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Induction of amyloid precursor protein by the neurotoxic peptide, amyloid-beta 25–35, causes retinal ganglion cell death

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Abstract

Patients with Alzheimer's disease (AD) show a significantly increased incidence of glaucoma. AD is also associated with the occurrence of the neurotoxic peptide amyloid beta (Aβ). Therefore, we investigated whether Aβ is associated with retinal cell death in a retinal ganglion cell line (RGC-5). Treatment with $Aβ_{25–35}$, a neurotoxic fragment of Aβ, induced cell death in RGC-5 in both a concentration- and time-dependent manner. The amount of amyloid precursor protein was increased by treatment of RGC-5 and primary culture of mouse cortical neurons with fibril $Aβ_{25–35}$ and $Aβ_{1–42}$, which is a putative physiological neurotoxic fragment of Aβ present in AD. Amyloid precursor protein knockdown inhibited the cell death induced by $Aβ_{25–35}$. Treatment with $Aβ_{25–35}$ increased the amount of intracellular $Aβ_{1–40}$ and $Aβ_{1–42}$, while β- and γ-secretase inhibitors reduced cell death. Thus, the regulation of Aβ can be viewed as a new therapeutic target for glaucoma, especially in patients with coincident AD.

Keywords: Alzheimer's disease, amyloid precursor protein, amyloid β, cell death, glaucoma, retinal ganglion cell.

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Glaucoma is a group of diseases of the optic nerve that involves the loss of retinal ganglion cells (RGC) in a characteristic pattern of optic neuropathy. Glaucoma is thought to cause visual field constriction and visual loss through RGC death. Although a rise in intraocular pressure is a known risk factor for glaucoma, 20–90% of patients are normal tension glaucoma (Klein *et al.* 1992; Bonomi *et al.* 1998; Iwase *et al.* 2004). Reducing intraocular pressure is a major therapeutic approach; however, other therapeutic methods, such as neuroprotective drugs and causal treatments, have been required in addition to treatment with anti-ocular hypertension drugs.

Many stimuli such as oxidative stress (Aslan *et al.* 2008), nitric oxide (Neufeld *et al.* 1999), glutamate (Dreyer *et al.* 1996), and endoplasmic reticulum stress (Shimazawa *et al.* 2007) have been reported to initiate retinal cell death. Bayer *et al.* reported that patients with Alzheimer's disease (AD) also had a significantly increased occurrence of glaucoma (Bayer *et al.* 2002a). This suggests that amyloid beta (Aβ), a known neurotoxic peptide that is one of the causes of pathogenesis of AD, may also be involved in this retinal cell death.

A reduction in Aβ is one of the candidates therapies for AD (i.e., through the use of β- and γ-secretase inhibitors or modulators and with anti-Aβ antibodies) (Brody and Holtzman 2008; Wolfe 2008; Hunt and Turner 2009), as it is believed that extracellular Aβ exerts neurotoxicity (Glabe 2006). However, intracellular Aβ also shows cell toxicity (LaFerla *et al.* 2007; Ohyagi 2008), and β- and γ-secretase inhibitors or modulators are also effective against this type of toxicity as well. Aβ has been reported to cause retinal cell death *in vivo* (Guo *et al.* 2007) and to increase by high intraocular pressure (McKinnon *et al.* 2002). Hence, it is assumed that Aβ leads to not only neuronal cell death in AD patients but also to retinal cell death in glaucoma patients in general. Thus, reduction of Aβ is a therapeutic target for glaucoma. However, the mechanism of retinal cell death by Aβ remains unclear.

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Abbreviations used: α7nAchR, α7 nicotinic acetylcholine receptor; Aβ, amyloid β; AD, Alzheimer's disease; APP, amyloid precursor protein; BACE1, β-site of APP cleaving enzyme 1; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase (mitogen-activated protein kinase kinase); PI3K, phosphatidylinositide 3-kinase; RAGE, receptor for advanced glycation end products; RGC, retinal ganglion cell; sAPPα, soluble APPα; siRNA, small interfering RNA.

Amyloid precursor protein (APP) is secreted by β- and γ-secretase and generates the cytotoxic Aβ peptide. The physiological function of APP is poorly understood. In addition to its association with Aβ production and cell toxicity, it also has a neuroprotection function in the form of soluble APP (sAPPα), secreted by α-secretase (Fahrenholz 2007). A number of cytokines, neurotrophic factors, and growth factors induce the expression of APP (Ge and Lahiri 2002). Moreover, APP induction is caused by Aβ in some cells (Davis-Salinas *et al.* 1995; Schmitt *et al.* 1997; Heredia *et al.* 2004). However, there is little known about the outcome beyond APP induction.

In the present study, we investigated the association of Aβ with retinal cell death, using a RGC-5, and further studied the involvement of APP and intracellular Aβ induction in RGC death induced by Aβ.

Materials and methods

Materials

$A\beta_{(25–35,\ 35–25,\ 1–40,\ 1–42,\ \text{and}\ 40–1)}$ peptides were obtained from Sigma-Aldrich (St Louis, MO, USA). These peptides were dissolved in phosphate buffered saline (pH 7.4). Aging of Aβ (fibril formation) was achieved by incubation of 1 mg/mL stock solution at 37°C for 4 days (Schmitt *et al.* 1997; Heredia *et al.* 2004; Schmidt *et al.* 2009), and used for all experiments. β-Secretase inhibitor (β-secretase inhibitor IV) and γ-secretase inhibitor (γ-secretase inhibitor XIV) were obtained from MERCK (Whitehouse Station, NJ, USA). mitogen-activated protein kinase kinase (MEK1/2) inhibitor (U0126) was obtained from Promega (Madison, WI, USA).

Cell culture

The RGC-5 line is a transformed cell line for retinal ganglion cells (RGCs) which was generously provided by Dr. Neeraj Agarwal (UNT Health Science Center, Fort Worth, TX, USA). The cells were maintained in Dulbecco's modified Eagles's medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2, as described in our previous report (Nakajima *et al.* 2009). Primary cultures of mouse cortical neurons were prepared from fetuses of pregnant ddY mice at embryonic day 17. Neuronal cells were confirmed by the expression of MAP-2 neuronal marker.

Preparation of small interfering RNAs

The following small interfering RNA (siRNA) sequences specific to mouse/rat APP were used:

#1, 5′-UACAAACUCACCAACUAGGCACCGG-3′ (sense), 5′-CCGGUGCCUAGUUGGUGAGUUUGUA-3′ (antisense),

#2, 5′-ACUUCUACGACUUUGUCUUCACCGC-3′ (sense), 5′-GCGGUGAAGACAAAGUCGUAGAAGU-3′ (antisense),

#3, 5′-UUACCACAGAACAUGGCGAUCUGGG-3′ (sense), 5′-CCCAGAUCGCCAUGUUCUGUGGUAA-3′ (antisense).

Stealth™ RNAi Negative Control Medium GC Duplex #2 was used as a control. All siRNAs were obtained from Invitrogen (Carlsbad, CA, USA). The RGC-5 were transfected with 50 pg of siRNA using Lipofectamine™ RNAi MAX Reagent (Invitrogen) according to the manufacturer's protocol.

Real-time PCR

TaqMan real-time PCR was performed as described below. Single-stranded cDNA was synthesized using a TaqMan Gene Expression Cell-to-CT Kit (Applied Biosystems, Carlsbad, CA, USA). Relative quantitative real-time PCR was performed using the TaKaRa Thermal Cycler Dice® Real Time System TP800 with a TaqMan Gene Expression Master Mix (Applied Biosystems), according to the manufacturer's protocol. The thermal cycler conditions were as follows: 2 min at 50°C and then 10 min at 95°C, followed by two-step PCR for 55 cycles consisting of 95°C for 15 s followed by 60°C for 1 min. All reactions were performed in duplicate. The results were expressed relative to the ribosomal RNA internal control.

Immunoblot analysis

Cells were washed with phosphate-buffered saline, harvested and lysed in ristocetin-induced platelet agglutination buffer (Sigma-Aldrich) with protease inhibitor cocktail and phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich). Lysates were centrifuged at

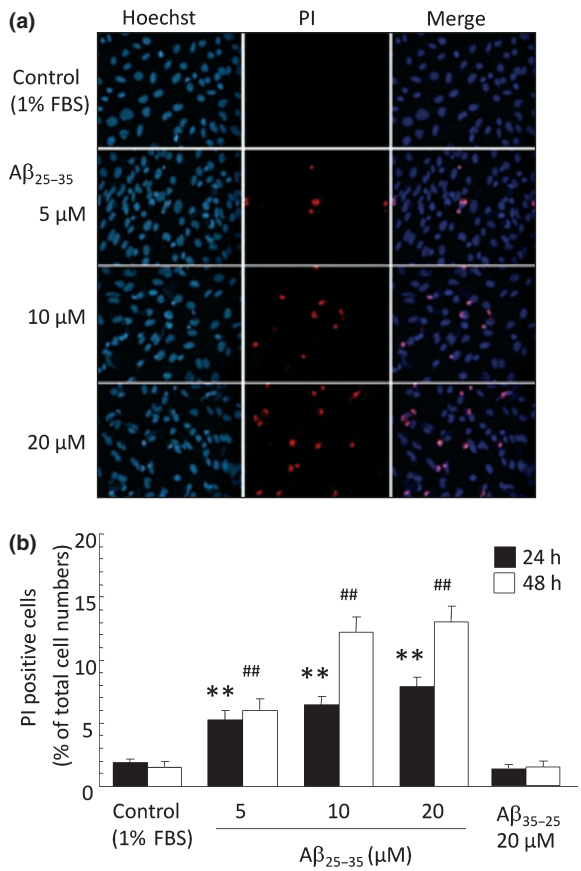

Fig. 1 $A\beta_{25–35}$ induced RGC death. (a) RGC-5 were treated with $A\beta_{25–35}$ at the indicated concentrations for 48 h. The cells were stained with Hoechst 33342 and propidium iodide (PI). (b) PI positive cells were counted and as described in Materials and Methods. **, ##$p < 0.01$ versus 24 and 48 h control, respectively (Dunnett's test, $n = 8$). Data are presented as means ± SEM.

12 000 *g* for 15 min at 4°C Surpernatants were collected and boiled for 5 min in sodium dodecyl sulfate sample buffer (Wako, Osaka, Japan). Total membrane and cytosolic fractions were prepared using Plasma Membrane Protein Extraction Kit (BioVision, Mountain View, CA, USA). Equal amounts of protein were subjected to 5–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gradient gels or 15–20% tricine gels and then transferred to poly(vinylidene difluoride) membranes. After blocking with Blocking One-P (Nakarai Tesque, Kyoto, Japan) for 30 min, membranes were incubated with the primary antibody [anti-APP (MILLIPORE, Billerica, MA, USA), anti-APP C99 (ProSci, Poway, CA, USA), anti-$A\beta_{1-40/42}$ (ALPHA DIAGNOSTIC, San Antonio, TX, USA), anti-β-actin (Sigma-Aldrich), anti-extracellular-regulated kinase (ERK) 1/2 and anti-pERK1/2 (Cell Signaling Technology, Danvers, MA, USA)]. Subsequently, the membranes were incubated with the secondary antibody [horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce Biotechnology, Rockford, IL, USA)]. The immunoreactive bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and then measured using LAS-4000 mini (FUJIFILM, Tokyo, Japan).

Cell death assay

Retinal ganglion cell-5 were seeded at 1×10^3 cells per well into a 96-well plate and then incubated for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The entire media were then replaced with fresh media containing 1% fetal bovine serum, and $A\beta_{25-35}$, with or without β- and γ-secretase inhibitors. Nuclear staining assays were carried out after a further 24- or 48-h incubation. Cell death was assessed by combination staining with Hoechst 33342 and propidium iodide. Images were collected using an Olympus IX70 inverted epifluorecence microscope (Olympus, Tokyo, Japan). A total of at least 300 cells per condition were counted in six randomly chosen fields.

Enzyme-linked immunosorbent assay

Retinal ganglion cell-5 were treated with 20 μM of $A\beta_{25-35}$ for 24 h. The culture media were then recovered in centrifuge tubes with 0.1% (final concentration) bovine serum albumin. The cells were washed with ice cold phosphate-buffered saline and lysed with HEPES buffer (10 mM HEPES, 1% NP-40 (Nacalai Tesque, Kyoto, Japan) and protease inhibitor cocktail). The media and lysate were then subjected to enzyme-linked immunosorbent assay (ELISA). ELISA for soluble $A\beta_{40}$ and $A\beta_{42}$ (Wako) were carried out according to the manufacturer's protocol using 20 μL (for $A\beta_{40}$) or 100 μL (for $A\beta_{42}$) of media or lysate containing 30–60 μg protein. The monoclonal antibodies, BA27 and BC5, contained in these kits specifically detect the C-terminal protein of $A\beta_{40}$ and $A\beta_{42}$, respectively. The absorbance was measured at 450 nm using

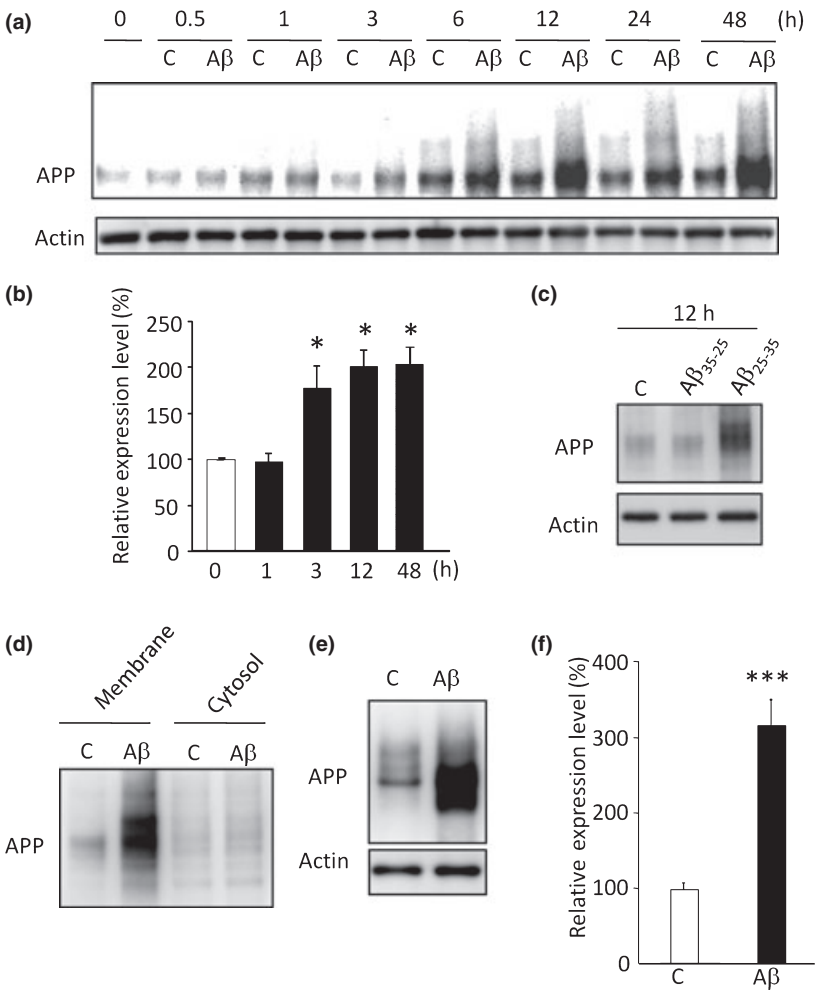

Fig. 2 APP was induced by $A\beta_{25-35}$ in RGC-5. (a) RGC-5 were treated with $A\beta_{25-35}$ (20 μM) for the indicated times. (b) Relative protein expression level of APP was calculated and described as a percentage based on the intensity of APP with Aβ divided by APP without Aβ (Control). Each sample was 1 μg protein and was corrected by the intensity of actin. (c) RGC-5 were treated with $A\beta_{25-35}$ or $A\beta_{35-25}$ (20 μM) for 12 h. (d) Membrane and cytosolic fractions of RGC-5 treated with $A\beta_{25-35}$ (20 μM) for 12 h were prepared as described in Materials and Methods and subjected to immunoblot analysis. (e, f) Mouse primary cortical neurons were treated with $A\beta_{25-35}$ (20 μM) for 12 h. Relative protein expression level of APP was calculated as described above. In the figure, C represents the control. $^*p < 0.05$ versus 0 h (Dunnett's test, $n = 3$). $^{***}p < 0.001$ versus control (Student's *t*-test, $n = 3$). Data are presented as means ± SEM.

VARIOSKAN FLASH (Thermo Fisher Scientific, Waltham, MA, USA). We confirmed that there were no ELISA reactions with $A\beta_{25-35}$ using media with or without 20 μM $A\beta_{25-35}$.

Statistical analyses

Data were presented as means ± SEM. Statistical comparisons were made using a one-way ANOVA followed by a Turkey's test, a Dunnett's test or a Student's *t*-test. A value of $p < 0.05$ was considered to indicate a statistical significance.

Results

Aβ leads to retinal cell death and APP induction

We investigated whether Aβ causes cell death in RGC-5, a ganglion cell-line. Cell death was significantly increased in response to aggregated $A\beta_{25-35}$ treatment in a concentration- and time-dependent manner, whereas $A\beta_{35-25}$ did not affect (Fig. 1a and b). TUNEL positive cells were seen less than 1% of total cells at 24 h after $A\beta_{25-35}$ treatment (data not shown), although propidium iodide positive cells were seen about 10% (Fig. 1b). Cell death was observed at 24 h after $A\beta_{25-35}$ treatment, and we assumed that intracellular alteration was caused within that time. The nature of the molecules associated with the RGC death induced by $A\beta_{25-35}$, was examined by immunoblotting analysis, which revealed alterations in expression level of several proteins and in protein phosphorylation. The amount of APP was increased after 3 h following treatment with aged $A\beta_{25-35}$ (Fig. 2a and b). On the other hand, $A\beta_{35-25}$ did not increase APP (Fig. 2c). APP induction was observed in total cell membrane fraction, although there was no effect in cytosolic fraction (Fig. 2d). APP was also induced in a primary culture of mouse cortical neurons within 12 h of $A\beta_{25-35}$ treatment (Fig. 2e and f).

APP induction was caused by various Aβ peptides and was concentration dependent

Next, we investigated whether APP induction was caused by other Aβ peptides such as $A\beta_{1-40}$ and $A\beta_{1-42}$, which are physiologically active Aβ fragments. Treatment with $A\beta_{1-42}$ induced significant expression of APP, as did $A\beta_{25-35}$. In

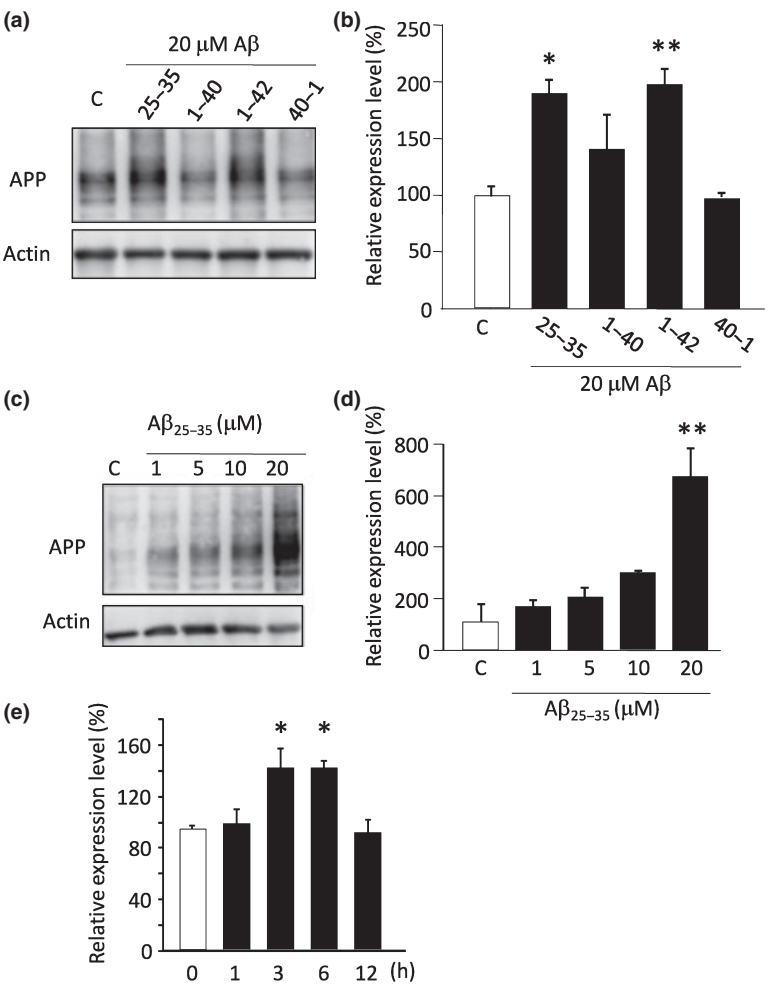

Fig. 3 APP induction was caused by various Aβ peptide and concentration dependency. (a, b) RGC-5 were treated with 20 μM of $A\beta_{25-35}$, $A\beta_{1-40}$, $A\beta_{1-42}$, and $A\beta_{40-1}$, respectively. (c, d) RGC-5 were treated with $A\beta_{25-35}$ as indicated concentrations for 12 h. (e) RGC-5 were treated with or without $A\beta_{25-35}$ (20 μM) for indicated times. Real-time PCR was performed as described in Materials and Methods. In figure, C means control. $^*p < 0.05$, $^{**}p < 0.01$ versus control (b, d, Dunnett's test; e, Student's *t*-test, $n = 3$). Data are presented as means ± SEM.

contrast, $A\beta_{1-40}$, which forms fewer aggregates and shows less toxicity than $A\beta_{25-35}$ and $A\beta_{1-42}$, caused only a slight induction of APP (Fig. 3a and b).

Amyloid precursor protein induction in response to active Aβ peptides occurred in a concentration-dependent manner (Fig. 3c and d), in agreement with the cell death results (Fig. 1b). To clarify whether this induction was caused by transcriptional activation, we measured APP mRNA levels using real-time PCR. APP mRNA was significantly increased at 3 and 6 h, but not after 12 h following $A\beta_{25-35}$ treatment (Fig. 3e), in agreement with the observed protein levels. Thus, APP induction by $A\beta_{25-35}$ was at least partially because of transcriptional activation.

APP induction occurred by an ERK independent mechanism

The observed increase in APP appeared to be a consequence of increased synthesis through transcription/translation activity rather than via accumulation because of disruption of APP degradation by the ubiquitin-proteasome system (Kumar *et al.* 2007), at least in the early time periods after induction. The α7 nicotinic acetylcholine receptor (α7nAchR), interacting with Aβ, has been reported to activate the ERK pathway (Dineley *et al.* 2001). The ERK pathway is also apparently involved in APP induction via some cellular mechanisms other than transcription (Liu *et al.* 2003). We hypothesized that APP induction was at least partially associated with ERK pathway. Immunoblot analysis showed $A\beta_{25-35}$ significantly activated ERK phosphorylation within 10 min in RGC-5 (Fig. 4a and b). As ERK activation was caused by $A\beta_{25-35}$ treatment before we observed any increase in APP mRNA expression, we examined whether ERK activation was involved in APP induction by using a MEK1/2 inhibitor, U0126. Although U0126 completely inhibited ERK phosphorylation, it had no effect on APP induction by $A\beta_{25-35}$ (Fig. 4c and d). Thus, APP induction by $A\beta_{25-35}$ was an ERK independent process.

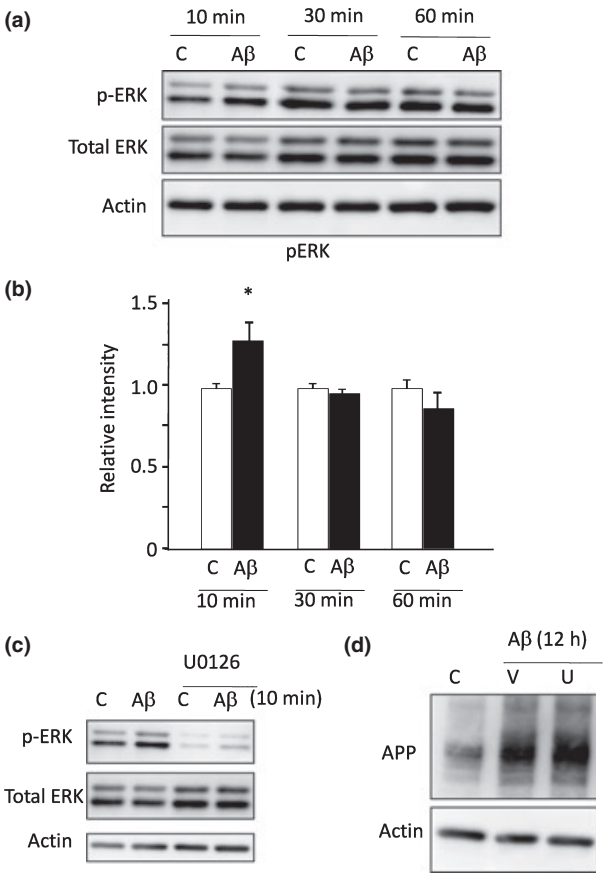

Fig. 4 APP induction occurred by an ERK-independent mechanism. (a, b) RGC-5 were treated with 20 μM of $A\beta_{25-35}$. Relative intensity was described by the intensity of phosphorylated ERK (p-ERK) divided by total ERK. (c, d) RGC-5 were treated with or without 1 mM of U0126 and then treated with 20 μM of $A\beta_{25-35}$ for indicated times. In figure, C, V and U represent the control, vehicle only, and U0126 treatment, respectively. $^*p < 0.05$ versus control (Student's *t*-test, $n = 3$). Data are presented as mean ± SEM.

APP induction was necessary for retinal cell death induced by $A\beta_{25-35}$

Understanding the physiological significance of APP induction is important. APP is cleaved by β- and γ-secretase to generate neurotoxic Aβ peptides. APP is also cleaved by α-secretase to generate neuroprotective sAPP. Therefore, to clarify whether APP induction mediates cell death directly in RGC-5, we performed an APP knockdown using siRNA transfection. As shown in Fig. 5(a) and (b), APP siRNAs reduced the amount of APP to about 20–40% compared with siRNA transfected cells treated with $A\beta_{25-35}$ (negative controls). All APP siRNAs also reduced cell death by $A\beta_{25-35}$, whereas siRNA containing #2 sequences were less effective at APP knockdown than were other sequences (Fig. 5b–d). Thus, it was the increase in APP itself, more than its constitutive expression, that potentiated RGC death by $A\beta_{25-35}$.

Intracellular Aβ fragments were increased by $A\beta_{25-35}$ stimulation

As APP induction by $A\beta_{25-35}$ is involved in the cytotoxic effect, we hypothesized that the amount of intracellular Aβs generated from increased APP levels would be increased by $A\beta_{25-35}$ treatment, and that these Aβs would then lead to cell death. To investigate whether the Aβ fragments were increased, we measured the quantity of Aβ in RGC-5 using immunoblotting analysis and ELISA. The intracellular $A\beta_{1-40}$, $A\beta_{1-42}$ and 99-amino acid carboxy-terminal fragment (C99), which is generated by β-secretase and serves as substrate for γ-secretase, were increased by $A\beta_{25-35}$ treatment (Fig. 6a–e). These inductions were suppressed by β- and γ-secretase inhibitors, although the amount of C99 fragment was not affected by a γ-secretase inhibitor (Fig. 6a–c). On the other hand, the extracellular Aβs were reduced (Fig. 6f and g), despite of the increase in both APP and intracellular Aβs.

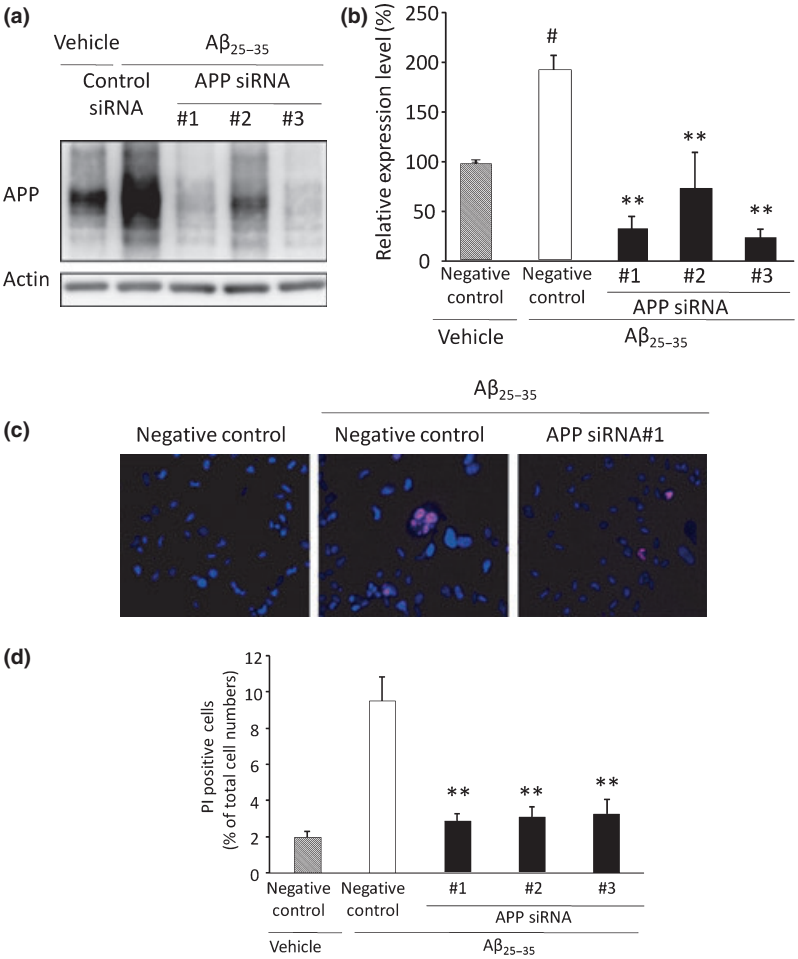

Fig. 5 APP knockdown reduced RGC death by $A\beta_{25–35}$. (a, b) RGC-5 were transfected with APP or negative control siRNAs for 24 h, and then treated with $A\beta_{25–35}$ (20 μM) for 48 h. Each cell was subjected to immunoblot analysis. Relative protein expression level of APP was calculated as described above. (c, d) siRNA-transfected RGC-5 were treated with $A\beta_{25–35}$ (20 μM) or vehicle for 48 h. Each panel is presented by merged Hoechst 33342 and propidium iodide (PI) staining cells. The percentage of apoptotic cells were calculated as described above. #$p < 0.05$ versus vehicle, **$p < 0.01$ versus negative control treated with $A\beta_{25–35}$ (b, Turkey's test, $n = 3$; d, Dunnett's test, $n = 6$). Data are presented as mean ± SEM.

β- and γ-Secretase inhibitors reduced retinal cell death caused by $A\beta_{25–35}$

Amyloid precursor protein knockdown reduced RGC death, and the quantity of intracellular Aβs was increased by $A\beta_{25–35}$ treatment. These intracellular Aβs were apparently derived from increased APP and were associated with the induction of RGC death by $A\beta_{25–35}$. Therefore, we investigated whether β- and γ-secretase inhibitors might reduce RGC death induced by $A\beta_{25–35}$ treatment. A β-secretase inhibitor significantly reduced Aβ-induced cell death at levels as low as 1 nM (Fig. 7a and b) and exerted its effects in a concentration-dependent manner, without toxicity (Fig. 7b). A γ-secretase inhibitor also suppressed RGC death by $A\beta_{25–35}$ (Fig. 7b). These findings suggested that APP induction by $A\beta_{25–35}$ may cause cell death as a result of increased Aβs.

Discussion

In this report, treatment with $A\beta_{25–35}$ induced cell death in RGC-5 in concentration- and time-dependent manners. The amount of APP and intracellular Aβ in both RGC-5 and mouse primary cortical neuronal cells was increased by Aβ treatment. Cell death induced by $A\beta_{25–35}$ could be reduced by treatment of RGC cells with β- and γ-secretase inhibitors, or by using siRNA.

Recently, the origin of the RGC-5 line has been re-characterized (Van Bergen *et al.* 2009). The RGC-5 line, designated as a transformed rat retinal ganglion cell line, is actually of mouse origin (Van Bergen *et al.* 2009). However, when differentiated by treated with staurosporine, the RGC-5 cells express some proteins characteristic to RGCs including Thy1 and NMDA receptor, which indicates that RGC-5 is a precursor cell line for retinal ganglion cells (Frassetto *et al.* 2006). Because the purpose of this study was to determine what kind of molecules participated in retinal damage by Aβ, the origin of the RGC-5 cell line was less relevant to our results.

Some receptors that can interact with Aβ, such as α7nAchR (Dineley *et al.* 2001), NMDA receptor (Shankar *et al.* 2007), and receptor for advanced glycation end products (RAGE) (Yan *et al.* 1996), are expressed in neurons. α7nAchR is thought to be associated with a disruption of synaptic function through ERK activation

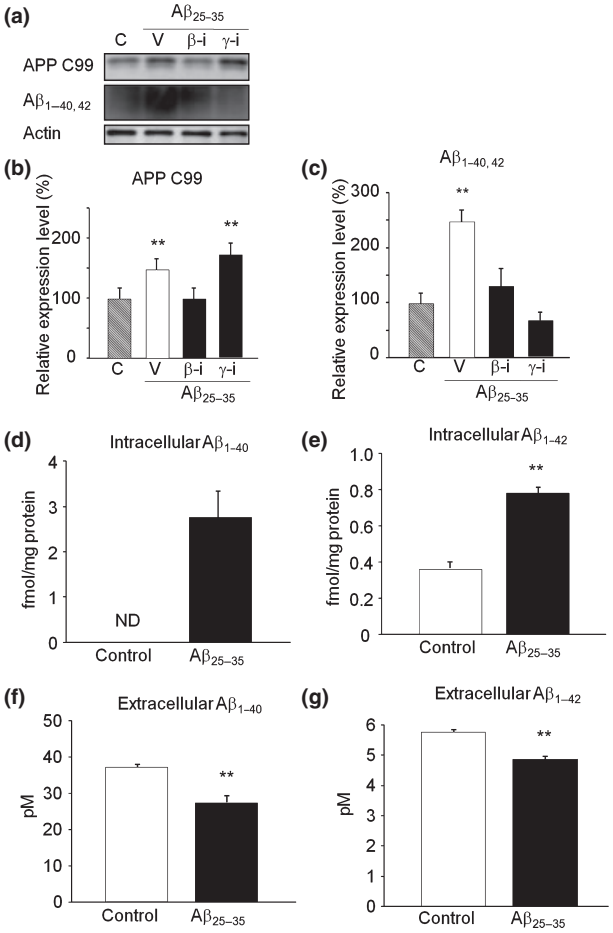

Fig. 6 Intracellular Aβ fragments were increased by $A\beta_{25–35}$ stimulation. RGC-5 were harvested at 24 h after $A\beta_{25–35}$ stimulation, and subjected to immunoblot analysis or ELISA described in Materials and Methods. (a–c) Relative protein expression level of APP C99 fragment and $A\beta_{1–40/42}$ were calculated as described above. β-I and γ-I mean β- and γ-secretase inhibitor, respectively; (d) the concentration of intracellular $A\beta_{1–40}$; (e) intracellular $A\beta_{1–42}$; (f) extracellular $A\beta_{1–40}$ and (g) extracellular $A\beta_{1–42}$, respectively. $^{**}p < 0.01$ versus control (Dunnett's test, $n = 3$, Student's *t*-test, $n = 4$, respectively). Data are presented as mean ± SEM.

(Dineley *et al.* 2001). Hepatocyte growth factor apparently activates the ERK pathway concerned with APP induction via a mechanism that does not depend on APP transcription (Liu *et al.* 2003).

The RAGE Aβ receptor has been reported to directly cause cell death (Yan *et al.* 1996) and Aβ interaction with RAGE has been associated with p38 MAPK signaling (Origlia *et al.* 2008). We therefore investigated whether Aβ stimulation activated ERK and p38 MAPK, using phosphorylation of ERK and p38 MAPK as a measurement of activation. ERK phosphorylation was seen within 10 min after Aβ stimulation, but the APP induction was not prevented by a MEK1/2 inhibitor, U0126, which prevented ERK phosphorylation (Fig. 4c and d). On the other hand, p38 MAPK activation was significantly increased at 24 h after stimulation (data not shown), although APP induction had been observed in an earlier time period. These signals were not associated with APP induction by $A\beta_{25–35}$ in RGC-5, although p38 MAPK activation may be involved in other aspects of RGC death. Therefore, other signaling pathways such as phosphatidylinositide 3-kinase (PI3K)/Akt pathway may be involved in APP induction by Aβ (Ruiz-Leon and Pascual 2004). $A\beta_{1–40}$ leads to APP induction and cell death (Heredia *et al.* 2004). However, our results suggested that APP induction by $A\beta_{1–40}$ was less effect than $A\beta_{25–35}$ and $A\beta_{1–42}$ in RGC-5 (Fig. 3a and b). APP induction was also observed in mouse primary cortical neurons (Fig. 2c and d). Aβs tend to aggregate and form complexes of varying size. We generated fibril formation of Aβs according to previous reports (Schmitt *et al.* 1997; Heredia *et al.* 2004; Schmidt *et al.* 2009). Further detailed analysis about Aβ formations such as monomer, soluble oligomer and fibril formation is required for ascertaining the association between APP induction and cell toxicity.

Amyloid precursor protein induction is caused by Aβ (Davis-Salinas *et al.* 1995; Schmitt *et al.* 1997; Heredia *et al.* 2004), various stimuli (Ge and Lahiri 2002) and brain injury (Loane *et al.* 2009) in various tissue and cells, and may be compensatory response to abnormal state for some cells. APP undergoes proteolytic cleavage by secretases to produce Aβ peptides and sAPPα. Considering the cell death response elicited by $A\beta_{25–35}$, it is assumed that the amount of Aβ peptides had increased, and that the increase in intracellular Aβ causes cell death, at least in part, because the effect of Aβs secreted extracellularly was masked by $A\beta_{25–35}$. Our immunoblotting analysis and ELISA results supported the possibility that an increase in intracellular Aβ leads to RGC death. The PI3K inhibitor, Wortmannin, has been reported to reduce the secretion of extracellular Aβ, and to increase the amount of intracellular Aβ in N2a mouse neuroblastoma cells (Petanceska and Gandy 1999). Accordingly, $A\beta_{25–35}$ stimulation may disturb intracellular APP and Aβ trafficking via PI3K.

In traumatic brain injury, APP, sAPPα, Aβ peptide, β-site of APP cleaving enzyme 1 (BACE1, i.e. β-secretase) and presenilin-1 (a component of γ-secretase) have been shown to increase, with β- and γ-secretase playing key roles in the severity of the injury (Loane *et al.* 2009). BACE1 knockout mice and those treated with a γ-secretase inhibitor showed reduced neuronal cell death. Therefore, we also used β- and γ-secretase inhibitors to clarify whether the increase in APP was involved in Aβ induction and RGC death. These inhibitors dramatically reduced in intracellular Aβs (Fig. 6a–e) and RGC death caused by $A\beta_{25–35}$ (Fig. 7b), indicating that RGC death induced by $A\beta_{25–35}$ is a consequence of increases in intracellular Aβ following APP induction. In another report, BACE1 activation via c-*jun* N-terminal

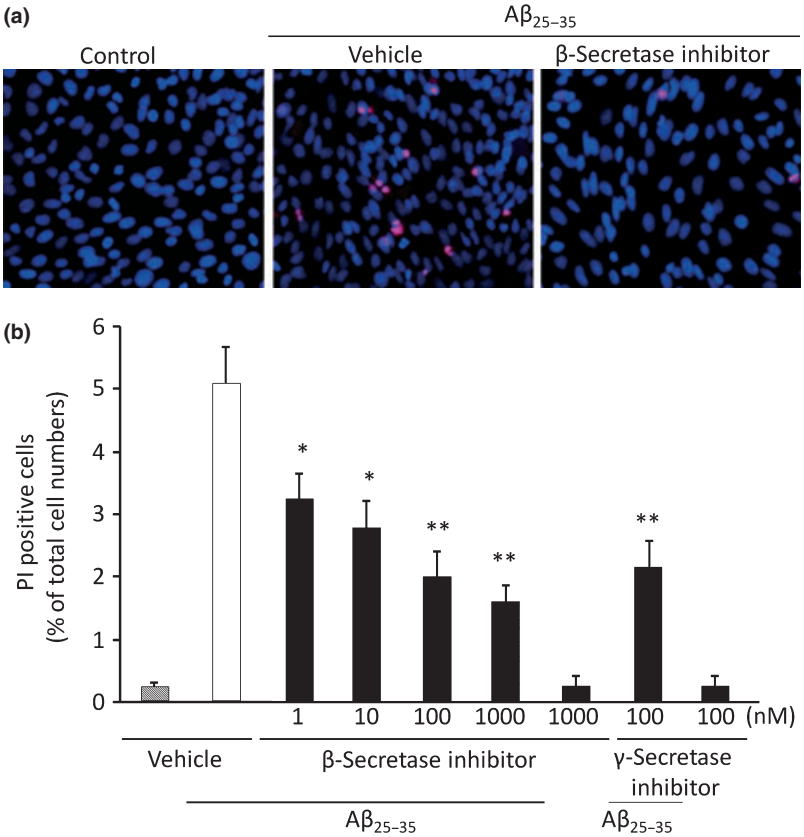

Fig. 7 β- and γ-Secretase inhibitor reduced RGC death by $A\beta_{25–35}$. (a) RGC-5 were treated with $A\beta_{25–35}$ (20 μM) or vehicle and β-secretase (1000 nM) for 48 h. Each panel is presented by merged Hoechst 33342 and propidium iodide (PI) staining cells. (b) RGC-5 were treated with $A\beta_{25–35}$ (20 μM), and β- or γ-secretase inhibitor as indicated concentrations for 48 h. $^*p < 0.05$, $^{**}p < 0.01$ versus vehicle treated with $A\beta_{25–35}$ (Dunnett's test, $n = 6$). Data are presented as mean ± SEM.

kinase/c-*jun* pathway was shown to lead to oxidative stress, which caused positive feedback (Tamagno *et al.* 2008). On the other hand, $A\beta_{1–42}$ was shown to mediate oxidative stress and up-regulation and activation of BACE1 (Tamagno *et al.* 2006). Jin *et al.* also demonstrated an association between the induction of oxidative stress by Aβ and an increase of γ-secretase activity (Jin *et al.* 2008). Considering these reports, it is possible that in RGC-5 treated with Aβ, the amount of intracellular Aβ increased, and β- and γ-secretase (i.e. presenilin) were simultaneously activated or up-regulated via the oxidative stress caused by intracellular and/or extracellular Aβ stimulation, with the end consequence being retinal cell death. Our siRNA study strongly suggested that up-regulation of APP by $A\beta_{25–35}$ was associated with cell death in RGC-5. APP siRNA reduced the total amount of APP and therefore also probably reduced the amount of sAPPα, whereas cell death was greatly reduced. Although it has been reported that sAPPα has a neuroprotective effect, sAPPα seemed to afford a little protection to RGC-5 treated with $A\beta_{25–35}$.

Glaucoma is recognized as a disease commonly associated with AD (Bayer *et al.* 2002a,b; Yoneda *et al.* 2005; Tamura *et al.* 2006). Moreover, for apolipoprotein-E, whose allele is known to be one of the risk factors for AD, the presence of single-nucleotide polymorphisms in its promoter is associated with increased optic nerve damage (Copin *et al.* 2002). In a previous study, we found that APP transgenic mice had reduced retinal function (Shimazawa *et al.* 2008), while other reports indicated that Aβ toxicity is apparently involved in retinal cell death *in vitro* (Yin *et al.* 2008) and *in vivo* (Guo *et al.* 2007). We reported that the levels of $A\beta_{42}$ in vitreous fluid of glaucoma and diabetic retinopathy patients were lower than in control (macular hole patients) (Yoneda *et al.* 2005). This result supported our data because the secretion of extracellular Aβ was decreased by $A\beta_{25–35}$ stimulation. On the other hand, high intraocular pressure leads to Aβ induction (McKinnon *et al.* 2002). Considering these findings, it is evident that some stimuli such as high intraocular pressure cause accumulation of Aβ in the retina, and this Aβ then gradually triggers retinal cell death upon the secretion of low amounts of Aβ in the early stages of glaucoma. The Aβ in the retina may be eliminated by some proteases, such as neprilysin, a rate-limiting peptidase involved in the physiological degradation of Aβ, which is secreted by other living cells (Iwata *et al.* 2000; Hara *et al.* 2006).

In conclusion, our findings suggest that the accumulation of intracellular Aβ associated with APP induction plays a pivotal role in RGC death caused by extracellular Aβ. Hence, modulation of Aβ induction and metabolism may be a therapeutic target for glaucoma, especially in patients with coincident AD.

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