subtracting the nitrocellulose membrane absorbance from the band absorbance and dividing by the GAPDH absorbance.

Statistical analysis

Results are expressed as mean \pm SEM. Statistical analysis was performed using SPSS 9.0 software (SPSS, Chicago, IL, USA). The differences were compared with analysis of variance followed by Dunnett's *t*-test. Differences with *P* < 0.05 were considered statistically significant.

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Author contributions: Heping Yang was responsible for study concept, implemented the experiments, performed data analysis and drafted the manuscript. All authors participated in the experiments, contributed to the interpretation of the analyses and article writing.

Conflicts of interest: None declared.

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