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Aβ-induced Ca^{2+} influx regulates astrocytic BACE1 expression via calcineurin/NFAT4 signals

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ABSTRACT

The β-site APP cleaving enzyme (BACE1) is required for the production of β-amyloid peptides, which give rise to β-amyloid (Aβ) deposits in the brains of Alzheimer's disease (AD) patients. In brains, BACE1 is primarily expressed by neurons, however BACE1 expression has also been observed in reactive astrocytes in close proximity to β-amyloid plaques in the brains of aged Tg2576 AD model mice. To date, the direct effects of Aβ on BACE1 gene expression in astrocytes is unknown. We found that Aβ42 or Aβ25–35 treatment induced BACE1 expression in primary astrocytes as well as human astrocytoma cell line. Aβ neurotoxicity has been associated with the disruption of intracellular calcium homeostasis both in neurons and in glial cells. Here, we demonstrated that NFAT4, a transcription factor tightly regulated by the calcium/calmodulin-dependent phosphatase, calcineurin, was activated in astrocytes applied with calcium ionophore or Aβ. Aβ-activated NFAT4 proteins were associated with astrocytic BACE1 gene expression via direct interaction with the BACE1 promoter region.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder of senile dementia in elderly people and is characterized by two major morpho-pathological hallmarks: deposition of extracellular amyloid plaques accompanied by intracellular neurofibrillary tangles (NFT) in the brain [1]. Amyloid-beta peptide (Aβ), the major component of plaques, is produced from amyloid precursor protein (APP) by proteolytic cleavage at its N- and C-termini by β- and γ-secretase, respectively [2]. β-Secretase has been identified by several groups and was named β-site APP cleaving enzyme 1 (BACE1), Asp2 or memapsin [3]. BACE1 mRNA shows the highest level of expression in the brain and is especially detected in neurons but not in glial cells [3,4]. However, recent studies have observed BACE1 immunoreactivity in reactive astrocytes around senile plaques in old Tg2576 mice [5,6]. It has been reported that various inflammatory cytokines and oxidative stress can induce astrocytic BACE1 expression [7,8] and studies by Cho et al. have shown that IFN-γ, pro-inflammatory cytokine, generates BACE1 expression and activity in astrocytes [9].

Since BACE1 levels were observed in astrocytes surrounding Aβ plaques, we hypothesized that Aβ42, the critical component of plaques may induce BACE1 gene expression in astrocytes. Aβ42 neurotoxicity has been associated with the destabilization of intracellular calcium homeostasis both in neurons and in glial cells, although the mechanisms of Aβ-induced disruption of calcium homeostasis remain unclear. Disrupted calcium homeostasis has been observed in the brains of AD patients. The levels of intracellular calcium and calcium-related enzyme activity were found to be elevated in the brains from AD patients [10]. Furthermore, our previous study showed that neuronal BACE1 expression was up-regulated by disrupted intracellular calcium homeostasis that was mediated by nuclear factor of activated T-cells 1 (NFAT1) [11]. In this study, we found that Aβ42 treatment stimulated astrocytic BACE1 gene expression at transcription level via activation of calcineurin (CaN)/NFAT4 signaling cascade. Furthermore, we found that Aβ-induced BACE1 expression occurs via a calcium permeable channel formed by membrane-inserted Aβ in astrocytes. Treatment with Zn^{2+} or clioquinol, which has been known to inhibit channels formed by Aβ, blocked Aβ-induced astrocytic BACE1 gene expression.

Our findings suggest that stimulation with Aβ42 potentiates astrocytic BACE1 gene expression via disturbance of intracellular calcium homeostasis that is mediated by Aβ-induced activation of CaN/NFAT4 signaling pathway in astrocytes.

2. Materials and methods

2.1. Cell cultures

U373MG cells were maintained in Dulbecco's modified Eagle medium (DMEM; 4.5 g/ml glucose with pyruvate; HyClone) with

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10% fetal bovine serum (FBS; HyClone) and 1% penicillin/streptomycin. Cortical astrocyte cultures were established from 2-day-old pups. Briefly, mouse brains were removed and the meningeal tissue was stripped off. Brains were dissociated mechanically and seeded in poly-D-lysin (Sigma)-coated dishes into medium consisting of DMEM with 10% FBS and then incubated at 37 °C in a humidified 5% CO_2 air atmosphere. After 2 weeks in culture, media were removed and the cells were trypsinized and replated at low density in 12-well plates for reagent treatment [5].

2.2. Peptides and treatment

Cells were treated with calcium ionophores, ionomycin or A23187 (Sigma) in DMEM supplemented with 1% FBS. Synthetic Aβ25–35 (U.S. Peptide, Inc.) and Aβ42 (Bachem) were dissolved in dimethylsulfoxide (DMSO; Amresco) and diluted to final concentration of 1 mM with water [11]. Prior to treatment with calcium ionophore or Aβ peptides, cells were treated with the following reagents as indicated in figures, cyclosporine A (CsA), BAPTA-AM, 2-amino-ethoxy diphenylborate (2-APB), dantrolene, nifedipine, EGTA, clioquinol, $ZnCl_2$ (all from Sigma) and U73122 (Calbiochem).

2.3. BACE1 promoter assay and cDNA construct

For analysis of BACE1 promoter activity, the following two constructs were used; uBACE-1Ka (−1 ∼ −994 bp) and uBACE-2K (+50 ∼ −2100 bp) of human BACE1 gene, as previously reported [9,11]. Numbering system followed previous report [12] (GenBank accession number AY542689). U373MG cells were transfected with the BACE1 promoter constructs using Lipofectamine reagent and Plus reagent (Invitrogen) [9]. For detection of the promoter activity, cell lysates were prepared in Passive Lysis Buffer (Promega) and luciferase assays were performed according to the manufacturer's instructions for Dual Luciferase Assay System (Promega). The signals were measured using auto microplate reader (Infinite M200, Tecan). Transfection efficiency was normalized as described previously [11]. For NFAT4 overexpression, cells were transiently transfected with cDNA constructs encoding NFAT4 (provided from Dr. HD Youn in Seoul National University, Seoul, Korea). DNA amounts were normalized with mock vector.

2.4. Western blot analysis

Cells were lyzed using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS and 50 mM Tris) supplemented with protease inhibitor cocktail and divalent cation chelators (EGTA 1 mM and EDTA 1 mM). Protein extracts were quantified using the bicinchoninic acid (BCA) protein assay solution (Amersham Pharmacia) and equally loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels [13]. For detection of BACE1 levels, anti-BACE1 polyclonal antibody (Calbiochem) or monoclonal antibodies against the C-terminus of BACE1 (Chemicon) were used. Anti-actin antibody (Sigma), anti-NFAT1 (Affinity BioReagents), anti-STAT1 (Upstate Biotechnology) and anti-NFAT4 monoclonal antibody (Santa Cruz Biotechnologies) were used.

2.5. Electrophoresis mobility shift assay (EMSA)

For binding reactions with nuclear extracts from U373MG cells, biotin-labeled probe against the NFAT-binding sequences (TGGAAAAAC) in the human BACE1 promoter region were generated [11]. The BACE1-NFAT probe (5′-biotin-TGCAGCCTG-GAAAAACTCTTC-3′). Nuclear extracts (5 μg) from U373MG cells treated with calcium ionophore were pre-incubated with poly (dI-dC) (1 μg; Sigma) and then reacted with biotin-labeled BACE1-NFAT probes for 30 min at room temperature. To confirm the specificity of interactions, competition assays using non-labeled cold probes were performed. A 100-fold molar excess of cold probes were pre-incubated with nuclear extracts. To detect the novel interaction between NFAT probes and NFAT4 proteins, anti-NFAT4 antibody (2 μg) was pre-added to the extracts for 30 min. Incubated mixtures were analyzed by 5% nondenaturing PAGE as previously described [9,11]. The signals were observed using a Light Super Shifted Module Kit (Pierce) in Bio-Imaging Analyzer (LAS-3000; Fuji).

2.6. Detection of sAPPβ levels

To identify BACE1 enzymatic function, the levels of sAPPβ in conditioned media (CM) were analyzed. For protein precipitation in CM, an equal volume of 20% trichloroacetic acid (Sigma) was subjected to CM and incubated for 24 h in 4 °C and then centrifuged at 10,000*g* for 5 min. The supernatant was removed and pellets were washed with ice-cold acetone (Sigma). Dried pellets were resuspended in RIPA buffer and the protein concentration was estimated using BCA solution (Pierce). The precipitated proteins were analyzed by 8% SDS–PAGE. To recognize the sAPPβ proteins, anti-sAPPβ polyclonal antibody (Signet Laboratories) was used.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software. Differences between groups were examined for statistical analysis using Tukey–Kramer Multiple Comparisons Tests.

3. Results

3.1. Aβ treatment induces BACE1 expression via calcium-related signaling in astrocytes

To test whether Aβ treatment regulates BACE1 promoter activity in astrocytes, the luciferase reporter assay for measuring BACE1 promoter activity was performed. We utilized the pGL3-Basic vector containing the promoter region of human BACE1 gene [9,11] and the human astrocytoma cell line U373MG. After 24 h, the BACE1 promoter was activated in U373MG cells that were stimulated with Aβ1–42 or Aβ25–35, a region of the Aβ peptide that is critically involved in neurotoxicity and aggregation [14] (Fig. 1A). BACE1 promoter activity was elevated by Aβ25–35 treatment in a dose-dependent manner in the concentration range tested (Fig. 1B). To investigate whether Aβ-induced BACE1 promoter activation is mediated by Aβ-stimulated increases in intracellular calcium levels, U373MG cells were pre-treated with BAPTA-AM to chelate intracellular calcium since Aβ has been shown to elevate intracellular calcium concentration in astrocytes [15]. As a result, pre-treatment with BAPTA-AM completely blocked Aβ-induced BACE1 promoter activity (Fig. 1C). We have previously shown that the CaN/NFAT1 signaling pathway, regulated by intracellular calcium levels, modulates BACE1 gene expression in neurons [11]. This led us to pre-treat cells with cyclosporine A (CsA), a calcineurin inhibitor. This treatment resulted in a blockage of Aβ-elicited BACE1 promoter activity (Fig. 1D). Next, to confirm whether Aβ treatment affects BACE1 protein levels in astrocytes, protein extracts from mouse primary astrocytes were analyzed using Western blot analysis. As expected, BACE1 protein expression was not detected in the absence of stimulation, however treatment with Aβ42 or Aβ25–35 potentiated BACE1 protein expression (arrow head, Fig. 1E) and CsA pre-treatment led to down-regulation of Aβ25–35-triggered BACE1 protein expression in astrocytes

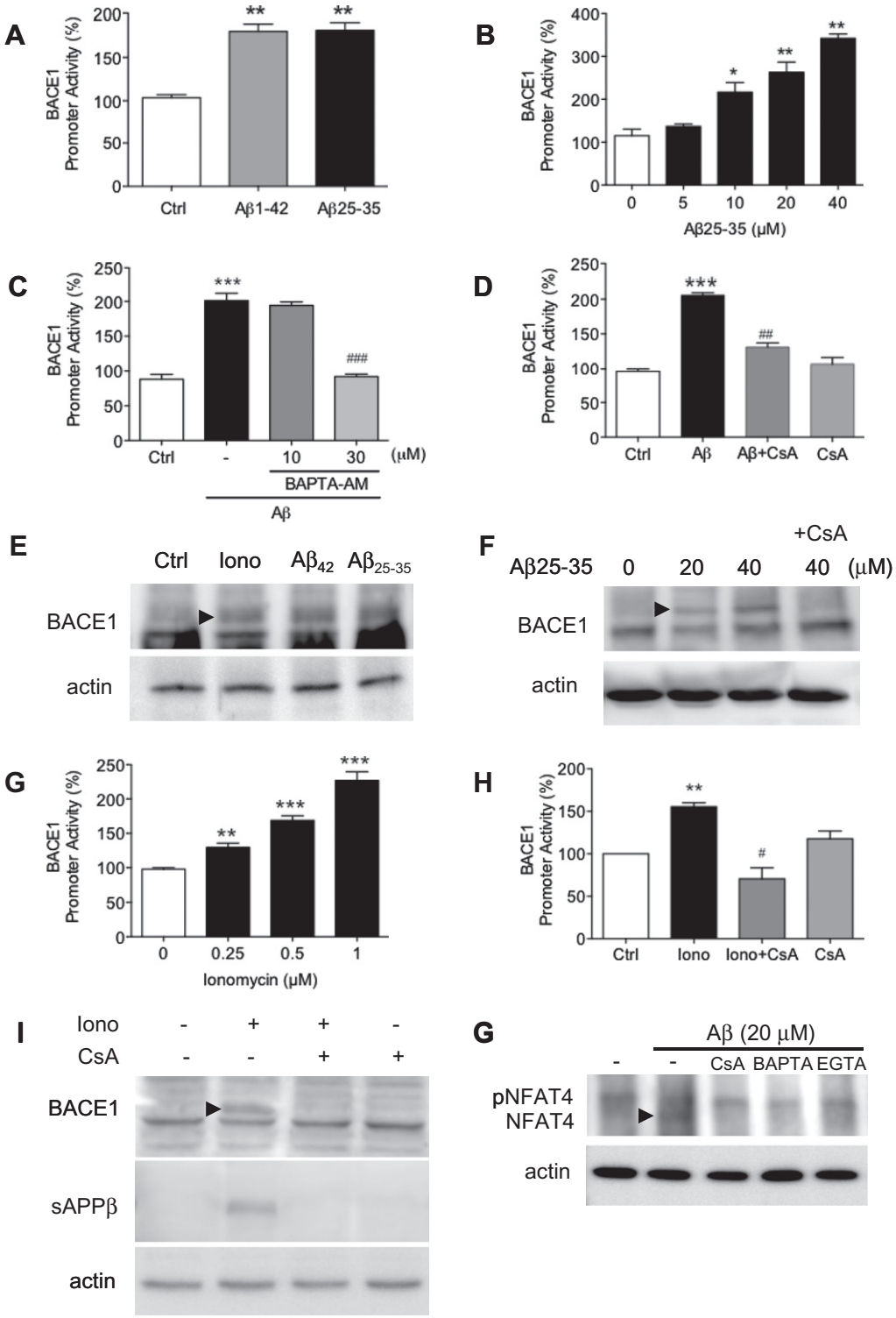

Fig. 1. Aβ treatment stimulates BACE1 gene expression via calcium-related signals in astrocytes. (A and B) BACE1 promoter activity was measured in cells treated for 24 h with either Aβ1–42 (1 μM) or Aβ25–35 (20 μM) (A) or with Aβ25–35 at different concentrations as indicated (B). (C and D) Reporter expressing cells were pre-treated with BAPTA-AM (30 μM) (C) or CsA (1 μM) (D) for 30 min followed by treatment with Aβ25–35 (20 μM) for 24 h and analyzed by luciferase reporter assay. (E and F) BACE1 protein level (arrow head) was analyzed by Western blotting in mouse primary astrocytes treated with 1 μM ionomycin (Iono), 1 μM Aβ1–42 or 20 μM Aβ25–35 for 24 h (E) or with Aβ25–35 with or without the pre-treatment of CsA (1 μM) (F). (G and H) BACE1 promoter activity was measured in cells treated for 24 h with increasing concentrations of ionomycin (Iono) as indicated (G) or with 1 μM ionomycin (Iono) with or without CsA (1 μM) pre-treatment for 30 min (H). (I) Primary astrocytes treated with calcium ionophore (Iono) with or without CsA (1 μM) pre-treatment were analyzed by Western blotting for BACE1 protein expression and sAPPβ secretion. (J) Activation of NFAT4 by dephosphorylation was assessed by Western blotting in Aβ-treated astrocytes with or without CsA or calcium chelators. Data are presented as mean ± SEM of three independent experiments performed in triplicate. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus vehicle-treated control (Ctrl) samples; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus Aβ25–35-treated samples.

(Fig. 1F), suggesting that Aβ-induced elevation of intracellular calcium levels potentiates BACE1 gene expression via calcineurin-related signaling in astrocytes.

Next, to confirm that BACE1 gene expression is stimulated by disrupted calcium homeostasis in U373MG cells, calcium ionophore was applied to the cells. BACE1 promoter activity was upregulated by calcium ionophore treatment in a dose-dependent manner (Fig. 1G) and was prevented by CsA pre-treatment (Fig. 1H). Similar results were observed using primary astrocytes where calcium ionophore treatment stimulated BACE1 protein expression and this stimulation was blocked by CsA pre-treatment (Fig. 1I). Furthermore, increased levels of sAPPβ, (secreted APP) a cleavage product of BACE1, were observed in CM from calcium ionophore-treated U373MG cells and it was abolished by CsA (Fig. 2I).

We next asked whether NFAT4, a transcription factor regulated by Ca^{2+}/CaN, is activated in U373MG cells when intracellular calcium homeostasis is disrupted by calcium ionophore or Aβ treatment. The NFAT4 isoform was increased by 20 μM Aβ25–35 treatment and this increase was prevented by CsA, BAPTA-AM or EGTA pre-treatment in U373MG cells (Fig. 1J), suggesting that astrocytic BACE1 gene expression elicited by Aβ treatment could be involved in NFAT4 activation.

3.2. NFAT4 modulates astrocytic BACE1 levels via direct interaction with human BACE1 promoter region

In our previous study we found that an NFAT binding site exists in the human BACE1 promoter region (−500 to −508 bp) and activated NFAT1 proteins can bind this region in neuronal cells [11]. Since calcium ionophore or Aβ treatment caused dephosphorylation of NFAT4 and not the NFAT1 isoform in astrocytoma cells, it was hypothesized that NFAT4 regulates astrocytic BACE1 gene expression via direct interaction with the specific region in BACE1 promoter. First, we investigated whether uBACE-1Ka (−1 ∼ −994 bp) containing NFAT1 binding sites (−500 to −508 bp) show calcium ionophore-induced BACE1 promoter activity in U373MG cells. We observed elevated promoter activity in U373MG cells transfected with uBACE-1Ka as well as uBACE-2K (+50 ∼ −994 bp) when cells were treated with calcium ionophore (Fig. 2A). Next, to asses whether NFAT4 directly interacts with the BACE1 promoter, EMSA assays were performed. A shifted band, indicative of a complex of NFAT4-DNA, was observed by incubation of labeled NFAT4 probe with nuclear extracts from calcium ionophore-treated U373MG cells (arrowhead in lane 2, Fig. 2B) and it was abolished by pre-incubation of competitor, non-labeled probe (arrow in lane 3, Fig. 2B). Also, the specific band of NFAT4-DNA was ablated in cells pre-treated with CsA (lane 3, Fig. 2C). Next, supershift analysis using specific antibody recognizing NFAT1 or NFAT4 proteins were performed to confirm the specificity of interaction of NFAT4 with the BACE1 promoter region in astrocytoma cells. The shifted band of protein-DNA complex was abolished by pre-incubation with NFAT4-specific antibody (lane 4, Fig. 2D), while incubation with anti-NFAT1 antibody showed no effects on the shifted band (lane 3, Fig. 2D), reflecting that NFAT4, but not NFAT1, interacts with the BACE1 promoter region in U373MG cells. To support this result, we co-transfected constructs encoding NFAT4 or vector alone with uBACE-2K in U373MG cells and observed that BACE1 promoter activity was increased in cells expressing NFAT4 compared with cells expressing vector alone (white bars, Fig. 2E). Furthermore, BACE1 promoter activity was enhanced when cells were stimulated with calcium ionophore (black bars, Fig. 2E). Taken together, these data suggest that astrocytic BACE1 gene

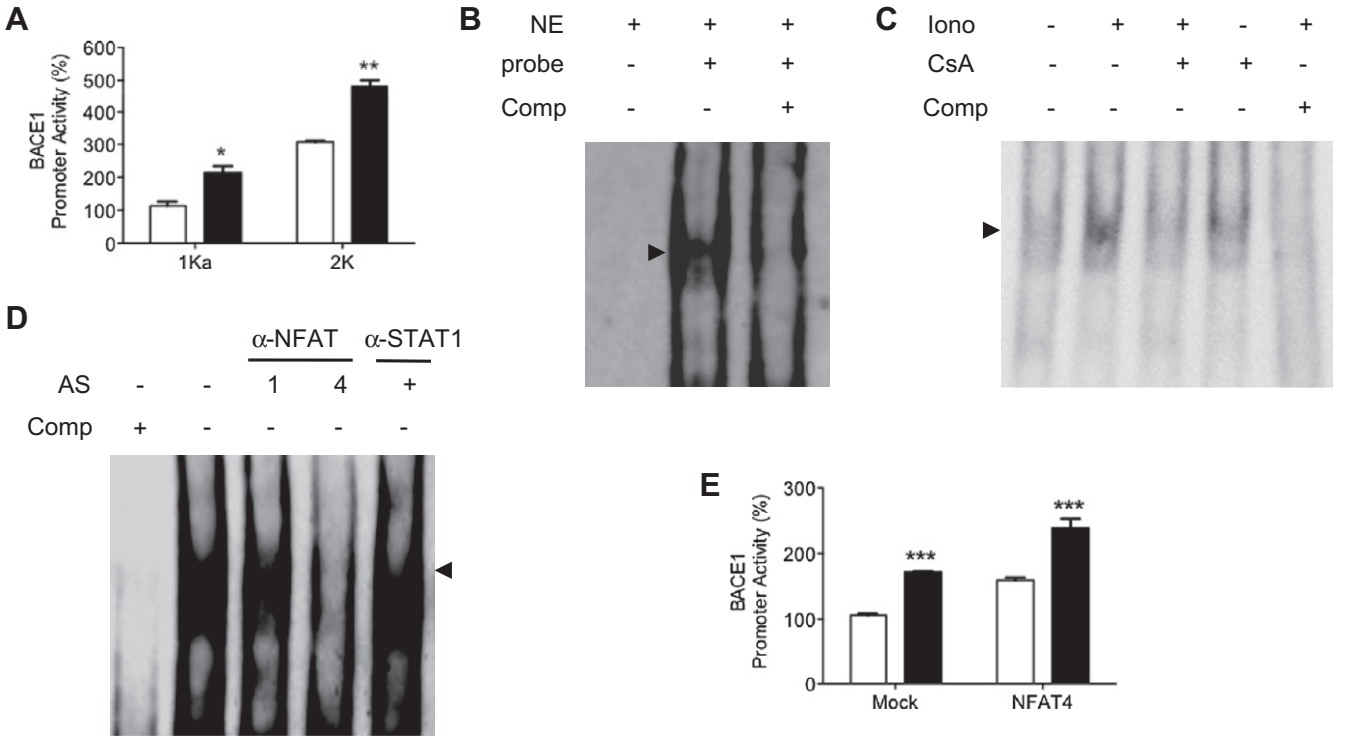

Fig. 2. NFAT4 promotes BACE1 expression via direct interaction with the BACE1 promoter. (A) BACE1 promoter activity was measured in cells transfected with reporter containing uBACE-1Ka (1Ka) or uBACE-2K (2 K) after ionomycin treatment (1 μM for 24 h). Open bars, vehicle-treated; solid bars, ionomycin treated. (B) Binding of NFAT to the BACE1 promoter region was analyzed by EMSA. NE, nuclear extracts; Comp, cold competitor; arrowhead, NFAF-probe complex. (C) NFAT-probe complex (arrowhead) was analyzed by EMSA using nuclear extracts from U373MG cells treated with ionomycin (Iono; 1 μM for 24 h) in the presence or absence of CsA (1 μM). (D) Nuclear extracts were pre-incubated with antibodies as indicated and then analyzed by EMSA. AS, antiserum. (E) BACE1 promoter activity was measured in NFAT4 overexpressing U373MG cells. Open bars, vehicle-treated; solid bars, ionomycin treated. Data are presented as mean ± SEM of three independent experiments performed in triplicate. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

expression induced by disruption of intracellular calcium homeostasis is regulated by NFAT4 activation.

3.3. Aβ-elicited BACE1 expression is mediated by calcium influx in U373MG cells

To demonstrate the mechanism of the Aβ-triggered astrocytic BACE1 gene expression, we asked whether Aβ treatment activates phospholipase C (PLC), which generates inositol trisphosphate (IP_3) that promotes calcium release from ER calcium stores to the cytosol following NFAT1 activation and BACE1 expression in U373MG cells. To inhibit PLC- and IP_3-mediated signaling, U373MG cells were pre-treated with 2APB, an inhibitor of IP_3-dependent calcium release, and U73122, an inhibitor of PLC (Fig. 3A). Aβ-induced BACE1 promoter activity showed no change by 2APB or U73122 pre-treatment, indicating that PLC- and IP_3-mediated signaling pathways do not play a critical role in the Aβ-induced BACE1 gene expression in U373MG cells. We also tested whether ryanodine receptors (RyRs) effect Aβ-induced BACE1 expression, although the expression and function of RYRs in astrocytes is unclear [16]. Cells pre-treated with Dantrolene, a RyR inhibitor, showed no effects on Aβ-elicited BACE1 promoter activity (Fig. 3A). Next, to examine whether Aβ-induced astrocytic BACE1 expression is mediated by calcium influx from external sources, U373MG cells were treated with the calcium chealator, EGTA, resulting in inhibition of Aβ-induced BACE1 promoter activity (Fig. 3B). However, calcium influx by Aβ treatment was not through voltage-dependent calcium channels (VDCC) under our conditions (Fig. 3B). Aβ has been recently shown to form pores in biological membranes as well as artificial membranes and some studies have reported that such pores act as a calcium-permeable channels [17]. These pores can be blocked by Zn^{2+} and clioquinol [15,18]. Abramov et al. found that Aβ25–35-induced calcium influx was abolished by Zn^{2+} and clioquinol in astrocytes [15]. Mirzabekov et al. demonstrated that Aβ25–35 inserted into lipid membranes to form ion-permeable channels that displayed channel activity [19]. In agreement with previous reports, we found that Aβ25–35-elicited BACE1 promoter activity was ablated by pre-treatment with $ZnCl_2$ or clioquinol in U373MG cells (Fig. 3C), suggesting that Aβ-induced astrocytic BACE1 expression is mediated by calcium influx through plasma membrane channels formed by Aβ.

Paralleling the BACE1 promoter activity studies above, $ZnCl_2$ or clioquinol exhibited inhibition effects on Aβ-induced NFAT4 activation but 2APB failed to block Aβ-triggered NFAT4 dephosphorylation in astrocytes (Fig. 3D). In addition, the BACE1 protein levels potentiated by Aβ25–35 treatment were down-regulated by $ZnCl_2$ but not 2APB in primary astrocytes (Fig. 3E), showing an association of BACE1 expression with calcium influx through channel formed by the membrane-inserted Aβ, not with calcium release from ER store in astrocytes.

3.4. Anti-BACE1 immunoreactivity in calcineurin-positive astrocytes surrounding amyloid plaques in APP/PS1 transgenic mice

To assess whether BACE1 protein expression in reactive astrocytes in close proximity to plaques is involved in calcineurin signaling, immunohistochemistry was performed using APP/PS1 double transgenic mouse brains. First we analyzed immunoreactivity for calcineurin in reactive astrocytes. We observed anti-calcineurin immuno-signals in cells showing reactive astrocyte like shape (Fig. 4A). Anti-BACE1 immuno-signals (arrows) were also detected in cells that have reactive astrocyte like shape near the plaque (star) (Fig. 4B). Also, using double immunostaining against calcineurin and BACE1, we found that calcineurin-positive reactive

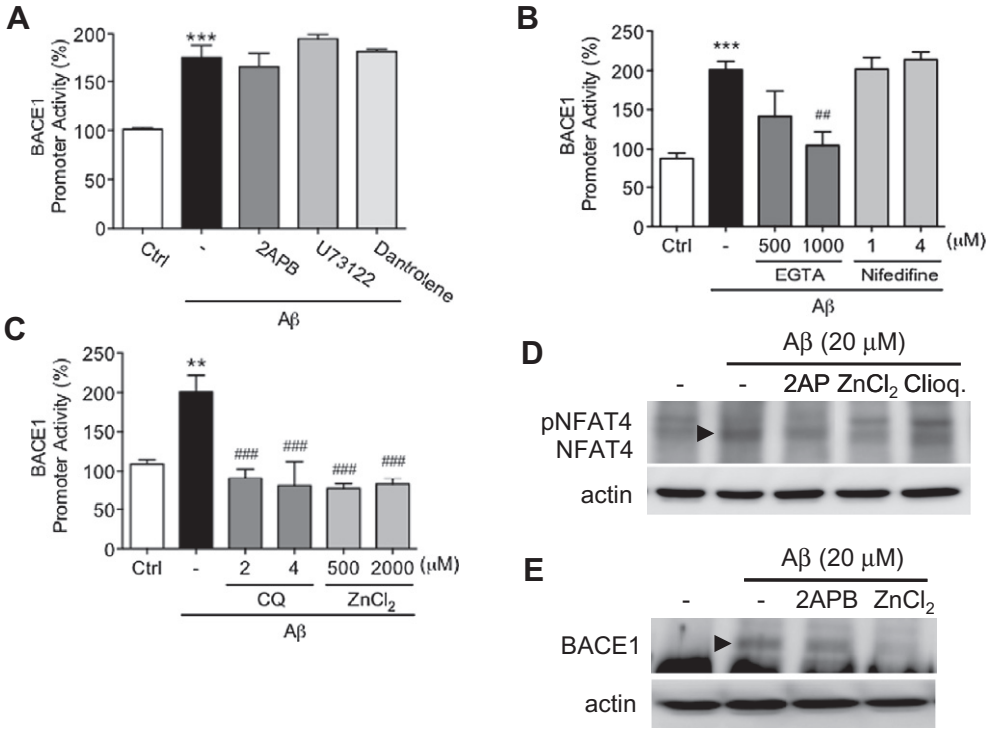

Fig. 3. Aβ-induced BACE1 expression and NFAT4 activation are mediated by calcium influx in astrocytes. (A–C) BACE1 promoter activity was measured in cells treated with Aβ25–35 (20 μM, 24 h) in combination with 2APB (PLC inhibitor), U73122 (IP_3R inhibitor), or dantrolene (RYR inhibitor) in the presence of EGTA or nifedipine (VDCC blocker) (A), clioquinol (CQ) (B) or $ZnCl_2$ (C). (D) NFAT4 activation was analyzed by Western blotting using primary astrocytes treated with Aβ25–35 (20 μM, 24 h) in combination with 2APB, $ZnCl_2$, or clioquinol. Arrowhead; active dephosphorylated NFAT4. (E) BACE1 protein levels were analyzed in primary astrocytes treated with Aβ25–35 (20 μM, 24 h) in combination with 2APB or $ZnCl_2$. Arrowhead; BACE1. Data are presented as mean ± SEM of three independent experiments performed in triplicate. $^{**}p < 0.01$; $^{***}p < 0.001$ versus vehicle-treated control (Ctrl) samples; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus Aβ25–35-treated samples.

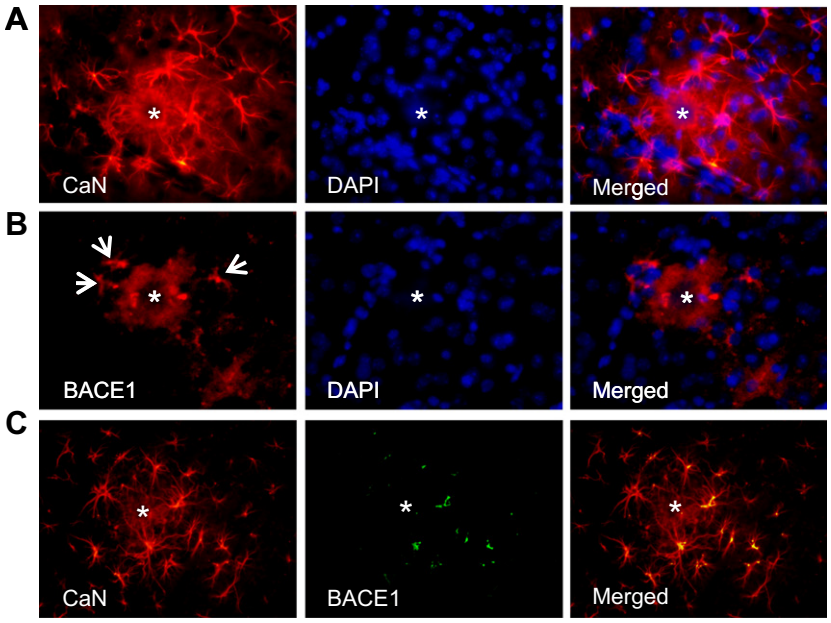

Fig. 4. Co-localization of calcineurin and BACE1 in reactive astrocytes from APP/PS1 Tg mice. (A–C) Brain sections from APP/PS1 double transgenic mouse (12 months) were immunostained with antibodies against calcineurin and/or BACE1 as indicated. Arrows, BACE1; stars, center of β-amyloid plaque.

astrocytes showed BACE1 protein expression in brains, indicating that the calcineurin signal is associated with astrocytic BACE1 protein expression *in vivo*.

4. Discussion

Several studies have found BACE1 expression in reactive astrocytes around senile plaques in AD model mouse brains [5]. Many groups have examined astrocytic BACE1 expression by indirect effects of plaques such as inflammatory responses or oxidative stress-related molecules, which are observed in close proximity to amyloid plaques [7–9]. Because astrocytes show BACE1 levels around Aβ plaques, we favor the hypothesis that Aβ directly triggers the astrocytic BACE1 expression. In this study, we found that both the full-length Aβ42 peptide and the neurotoxic Aβ25–35 fragment stimulated BACE1 gene expression in primary astrocytes as well as the U373MG cell line at the transcription level. Aβ has been shown to disturb intracellular calcium homeostasis that may alter calcium-related signal transduction pathways [20]. Abramov et al. have showed that intracellular calcium levels are increased in astrocytes exposed to Aβ [15]. Moreover, we addressed whether Aβ-induced BACE1 gene expression is mediated by the calcium-related cellular signaling pathway CaN/NFAT4 in astrocytes. Aβ has been reported to increase intracellular calcium concentration by calcium influx through calcium-permeable channels formed by Aβ and those channels are Zn^{2+}-sensitive [17,18]. The mechanism Aβ pore blockage by Zn^{2+} in astrocytes is unclear, however some studies have shown that Zn^{2+} may block the channels or prevent their formation [15,18]. In addition to Zn^{2+}, clioquinol, a chelator of Cu^{2+}, Zn^{2+}, and Fe^{2+}, is known to prevent Aβ aggregation following inhibition of Aβ pore generation [21,22]. Abramov et al. also reported that Aβ25–35 treatment stimulates calcium influx that was blocked by Zn^{2+} or clioquinol in astrocytes [15]. In our experiments, Zn^{2+} or clioquinol showed striking blocking effect on Aβ-stimulated NFAT4 activation in primary astrocytes as well as U373MG cells. Also, Aβ-induced astrocytic BACE1 gene expression was completely inhibited by Zn^{2+} or clioquinol. Although conformation of Aβ pore formation in our experimental conditions was not assessed, our data are consistent with previous reports and suggest that Aβ treatment modulates NFAT4 activation and BACE1 gene expression via calcium influx through calcium permeable channels formed by Aβ in astrocytes. In contrast, inhibitors of PLC/IP_3 signaling cascade such as 2APB and U73122, failed to block Aβ-induced BACE1 expression and NFAT4 activation. Also nifedipine pre-treatment showed no effects on Aβ-induced events.

Liu et al. showed activation of calcineurin in the brains of AD patients suggesting that CaN/NFAT signaling cascade plays an important role in AD pathogenesis [23]. Indeed, Norris et al. found that the levels of calcineurin were up-regulated in reactive astrocytes around senile plaques in AD model mouse brains [24], supporting our results that neurotoxic Aβ42 or Aβ25–35 stimulates NFAT4 activation and BACE1 gene expression in astrocytes.

Previously, we found that the CaN/NFAT1 signal pathway modulated BACE1 gene expression in neurons [11]. Neuronal BACE1 was also up-regulated by Aβ-induced calcineurin/NFAT1 activation, although we did not identify the source of increased calcium levels [11]. Taken together, stimulation with Aβ appears to elevate BACE1 levels in both neurons and astrocytes at the transcriptional level. CaN/NFAT signaling pathways play a central role in Aβ-induced BACE1 expression following Aβ generation, although the Aβ-activated NFAT isoform showed cell-type specificity. Even though cell-type specific NFAT isoforms exist, both NFAT1 and NFAT4 are modulated by specific inhibitors of calcineurin, CsA and FK506. These observations identify an important function of CaN/NFAT signal transduction in BACE1 expression and suggest a new drug target for AD. Since it has been reported that disruption of intracellular calcium homeostasis in neurons and astrocytes is a crucial factor in AD pathogenesis, preventing disturbances in calcium-related signals may be an attractive drug target for AD therapy and prevention.

Acknowledgments

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References

[1] M. Citron, Strategies for disease modification in Alzheimer's disease, Nat. Rev. Neurosci. 5 (2004) 677–685.
[2] B. De Strooper, W. Annaert, Proteolytic processing and cell biological functions of the amyloid precursor protein, J. Cell Sci. 113 (Pt 11) (2000) 1857–1870.
[3] S. Sinha, J.P. Anderson, R. Barbour, et al., Purification and cloning of amyloid precursor protein beta-secretase from human brain, Nature 402 (1999) 537–540.
[4] R. Yan, M.J. Bienkowski, M.E. Shuck, et al., Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity, Nature 402 (1999) 533–537.
[5] H.S. Hong, E.M. Hwang, H.J. Sim, et al., Interferon gamma stimulates beta-secretase expression and sAPPbeta production in astrocytes, Biochem. Biophys. Res. Commun. 307 (2003) 922–927.
[6] S. Rossner, J. Apelt, R. Schliebs, et al., Neuronal and glial beta-secretase (BACE) protein expression in transgenic Tg2576 mice with amyloid plaque pathology, J. Neurosci. Res. 64 (2001) 437–446.
[7] I. Blasko, R. Veerhuis, M. Stampfer-Kountchev, et al., Costimulatory effects of interferon-gamma and interleukin-1beta or tumor necrosis factor alpha on the synthesis of Abeta1-40 and Abeta1-42 by human astrocytes, Neurobiol. Dis. 7 (2000) 682–689.
[8] M. Sastre, I. Dewachter, G.E. Landreth, et al., Nonsteroidal anti-inflammatory drugs and peroxisome proliferator-activated receptor-gamma agonists modulate immunostimulated processing of amyloid precursor protein through regulation of beta-secretase, J. Neurosci. 23 (2003) 9796–9804.
[9] H.J. Cho, S.K. Kim, S.M. Jin, et al., IFN-gamma-induced BACE1 expression is mediated by activation of JAK2 and ERK1/2 signaling pathways and direct binding of STAT1 to BACE1 promoter in astrocytes, Glia 55 (2007) 253–262.
[10] A. Palotas, J. Kalman, M. Palotas, et al., Fibroblasts and lymphocytes from Alzheimer patients are resistant to beta-amyloid-induced increase in the intracellular calcium concentration, Prog. Neuropsychopharmacol. Biol. Psychiatry 26 (2002) 971–974.
[11] H.J. Cho, S.M. Jin, H.D. Youn, et al., Disrupted intracellular calcium regulates BACE1 gene expression via nuclear factor of activated T cells 1 (NFAT 1) signaling, Aging Cell 7 (2008) 137–147.
[12] Y.W. Ge, B. Maloney, K. Sambamurti, et al., Functional characterization of the 5′ flanking region of the BACE gene: identification of a 91 bp fragment involved in basal level of BACE promoter expression, FASEB J. 18 (2004) 1037–1039.
[13] S.M. Jin, H.J. Cho, E.S. Jung, et al., DNA damage-inducing agents elicit gamma-secretase activation mediated by oxidative stress, Cell Death Differ. 15 (2008) 1375–1384.
[14] C.J. Pike, A.J. Walencewicz-Wasserman, J. Kosmoski, et al., Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25–35 region to aggregation and neurotoxicity, J. Neurochem. 64 (1995) 253–265.
[15] A.Y. Abramov, L. Canevari, M.R. Duchen, Changes in intracellular calcium and glutathione in astrocytes as the primary mechanism of amyloid neurotoxicity, J. Neurosci. 23 (2003) 5088–5095.
[16] M. Matyash, V. Matyash, C. Nolte, et al., Requirement of functional ryanodine receptor type 3 for astrocyte migration, FASEB J. 16 (2002) 84–86.
[17] H. Lin, R. Bhatia, R. Lal, Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology, FASEB J. 15 (2001) 2433–2444.
[18] N. Arispe, H.B. Pollard, E. Rojas, Zn^{2+} interaction with Alzheimer amyloid beta protein calcium channels, Proc. Natl. Acad. Sci. USA 93 (1996) 1710–1715.
[19] T. Mirzabekov, M.C. Lin, W.L. Yuan, et al., Channel formation in planar lipid bilayers by a neurotoxic fragment of the beta-amyloid peptide, Biochem. Biophys. Res. Commun. 202 (1994) 1142–1148.
[20] M.P. Mattson, S.L. Chan, Dysregulation of cellular calcium homeostasis in Alzheimer's disease: bad genes and bad habits, J. Mol. Neurosci. 17 (2001) 205–224.
[21] R.A. Cherny, C.S. Atwood, M.E. Xilinas, et al., Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice, Neuron 30 (2001) 665–676.
[22] S. Melov, '...and C is for Clioquinol' – the AbetaCs of Alzheimer's disease, Trends Neurosci. 25 (2002) 121–123. discussion 123–124..
[23] F. Liu, I. Grundke-Iqbal, K. Iqbal, et al., Truncation and activation of calcineurin A by calpain I in Alzheimer disease brain, J. Biol. Chem. 280 (2005) 37755–37762.
[24] C.M. Norris, I. Kadish, E.M. Blalock, et al., Calcineurin triggers reactive/inflammatory processes in astrocytes and is upregulated in aging and Alzheimer's models, J. Neurosci. 25 (2005) 4649–4658.