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Caspr interaction with Amyloid Precursor Protein reduces amyloid-β generation in vitro

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HIGHLIGHTS

- Caspr is abnormally expressed in the cerebral cortex of APP/PS1 mice.
- Caspr overexpression reduces level of APP, while it does not alter APP mRNA.
- Caspr interacts with APP.
- Caspr decreases Aβ40 and Aβ42 generation.

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ABSTRACT

Contactin associated protein (Caspr), an adhesion molecule, plays roles in formation of paranodal junctions in myelinated axons, neurite outgrowth, synaptic plasticity in nervous system. Here we have shown a novel function of Caspr in pathogenesis of Alzheimer's disease (AD). Caspr distributes around amyloid plaques in APP/PS1 mice. Levels of Caspr increase in the cerebral cortex of 7-month-old APP/PS1 mice comparing to wild-type littermates. Caspr decreased protein levels of APP in both HEK-293 cells stably transfected with Indiana mutant APP (V717F; HEK-APP) and CHO cells which express endogenous APP, while it did not alter mRNA levels of APP. Furthermore, Caspr co-localizes and interacts with APP. Amyloid-β (Aβ) 40 and Aβ42 generation were also reduced in HEK-APP cells by Caspr overexpression.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that results in loss of memory and cognitive function. Amyloid plaques and neurofibrillar tangles are two pathological makers of AD. Amyloid plaques are formed by aggregation of amyloid-β (Aβ) peptides [12]. Aβ induces neurotoxicity through induction of apoptosis and inflammation, disruption of calcium homeostasis, oxidative stress and activation of complement [12]. Recent reports indicate that oligomeric Aβ reduces density of spines and suppresses Long-Term Potentiation (LTP) [21]. Therefore, reduction of Aβ generation or Aβ-induced cell toxicity is considered as one of the prime strategies of AD therapy [12]. Aβ is generated from the proteolytic cleavage of Amyloid Precursor Protein (APP). APP is cleaved by β-secretase at its extracellular domain and generates a soluble extracellular fragment called sAPPβ and a transmembrane fragment CTFβ (C-terminal fragment). CTFβ is further cleaved by γ-secretase and releases Aβ [12]. The cleavage of APP by β- and γ-secretase is termed the amyloidgenic processing pathway. Proteolytic cleavage by α- and γ-secretase precludes Aβ generation and so is known as the non-amyloidogenic processing pathway [3]. Therefore, identification of proteins which modulate cleavage or expression of APP may provide potential drug targets for AD therapy.

Contactin associated protein (Caspr) is a transmembrane protein with a large extracellular domain that contains a series of laminin-G-like domains and EGF repeats and a short intracellular domain [19]. Caspr is well known for its function in the formation of axoglial paranodal junctions surrounding the nodes of Ranvier in myelinated axons through interaction with F3/Contactin and neurofascin 155 [20]. Caspr interacts with Nogo-A at the paranodes in myelinated axons [17]. Caspr also promotes neurite outgrowth by binding to Prion protein (PrP) [4]. Caspr interacts with

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AMPA receptors and regulates the trafficking of AMPA receptors to synapses [22]. Caspr was recently identified in a γ-secretase associated complex, suggesting its possible function in AD pathology [11]. This report demonstrates that Caspr distributes surrounding amyloid plaques in the cortex of APPswe/PSEN-1 (Δ9) double transgenic mice (APP/PS1). Levels of Caspr in the cerebral cortex of 7-month-old APP/PS1 mice increase when comparing compared to wild-type littermates. Co-immunoprecipitation shows that Caspr interacts with APP. Furthermore, over expression of Caspr in HEK-293 cells stably transfected with Indiana mutant APP (V717F; HEK-APP) results in a significant reduction of APP levels compared to the control cells. However, over expression of Caspr in HEK-293 cells does not affect levels of APP mRNA. Moreover, the amounts of Aβ40 and Aβ42 in HEK-APP are reduced significantly by Caspr over expression. Thus, Caspr may be a novel regulator of Aβ generation.

2. Materials and methods

2.1. Plasmids and mice

PCMV-Caspr-myc plasmid was purchased from Origene. pcDNA-Caspr4 and PCMV-Tead2 plasmids were purchased from Shanghai NiuEn Biotech., Ltd. APP/PS1 mice were obtained from the Jackson Laboratory (stock number 004462).

2.2. Antibodies

Rabbit anti-Caspr antibody was generously provided by Prof. Melitta Schachner. MAB348 (Millipore), A8717 (Sigma), 6E10 (Covance), anti-γ-tubulin (Sigma), anti-β-actin (Sigma).

2.3. Immunofluorescence staining

Immunofluorescence staining was performed as described previously [15].

2.4. Immunoprecipitation

Mouse brains were lysed with RIPA buffer (50 mM Tris–HCl, PH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors and phosphorylation inhibitors. Brain lysates was incubated with antibodies together with protein A-coupled sepharose. The immunocomplexes were washed with PBS containing 0.5% NP-40, and re-suspended and boiled in 2× Laemmli sample buffer. The supernatant was subjected to immunoblotting analysis.

2.5. Aβ ELISA analysis

Aβ ELISA assays were performed using human Aβ40/Aβ42 ELISA kits according to the instructions of the manufacturer (Invitrogen).

2.6. Cell viability determination

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [25].

2.7. Quantitative real-time PCR

Quantitative RT-PCR (qPCR) was performed using FastStart Universal SYBR Green Master (Roche) in combination with target-specific primers for human Caspr (5′-CCCTGAAGCCATTTGTAGTGT-3′, 5′-GGAGCAGAGGTCCTGAAGTA-3′), human APP (5′-GAGGAGGATGACTCGGATGTCT-3′, 5′-AGCCACTTCTTCCTCCTCTGCT-3′), and human GAPDH (5′-CAAGGTCATCCATGACAACTTTG-3′, 5′-GTCCACCACCCTGTTGCTGTAG-3′). APP mRNA level was calculated relative to GAPDH using the delta-delta computed tomography method.

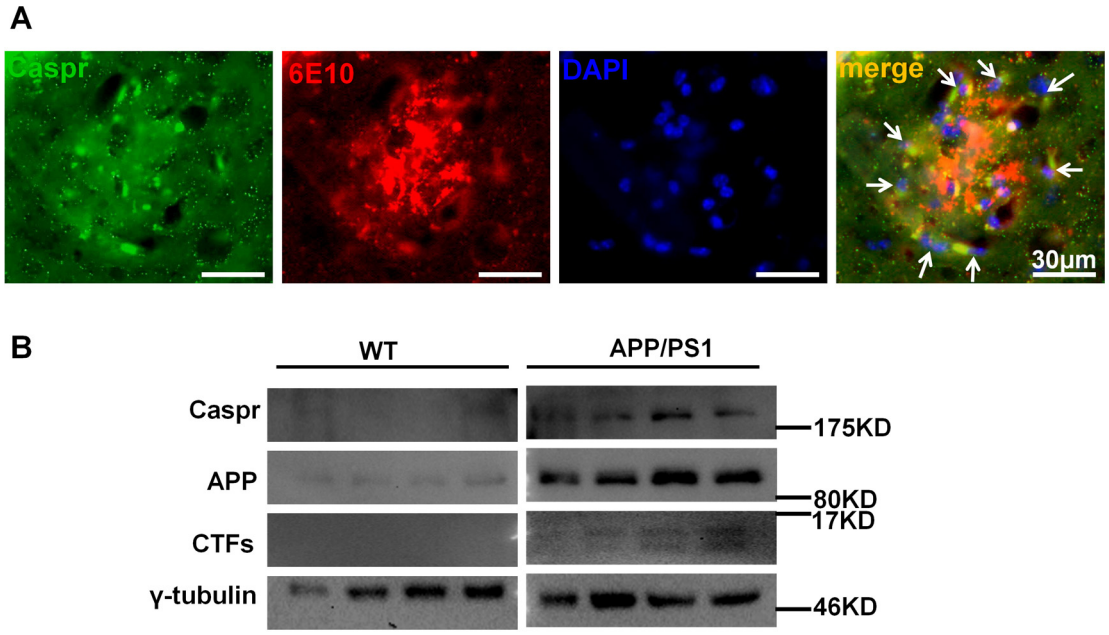

Fig. 1. Abnormal expression of Caspr in the cerebral cortex of APP/PS1 mice. (A) Immunofluorescence staining with antibodies against Caspr (Green) and Aβ (6E10, red) in the coronal cerebral cortex of 7-month-old APP/PS1 mice. Scale bar: 30 μm. (B) Immunoblotting analysis of Caspr expression in the cerebral cortex of 7-month-old APP/PS1 and wild-type littermates. γ-Tubulin was probed as the loading control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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2.8. Statistical analysis

The data was analyzed using the Statistical Product and Service Solution program (SPSS). An unpaired independent Student's *t*-test was used to compare two independent samples. All data were presented as mean ± S.E.M. All sample numbers were more than 5 ($n > 5$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS: non-significant.

3. Results

3.1. Caspr is abnormally expressed in the cerebral cortex of APP/PS1 mice

We first examined the localization and expression of Caspr in the brains of APP/PS1 mice, which begin to have Aβ plaques as early

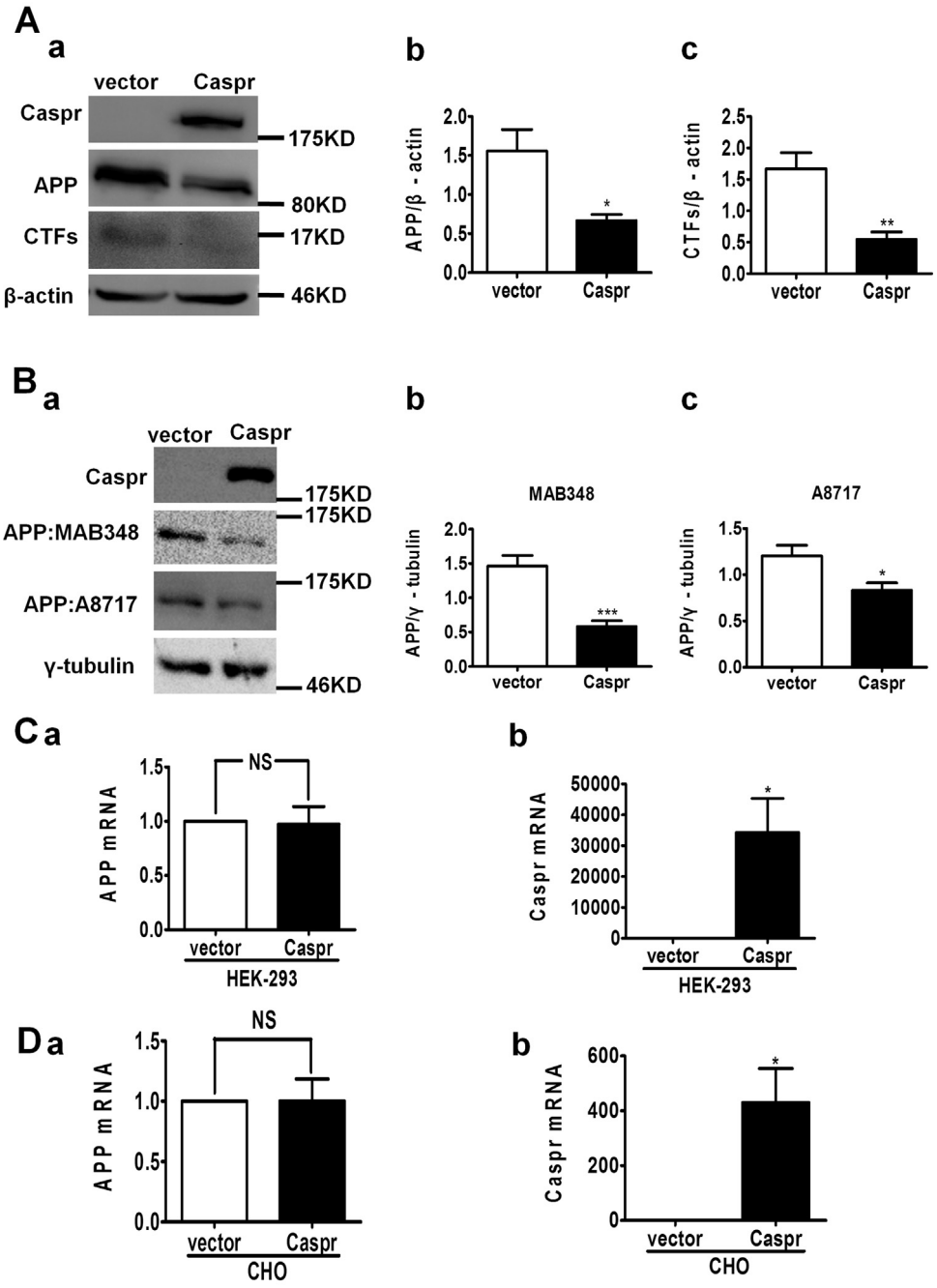

Fig. 2. Caspr overexpression reduces level of APP, while it has no effect on level of APP mRNA. (A) HEK-APP cells were transfected with either PCMV-Caspr-myc or the vector. Cell lysates were collected at 48 h after transfection and were subjected to immunoblotting analysis. Caspr antibody was used to detect Caspr (~180 kDa). APP antibody recognizing the N-terminus of APP (MAB348) was used to detect APP (~100 kDa) and an antibody recognizing the C-terminus of APP (A8717) was used to detect CTFs (~10 kDa). β-Actin was probed as the loading control (a). Densitometric analysis of immunoblots for levels of APP (b) and CTFs (c). (B) Overexpression of Caspr reduces endogenous APP expression in CHO cells. Cell lysates were collected at 48 h after transfection and were subjected to immunoblotting analysis. Level of APP was analyzed with two anti-APP antibodies (A8717, MAB348). Probing for γ-tubulin was used to verify equal loading (a). Densitometric analysis of immunoblots for APP expression detected by MAB348 (b) and A8717 (c). (C and D) Caspr overexpression does not affect APP mRNA. HEK-293 cells (C) or CHO cells (D) were transfected with either PCMV-Caspr-myc or the vector. mRNA was extracted at 48 h after transfection and subjected to qPCR with specific primers for APP and Caspr. GAPDH was detected as the internal control. Relative mRNA level of APP or Caspr in either vector-transfected HEK-293 cells (C) or vector-transfected CHO cells (D) was normalized to 1. Quantification of relative mRNA levels of APP (Ca, Da) and Caspr (Cb, Db).

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as 2.5 months of age and have a high Aβ load in the hippocampus and the cerebral cortex from 6 months of age [1,23]. We performed double immunofluorescence staining with antibodies against Caspr [4] and Aβ (6E10) in the cerebral cortex of 7-month-old APP/PS1 mice. Caspr-immunoreactivity distributed in a clustering pattern around Aβ plaques (Fig. 1A). Levels of Caspr in the cerebral cortex of 7-month-old APP/PS1 were analyzed by immunoblotting analysis. Consistent with a previous report [14], APP and CTFs (the fragments of APP cleaved by α/β-secretase) levels were higher in APP/PS1 mice (Fig. 1B). Increased levels of Caspr were detected in the cerebral cortex of APP/PS1 mice compared with wild-type mice (Fig. 1B). The specificity of Caspr antibody has been described previously [4]. To further confirm the specificity of Caspr antibody, we used this antibody to stain for Caspr on longitudinal sections of rat spinal cord (Suppl. 1). Caspr immunoreactivity displayed a symmetrical distribution with a pattern like its paranodal location as described previously [5]. These results suggest that Caspr may be involved in AD pathology.

3.2. Caspr over expression reduces level of APP

We first investigated whether Caspr might modulate the expression of APP. PCMV-Caspr-myc was transfected into HEK-APP cells. The cell lysates were prepared 48 h after transfection and analyzed by immunoblotting. A band at ~100 kDa corresponding to APP and a band at ~180 kDa corresponding to Caspr were detected with anti-APP and anti-Caspr antibody respectively (Fig. 2Aa). The endogenous Caspr level in HEK-APP cells was too low to be detectable by immunoblotting analysis (Fig. 2Aa). Densitometric analysis showed that APP levels in Caspr-transfected HEK-APP cells decreased compared to vector-transfected cells (Fig. 2Aa, b). However, transfection of Caspr4 or Tead2 failed to alter APP levels in HEK-APP cells (Suppl. 4). We further investigated whether Caspr affects levels of CTFs by immunoblotting analysis with an antibody recognizing the intracellular domain of APP (A8717). Decreased levels of CTFs in Caspr-transfected HEK-APP cells were detected in comparison with vector-transfected cells (Fig. 2Aa, c).

We further examined whether Caspr affects endogenous APP levels. We transfected PCMV-Caspr-myc into CHO cells which express endogenous APP. APP levels were analyzed by immuoblotting with two APP antibodies, MAB348 and A8717, which recognize the extracellular domain and the intracellular domain of APP respectively. Immunoblotting analysis by either MAB348 or A8717 showed that APP levels in Caspr-transfected CHO cells were decreased significantly compared to vector-transfected cells (Fig. 2B). These results indicate that Caspr decreases levels of APP.

We considered whether Caspr might modulate levels of APP mRNA. Levels of APP and Caspr mRNA were analyzed with qPCR in either HEK-293 cells (Fig. 2C) or CHO cells (Fig. 2D) transfected with PCMV-Caspr-myc. Compared to vector-transfected cells, transfection of PCMV-Caspr increased Caspr mRNA, but not APP mRNA levels, in either HEK293 cells (Fig. 2C) or CHO cells (Fig. 2D). Thus, Caspr reduces levels of APP protein, while it does not alter levels of APP mRNA.

3.3. Caspr interacts with APP

Caspr associates with F3/Contactin, which shares high sequence homology with TAG1/Contactin2 [27]. The latter regulates the proteolytic cleavage of APP [15]. We therefore asked whether Caspr might interact with APP. We transfected PCMV-Caspr-myc in HEK-APP. Double immunofluorescence staining for myc and APP in

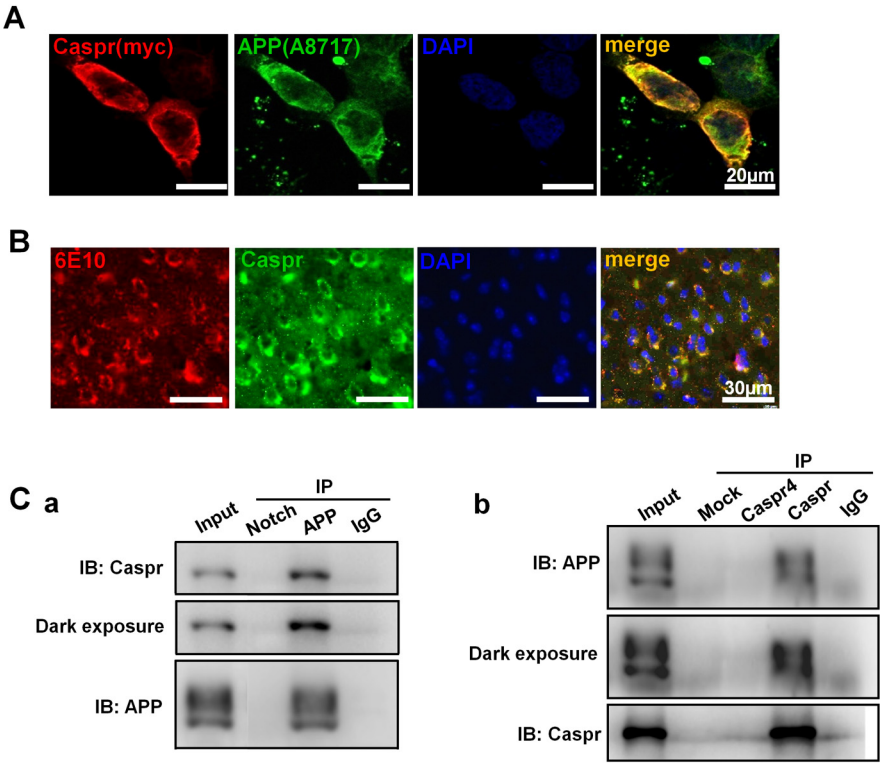

Fig. 3. Caspr interacts with APP. (A) HEK-APP cells were transfected with PCMV-Caspr-myc plasmid. Double immunofluorescence staining was performed around 24 h after transfection with antibodies against myc and APP (A8717). Scale bar: 20 μm. (B) Double immunofluorescence staining was performed with antibodies against Caspr and APP (6E10) on the coronal section of front cortex of adult C57/6L mouse. Scale bar: 30 μm. (C) Lysates prepared from C57BL/6J mouse brain were immunoprecipitated using antibodies as indicated and probed with anti-Caspr and anti-APP antibodies. A band around 250 kDa corresponding to Caspr was detected by anti-Caspr antibody. Bands around 110 kDa corresponding to APP was detected by anti-APP antibody (A8717). 1/30 brain lysates used in immunoprecipitation were subjected to western immunoblotting as the input. Mock: PBS; IgG: non-immunoglubulin; Caspr4: Contactin associated protein 4.

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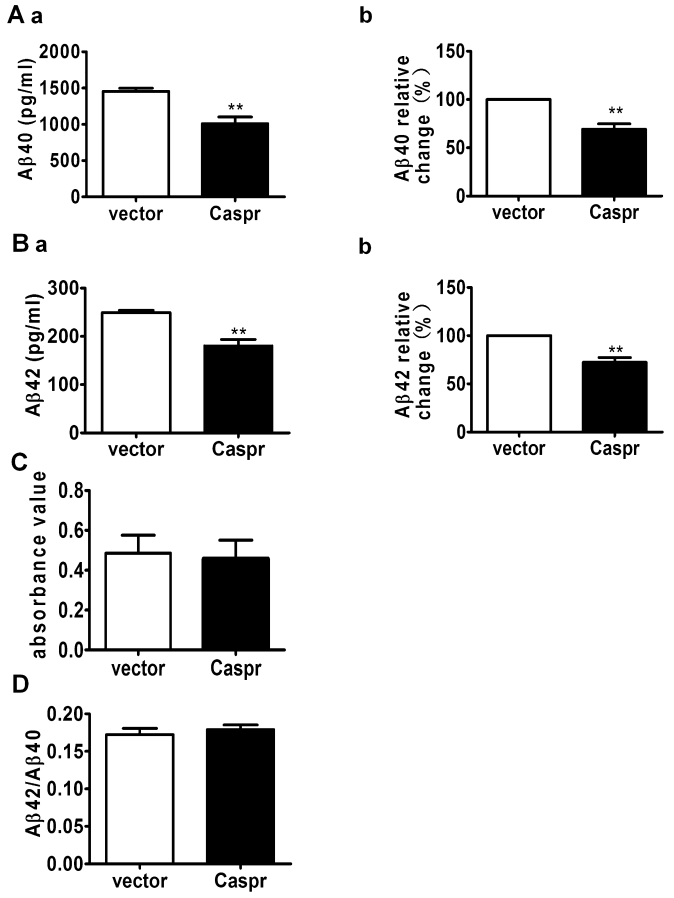

Fig. 4. Caspr Over expression decreases Aβ40 and Aβ42 generation. (A and B) HEK-APP cells were transfected with either PCMV-Caspr-myc or the vector. Conditioned medium was collected and subjected to Aβ ELISA to quantify levels of Aβ40 (Aa) and Aβ42 (Ba) at 48 h after transfection. Levels of Aβ40 (Ab) and Aβ42 (Bb) in vector-transfected cells were normalized as 100%. Quantification of relative levels of Aβ40 (Ab) and Aβ42 (Bb) in Caspr-transfected cells. (C) Caspr over expression does not affect cell viability. MTT assay was performed in Caspr-transfected and vector-transfected HEK-APP cells. (D) Caspr overexpression does not affect the ratio of Aβ42/Aβ40. The ratio of Aβ42/Aβ40 was calculated and compared between Caspr-transfected and vector-transfected HEK-APP cells.

either Caspr-transfected HEK-APP cells (Fig. 3A) or for Caspr and APP in the cerebral cortex of wild-type mice (Fig. 3B) showed that Caspr colocalized with APP. The association of endogenous Caspr and APP was further examined in mouse brain homogenates by co-immunoprecipitation. APP, but not Notch, co-immunoprecipitated with Caspr from lysates of adult mouse brain. Conversely, Caspr, but not Caspr4, co-immunoprecipitated with APP from lysates of adult mouse brain. These observations thus suggest that the two proteins interact with each other physiologically in adult mouse brains (Fig. 3C).

3.4. Caspr overexpression inhibits generation of Aβ

Proteolytic production of Aβ from APP is one of the critical steps of AD pathology. Caspr interacts with APP and reduces expression of APP. We therefore considered whether Caspr might affect generation of Aβ40 and Aβ42. Levels of Aβ40 and Aβ42 in conditioned medium of HEK-APP cells transfected with Caspr were analyzed by ELISA assay. Aβ40 (Fig. 4Aa) and Aβ42 (Fig. 4Ba) levels in conditioned medium of Caspr-transfected HEK-APP cells decreased significantly in comparison to vector-transfected cells. As Aβ40 and Aβ42 levels in vector-transfected cells were normalized as 100%, levels of Aβ40 and Aβ42 in Caspr-transfected cells were reduced to 69.72 ± 5.12% and 72.68 ± 4.64% respectively (Fig. 4Ab, Bb). Cell viability may affect Aβ40 and Aβ42 levels in conditioned medium. MTT analysis indicated that cell viability in Caspr-transfected cells was not different from vector-transfected cells (Fig. 4C). Aβ42 is easier to aggregate and more toxic than Aβ40. Furthermore, γ-secretase cleavage modulates the ratio of Aβ42/Aβ40 [26]. We thus analyzed whether Caspr overexpression modulates the ratio of Aβ42/Aβ40. No significant difference in the ratio of Aβ42/Aβ40 was detected between Caspr-transfected cells and vector-transfected cells (Fig. 4D). Therefore, Caspr overexpression suppresses Aβ42 and Aβ40 generation, while it does not change the ratio of Aβ42/Aβ40.

4. Discussion

Caspr is an adhesion molecule with a large extracellular domain and a short intracellular domain. Caspr interacts with F3/Contactin and neurofascin 155, that is required for the formation of axoglial paranodal junctions in myelinated axons [7]. Caspr interacts with Nogo-A, a molecule that has been extensively studied in the cortex of the CNS during regeneration [17]. A recent study indicates PrP as a novel binding partner of Caspr [4]. The extracellular domain of Caspr is released from the full-length receptor after binding to PrP. Through this process, Caspr inhibits neurite outgrowth of CNS neurons [4]. Interestingly, both Nogo-A and PrP reduce Aβ generation by preventing the access of BACE1 to APP [10]. PrP binds to the pro-domain of BACE1, the rate-limiting step in this amyloidogenic processing of APP, and modulates BACE1 cleavage of APP [9]. Moreover, PrP plays essential roles in Aβ-induced toxicity [6,13,18,24]. These results indicate a possibility that Caspr may be involved in AD pathology. We show here that Caspr distributes around amyloid plaques and that Caspr levels increase in the cerebral cortex of 7-month-old APP/PS1 mice. These results provide further evidences that Capsr may play roles in AD pathology. However, the distribution and expression of Caspr needs to be further studied in the brains of AD patients.

We have found that Caspr reduces protein level of APP, while it does not alter mRNA level of APP. These results indicate that Caspr decreases level of APP through a post-transcriptional mechanism. There are several possible mechanisms underlying this: (1) Caspr decreases the translation or the stability of APP; (2) Caspr increases proteolytic cleavage of APP, thus it decreases levels of full-length APP. However, levels of CTFs have been decreased by over expression of Caspr. It is possible that Caspr decreases levels of full length APP by enhancing proteolytic cleavage of APP. Therefore, we propose a possible function of Caspr in modulating either the translation or the stability of APP, and therefore reducing generation of Aβ. However, further studies should be performed to examine these possibilities.

Caspr has been identified as a novel γ-secretase-associated protein in detergent-resistant membranes [11]. Downregulation of Caspr in HEK-293 cells stably expressing wild-type APP with siRNA reduces generation of Aβ40 and Aβ42 [11]. However, our results indicate that Caspr decreases generation of Aβ40 and Aβ42. The difference in our experiments is that we have used HEK-293 cells stably expressing the Indiana mutant APP (V717F) [16]. Mutations in APP may affect the cleavage of APP. For example, mutations at codons 716 (I716V) and 717 (V717I and V717L) predominantly affect Aβ42 generation by γ-secretase cleavage. While mutations at codons 714 (T714I) and 715 (V715A and V715M) primarily affect generation of Aβ40 by γ-secretase cleavage [2]. PrP inhibits cleavage of wild-type APP by BACE1, while it exerts no control on cleavage of Swedish mutant APP [8]. Downregulation of Caspr

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expression on AD transgenic mice which express different mutant APP proteins may help to address this question.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2013.05.055.

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