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Up-regulation of calsyntenin-3 by β-amyloid increases vulnerability of cortical neurons

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ABSTRACT

β-Amyloid (Aβ) may play an important role in the pathogenesis of Alzheimer's disease. However, a causal relationship between Aβ oligomers and layer-specific neurodegeneration has not been clarified. Here we show up-regulation of calsyntenin (Cst)-3 in cultured neurons treated with Aβ oligomers and in Tg2576 mice. Cst-3 is distributed in large neurons in layers 2–3 and 5 of the cerebral cortex, and accumulated in dystrophic neurites surrounding Aβ-plaques. Overexpression of Cst-3 accelerates neuronal death. These results indicate that up-regulation of Cst-3 in cortical neurons in layers 2–3 and 5 by Aβ oligomers may lead to increase in vulnerability of neurons.

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1. Introduction

Alzheimer's disease (AD) is characterized by the accumulation of β-amyloid (Aβ) plaques and neurofibrillary tangles, and neuronal loss in the neocortex. Genetic, neuropathological, and biological evidence indicates that Aβ plays an important role in the early pathogenesis of AD: cognitive impairment caused by synaptic dysfunction [1–3]. Despite a poor correlation between Aβ-plaque density and cognitive decline in very mild AD patients, the amount of soluble Aβ oligomer in neocortex correlates well with cognitive decline or synaptic loss in humans and transgenic mice expressing mutant human amyloid precursor protein (APP) [4–8]. However, the molecular mechanisms of synaptic dysfunction by soluble Aβ oligomers remain to be fully elucidated. The identification of Aβ-induced genes that mediate synaptic dysfunction would provide considerable insight into the mechanisms of AD.

Calsyntenins (Cst, also termed alcadeins), type-1 transmembrane proteins of the cadherin superfamily, are found in the synaptic membrane in the adult brain [9]. Three Csts, Cst-1, Cst-2, and Cst-3, identified in human, mouse and rat [10,11], have the WDDS motif, which binds to the light chain of kinesin-1. In the past few years, biological evidence restricted to Cst-1 has been accumulated; Cst-1 acts as a cargo-docking protein for kinesin-1-mediated vesicular transport [12,13]. CASY-1, an ortholog of Csts in *Caenorhabditis elegans*, plays an important role in learning [14,15]. The expression of the Cst-1 gene is reduced in amyloid-rich areas in APP + PS1 Tg mouse brain [16]. In contrast, little is known about the regulation and the function of other Csts, especially Cst-3, except their distribution in cerebral cortex [11].

To address these issues, we examined the expression of Cst genes in Aβ-treated neuronal culture, and in the cerebral cortex from an AD mouse model (APP_{SW}, Tg2576). We also examined whether altered gene expression causes enhanced vulnerability of neurons to stimuli leading to neurodegeneration.

2. Materials and methods

2.1. Neuronal cell culture

Cerebral cortices dissected from day E17 embryonic rats were dissociated by incubation with 0.08% trypsin/0.008% DNase I at 37 °C for 10 min, and passed through a 62-μm nylon mesh. The cells (10^5 cells/dish for immunofluorescence or 4.5×10^6 cells/dish for preparation of RNA or protein) were seeded in gelatin–polyornithine-coated dishes with 3.5- or 6-cm diameter, respectively, and were cultured in MEM–5% fetal bovine serum–10 μM β-mercaptoethanol.

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; Cst, calsyntenin; MEN-N2, minimum essential medium with N2 supplement.

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2.2. Treatment with Aβ

Peptides Aβ1-42 and Aβ42-1 (Bachem Inc.) were treated with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma), and then dried under vacuum in a SpeedVac. Freshly prepared Aβ1-42 or Aβ42-1 was prepared by dissolving the HFIP-treated peptides at 250 μM in 0.05 N HCl, filtering through a 0.45-μm membrane filter, and diluting to 5 μM with minimum essential medium with N2 supplement (MEM-N2). The peptide solution (2 or 5 μM) was added to the 7 DIV cultures immediately after preparation. Aβ1-42 oligomers and fibrils were prepared according to the methods of Stine et al. [17]. Briefly, Aβ1-42 oligomers were prepared by diluting 5 mM Aβ1-42 in DMSO to 100 μM in ice-cold Ham's F12, and incubating at 4 °C for 4 days. Aβ1-42 fibrils were prepared by diluting 5 mM Aβ1-42 in DMSO to 100 μM in 10 mM HCl, and incubating at 37 °C for 4 days. The peptide solution (2 μM) was added to the 7 DIV cultures.

2.3. Mice

Heterozygous Tg2576 (APP_{SW}) mice and wild-type littermates were purchased from Taconic Farms Inc. Mice were killed by anesthesia overdose and perfused transcardially with saline followed by ice-cold phosphate-buffered 4% paraformaldehyde. After post-fixation in phosphate-buffered 4% paraformaldehyde for 24 h at 4 °C and cryoprotection with 20% sucrose, brains were sectioned coronally at 10 μm through the entire hippocampus on a cryostat.

2.4. Suppressive subtractive hybridization

Suppressive subtractive hybridization screening was performed according to the manufacturer's instructions (Clontech). Briefly, poly-$(A)^+$RNA from cultured cortical neurons treated with 5 μM Aβ1-42 for 15 h was used as a "tester" and poly-$(A)^+$RNA from cultured cortical neurons treated with MEM-N2 was used as a "driver". From this screening, we identified a partial cDNA sequence (590 bp) corresponding to rat Cst-3 (accession number AJ431642).

2.5. Northern blot analysis

Poly-$(A)^+$RNA from cultured cortical neurons or mouse cerebral cortex was isolated using a Micro-FastTrack 2.0 Kit (Invitrogen). Aliquots of 2 μg of poly-$(A)^+$RNA were denatured, electrophoretically fractionated on a 1.4% agarose/formaldehyde gel, and transferred to a nylon membrane. Hybridization was performed in a solution containing cloned cDNA labeled with ^{32}P-dCTP using a random labeling kit (Roche Applied Science). Radioactivities of the bands were measured using a Bioimage analyzer BAS 2500.

2.6. Quantitative real-time PCR (QRT-PCR)

First-strand cDNA was synthesized from poly-$(A)^+$RNA of cultured cortical neurons using SuperScript II and oligo (dT) primers (Invitrogen). Quantitative RT-PCR analysis was performed using an iCycler iQ Detection System (Bio-Rad) with ELONGase Enzyme Mix (Invitrogen), SYBR Green I (1/50 000 dilution, Takara), and 400 nM gene-specific primers (nucleotides 37–60 and 242–219 for Cst-3; nucleotides 201–231 and 284–261 for ACTB). Results were evaluated with the ICYCLER IQ REAL-TIME DETECTION SYSTEM software (Bio-Rad).

2.7. Plasmid constructs

Cst-3 and Cst-2 were constructed by inserting PCR amplified fragments into pCMV-Tag5 containing a c-myc epitope (Stratagene). Detailed construct information is available upon request. Mouse Cst-1 cDNA clone (clone ID 6315355) was purchased from OPEN BIOSYSTEMS. The coding fragment of Cst-1 was cloned into pCMV-Tag5 containing a c-myc epitope (Stratagene).

2.8. Transfection

The constructs were transfected into 5 DIV cortical neurons with Lipofectamine 2000 according to the manufacturer's manual (Invitrogen). For serum withdrawal experiments, the culture medium was replaced with MEN-N2 18 h after transfection and the cells were cultured for an additional 6, 12, or 24 h.

2.9. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, treated with 0.3% triton X-100 for 10 min, blocked with 2% skim milk for 30 min, and reacted with the primary antibodies for 1 h, followed by a 1 h reaction with the secondary antibodies. Cell nuclei were stained with 10 μM Hoechst 33342 in PBS (−) for 10 min. Apoptotic neurons were counted in at least 60 transfected cells for each construct and in each transfection experiment. Immunofluorescence of frozen mouse brain was performed in the same manner as for cultured cells but followed by treatment with 10 mM $CuSO_4$ in 50 mM ammonium acetate buffer (pH 5.0) for 10 min for quenching autofluorescence of lipofuscin pigments inside neurons in aged mouse brain [18]. Immunofluorescence was visualized with an Olympus epifluorescence microscope.

2.10. Immunoblotting

Cultured cells were homogenized in 1% NP-40 containing 2 mM EDTA and protease inhibitors, and centrifuged for 20 min (14 000 rpm, 4 °C). Lysates were analyzed by SDS–PAGE (a 5–15% acrylamide linear gradient gel). After transferring to Immobilon, proteins were detected with specific antibodies using the enhanced chemiluminescence method.

2.11. Antibodies

Anti-Cst-3 polyclonal rabbit antibodies were raised against the peptide SSDERRIIETPPHRY (Cst-3 931–956) conjugated with KLH at the N-terminus of the peptide. The peptide antibodies were purified on the ligand-immobilized EAH Sepharose 4B (GE Healthcare). The polyclonal anti-myc tag antibodies (MBL), polyclonal anti-APP T668 (Cell Signaling), and a monoclonal anti-PHF antibody (AT8, Innogenetics) were purchased. Secondary antibodies were Texas red-conjugated anti-rabbit IgG (Vector), Alexa 488-conjugated goat anti-rabbit IgG, and Alexa 594-conjugated goat anti-mouse IgG_1 (Molecular Probe).

3. Results

3.1. Differential regulation of Csts by Aβ1-42

The cell viability of rat cortical neurons treated with freshly prepared Aβ1-42 assessed by trypan blue exclusion were 91%, 87%, 85%, and 66% of the untreated control after 6, 15, 24, and 48 h of treatment, respectively [19]. RNA isolated from neuronal cultures 15 h (slight neurodegeneration) after treatment with freshly prepared Aβ1-42 was used to generate an Aβ-inducing gene library using PCR-based suppression subtraction hybridization. We identified Cst-3 as a gene up-regulated by Aβ (Fig. 1A and B). Next, to determine whether other Csts are also up-regulated by Aβ, we assessed the expression of Cst-1 and Cst-2 in cortical neurons treated with freshly prepared Aβ1-42 for 15 h. Northern blot analyses

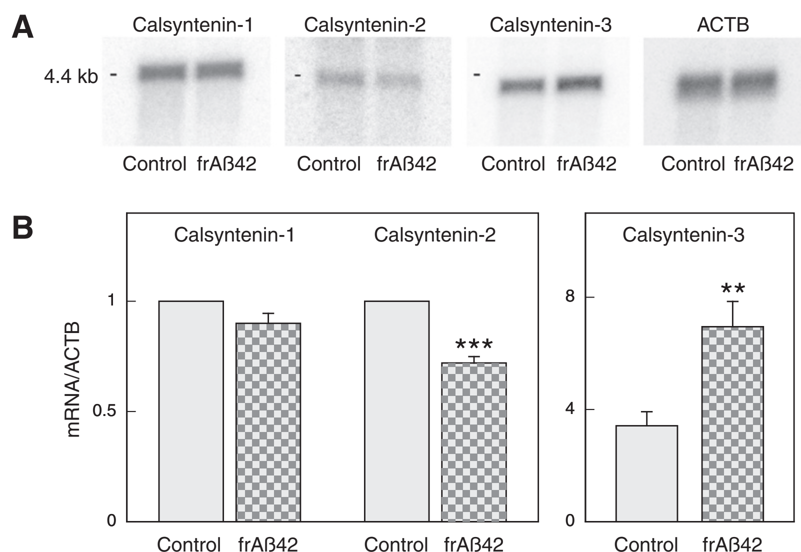

Fig. 1. Differential regulation of calsyntenin mRNAs by Aβ1-42. (A) Northern blot analysis of calsyntenin-1, calsyntenin-2, and calsyntenin-3 in cortical neurons treated with Aβ1-42 for 15 h. (B) Quantification of Cst-1 and Cst-2 mRNAs by Northern blotting, and of Cst-3 mRNA by quantitative RT-PCR. Data are the mean ± S.E.M. of three experiments for Northern blotting and of six experiments for quantitative RT-PCR. $^{**}P < 0.001$; $^{***}P < 0.001$ with Student's t-test compared with the untreated control.

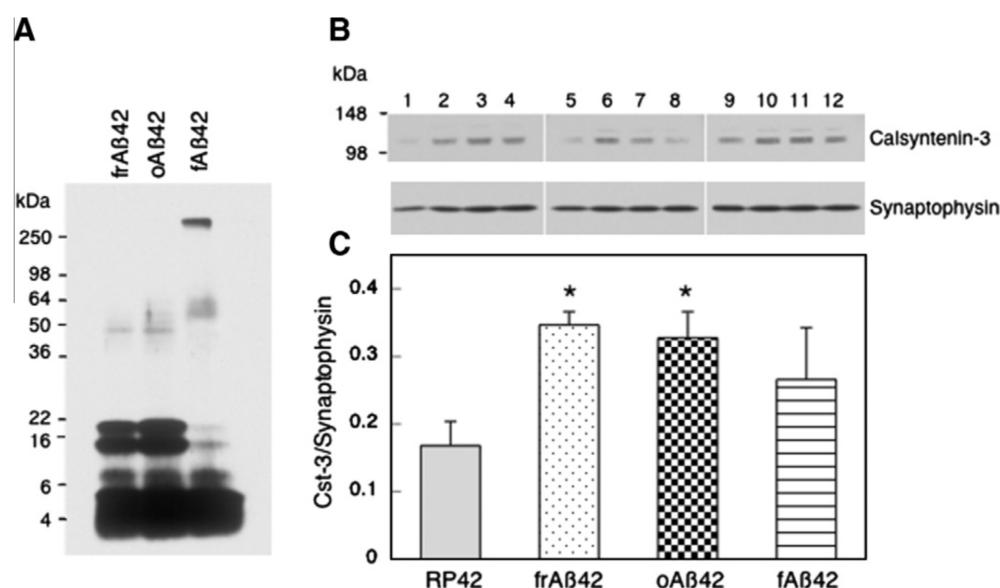

Fig. 2. Oligomeric Aβ1-42 increases with the level of calsyntenin-3 protein. (A) Western blot analysis of Aβ1-42 preparations. One microgram aliquots of fresh, oligomeric, or fibrillar preparations were separated by SDS–PAGE on a 10–20% linear gradient gel and analyzed by immunoblotting with anti-Aβ antibody 82E2 (recognizing residues 1–16 of Aβ). (B) Western blot analysis of calsyntenin-3 in cortical neurons treated with either Aβ 42-1 (lanes 1, 5, and 9), fresh (lanes 2, 6, and 10), oligomeric (lanes 3, 7, and 11), or fibrillar (lanes 4, 8, and 12) Aβ1-42 preparations (final concentration 2 μM) for 18 h. Synaptophysin was used for the control of equal loading. (C) Quantification of calsyntenin-3 in cerebral cortical neurons treated with Aβ42-1 (RP42), fresh (frAβ42), oligomeric (oAβ42), or fibrillar (fAβ42) preparations of Aβ1-42 by Western blotting. Data are the mean ± S.E.M. of three experiments. Statistical significance was analyzed by analysis of variance and a post hoc test, $^{*}P < 0.05$.

showed down-regulation of Cst-2 and no alteration of Cst-1 in freshly prepared Aβ1-42-treated cortical neurons (Fig. 1A and B).

3.2. Aggregation state of Aβ is critical for the up-regulation of Cst-3

We examined whether Aβ1-42 increases the protein level of Cst-3 in cortical neurons. The experiments described in the previous section were conducted using Aβ1-42 peptide freshly prepared in 50 mM HCl. In this section, we examined the ability of Aβ1-42 peptide freshly prepared, oligomeric, or fibrillar preparations to induce the expression of Cst-3 protein in cortical neurons. Western blot analysis of Aβ1-42 preparations revealed abundant Aβ1-42 trimer and tetramer, and a lesser amount of 50 kDa oligomer in both freshly prepared and oligomeric preparations. The fibrillar preparation contained high-molecular-weight immunoreactivity that did not enter the resolving gel, but quite little amounts of trimer and tetramer (Fig. 2A). The treatment of cortical neurons with freshly prepared or oligomeric but not fibrillar Aβ1-42 caused a significant increase in the level of Cst-3 protein (Fig. 2B and C).

3.3. Up-regulation of Cst-3 gene in APP_{SW} transgenic mice

Next, we determined whether the Cst-3 gene is up-regulated in aged APP mutant mice (APP_{SW}, Tg2576), which contained numerous Aβ plaques in the neocortex and hippocampus. Northern blot analyses revealed up-regulation of Cst-3 but no altered expres-

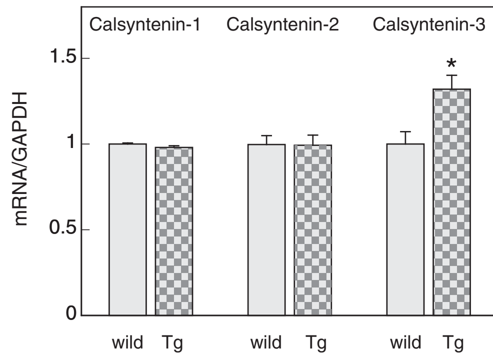

Fig. 3. Northern blot analysis of calsyntenin-1, calsyntenin-2, and calsyntenin-3 in neocortex of 24-month-old wild-type and Tg2576 mice. Data are the mean ± S.E.M. of three experiments. $^{*}P < 0.05$ with Student's t-test compared with wild-type mice.

sion of Cst-1 or Cst-2 in aged (24-month-old) Tg2576 neocortex (Fig. 3).

To investigate the cellular distribution of Cst-3 in brains from Tg2576 and wild-type mice, we performed immunofluorescence of serial frozen tissue sections with antibody against Cst-3. In aged (21-month-old) wild-type mice, immunofluorescence was detected in neurons in layers 2–6 of neocortex with different fluorescence intensities: Intense fluorescence was found in large cortical neurons in layer 5, with moderate immunoreactivity in large neurons in layers 2–3 and weak immunoreactivity in layers 4 and 6 (Fig. 4A and C). In aged (21-month-old) Tg2576 neocortex, strong immunofluorescence was observed in dystrophic neurites surrounding Aβ plaques, in addition to neuronal distribution (Fig. 4B, D, and E). Double immunofluorescent analysis revealed that the distribution pattern of Cst-3 in dystrophic neurites is similar to that of phospho-APP (T668) but different from that of phosphorylated-Tau (AT8) (Fig. 4F and G), suggesting that Cst-3 may be accumulated in dystrophic neurites of early-stage Aβ plaques [20].

3.4. Overexpression of Cst-3 increases vulnerability of cultured cortical neurons

We examined whether overexpression of Cst-3 in cortical neurons leads to an increase in the vulnerability of cortical neurons in vitro. Cst-3 with a flag-tag at the N-terminus or a myc-tag at the C-terminus (Fig. 5A) was generated and expressed in 5 DIV cortical neurons. The expression of exogenous Cst-3 protein was confirmed by Western blotting and immunofluorescence using antibodies against c-myc and flag (Fig. 5B and C). To determine whether the overexpression of Cst-3 accelerates cell death after serum withdrawal, cortical neurons expressing myc- or flag-tagged Cst-3 and showing DNA fragmentation were visualized by staining with anti-myc or anti-flag antibodies and Hoechst 33342 (Fig. 5C). Fig. 5D shows that cortical neurons expressing Cst-3 with either myc-tag or flag-tag were more sensitive to serum withdrawal than those expressing non-toxic MAP1B fragments [19] or untransfected neurons. It is unlikely that the induction of cell death in Cst-3-overexpressing neurons was due to a toxic effect of transfection per se because there was no significant difference in cell death between untransfected neurons and Cst-3-overexpressing neurons before serum withdrawal (untransfected neurons, 13.8 ± 1.3% cells were apoptotic 18 h after transfection; overexpression of myc-tagged Cst-3, 16.9 ± 1.6% cells were apoptotic; overexpression of flag-tagged Cst-3, 19.4 ± 3.0% cells were apoptotic, mean ± S.E., $n = 4$).

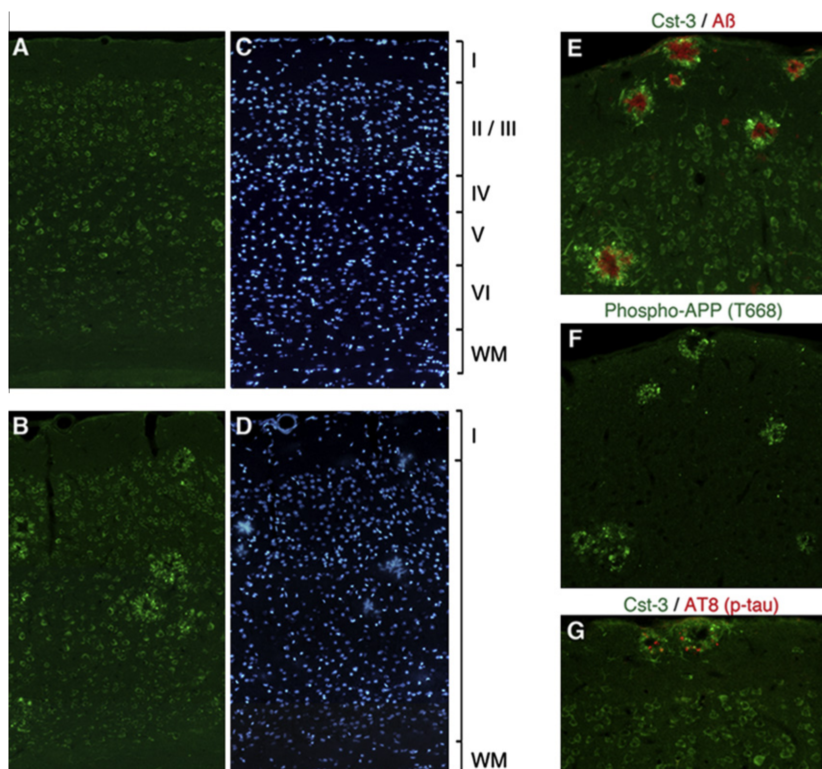

Fig. 4. Accumulation of calsyntenin-3 immunoreactivity in dystrophic neurites surrounding Aβ plaques in neocortex of APP_{SW} (Tg2576) mice. (A–D) Immunolabeling of the neocortex from 21-month-old wild-type (A) and Tg2576 mice (B) for calsyntenin-3 (green). Nuclei and Aβ plaques were counterstained with Hoechst 33342 (blue) in the neocortex from 21-month-old wild-type (C) and Tg2576 mice (D). (E–G) Immunolabeling of the neocortex from 21-month-old Tg2576 mice. (E) Double labeling of neocortex for calsyntenin-3 (green) and Aβ (red). (F) Immunolabeling of section adjacent to E with anti-phospho-APP (T668). (G) Double labeling of neocortex for calsyntenin-3 (green) and p-tau (AT8, red).

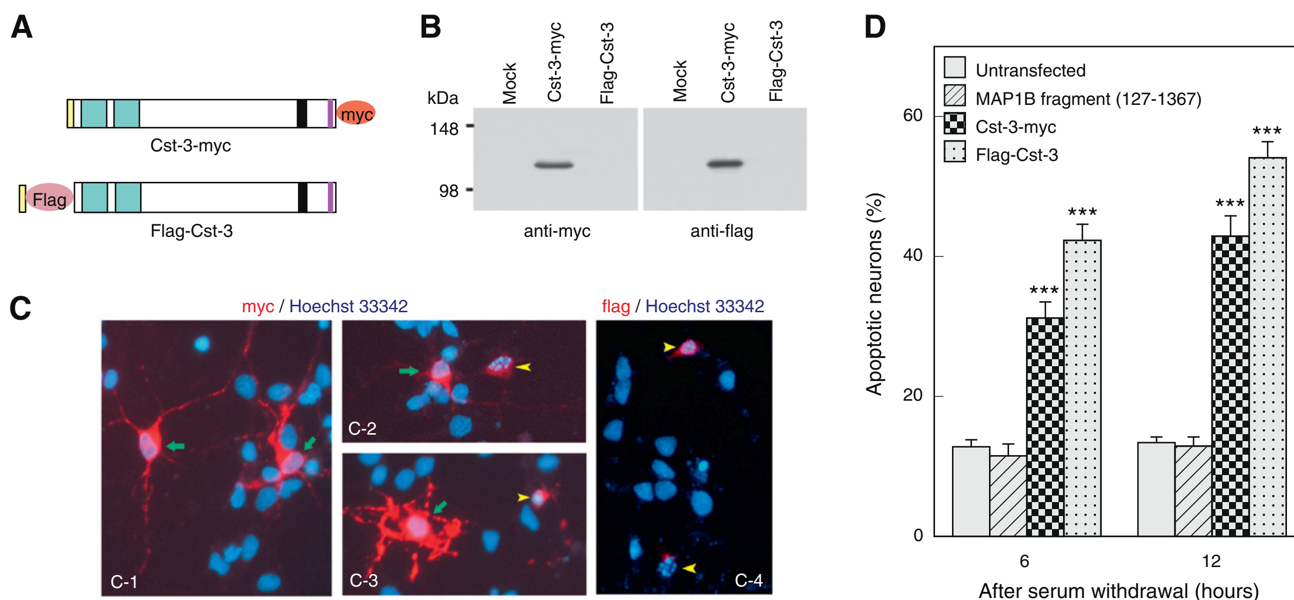

Fig. 5. Overexpression of calsyntenin-3 in cerebral cortical neurons accelerates neuronal death. (A) Schematic representation of calsyntenin-3 constructs used in this study. The myc-tag was attached to the C-terminus of calsyntenin-3, and the flag-tag was attached below the signal peptide sequence of calsyntenin-3. (B) Western blot of calsyntenin-3 expressed in 5 DIV cortical neurons. The lysates of cortical neurons transfected with the indicated constructs were fractioned by SDS–PAGE on a 5–15% linear gradient gel and analyzed by immunoblotting with the indicated antibodies. (C) Distribution of Cst-3-myc and flag-Cst-3 overexpressed in cortical neurons. Five DIV cortical neurons 18 h after transfection with Cst-3-myc (C, 1–3) or flag-Cst-3 (C-4) vectors were examined for immunofluorescence of anti-myc or anti-flag antibodies, respectively. Nuclei were counterstained with Hoechst 33342. Green arrows indicate intact nuclei and yellow arrowheads indicate apoptotic nuclei. (D) Five DIV cortical neurons were transfected with Cst-3-myc or flag-Cst-3. Cultured medium was replaced with serum-free medium 18 h after transfection, and cells were cultured for an additional 6 or 12 h. The number of transfected cells undergoing apoptosis was determined after immunostaining for the myc-tag and Hoechst 33342 staining in at least 60 transfected cells for each construct in each determination. Data are the mean ± S.E.M. of two independent experiments with five determinations each. Results are expressed as the percentage of apoptotic cells among myc-positive cells. $^{***}P < 0.0001$ by ANOVA and a post hoc test compared with cells transfected with non-toxic MAP1B fragment.

4. Discussion

This study provides evidence for the first time that up-regulation of Cst-3 by Aβ increases the vulnerability of cortical neurons. Cst-3 is a type-1 transmembrane protein of the cadherin superfamily and its distribution pattern in cerebral cortex is quite different from those of other Csts [11]. Cst-3 mRNA is highly expressed in a subpopulation of neurons in cortical layer 5. In contrast, Cst-1 mRNA is strongly expressed in most neurons throughout the cortical layers, and Cst-2 mRNA is highly expressed in a subpopulation of neurons in layers 5 and 6. Our immunofluorescence observations indicated that the most intensive immunoreactivity of Cst-3 is distributed in large neurons in layer 5, with moderate immunoreactivity in large neurons in layers 2–3 and weak immunoreactivity in layers 4 and 6 in wild-type mice. In AD, the pyramidal neurons in layers 2, 3, and 5 of neocortex are vulnerable to neuronal degeneration [21], despite of Aβ-deposits in all six layers. Although it is exactly unclear whether the layer heterogeneity of the Aβ-susceptibility depends on intrinsic factors in neuron themselves, it is possible that cortical neurons expressing a high level of Cst-3 may be vulnerable to AD degeneration. Indeed, dystrophic neurites surrounding Aβ plaques contained accumulated Cst-3 immunoreactivity.

Our in vitro finding indicates that Aβ oligomer but not fibril up-regulates Cst-3 expression. This does not conflict with the in vivo findings showing Cst-3 accumulation in dystrophic neurites surrounding Aβ plaques because Aβ oligomers are distributed surrounding plaque cores and induce dendritic spine loss and dystrophic neurites near Aβ plaques [22,23]. Thus, it is reasonable to speculate that Aβ oligomers initiates these synaptic degeneration via the up-regulation of Cst-3 in neurons in layers 2–3 and 5 of the cerebral cortex.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.01.025.

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