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Calnuc binds to Alzheimer's β-amyloid precursor protein and affects its biogenesis

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Abstract

Calnuc, a Golgi calcium binding protein, plays a key role in the constitution of calcium storage. Abnormal calcium homeostasis has been linked to Alzheimer's disease (AD). Excessive production and/or accumulation of β-amyloid (Aβ) peptides that are proteolytically derived from the β-amyloid precursor protein (APP) have been linked to the pathogenesis of AD. APP has also been indicated to play multiple physiological functions. In this study, we demonstrate that calnuc interacts with APP through direct binding to the carboxyl-terminal region of APP, possibly in a calcium-sensitive manner. Immunofluorescence study revealed that the two proteins co-localize in the Golgi in both cultured cells and mouse brains. Over-expression of calnuc in neuroblastoma cells significantly reduces the level of endogenous APP. Conversely, down-regulation of calnuc by siRNA increases cellular levels of APP. Additionally, we show that over-expression of calnuc down-regulates the APP mRNA level and inhibits APP biosynthesis, which in turn results in a parallel reduction of APP proteolytic metabolites, sAPP, CTFs and Aβ. Furthermore, we found that the level of calnuc was significantly decreased in the brain of AD patients as compared with that of age-matched non-AD controls. Our results suggest a novel function of calnuc in modulating the levels of APP and its proteolytic metabolites, which may further affect the patho/physiological functions of APP including AD pathogenesis.

Keywords: Alzheimer's disease, amyloid precursor protein, calcium, calnuc, Golgi.
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One key pathologic feature of Alzheimer's disease (AD) is the formation of extracellular senile plaques whose major components are the heterogeneous β-amyloid (Aβ) species. Aβ is proteolytically derived from the β-amyloid precursor protein (APP) through sequential cleavage by β-secretase (BACE1) and the presenilin/γ-secretase complex. Alternatively, APP can be cleaved by α-secretase to generate non-amyloidogenic soluble APPα (Greenfield *et al.* 2000; Cao and Sudhof 2001; Selkoe 2001; von Rotz *et al.* 2004; Vetrivel *et al.* 2006). Along with Aβ generation, cleavages by the α-secretase, BACE1 and presenilin/γ-secretase also produce APP C-terminal fragments (CTF), namely αCTF, βCTF, and γCTF (a non-membrane anchored APP intracellular domain, AICD), respectively. Full-length APP is synthesized in the endoplasmic reticulum (ER) and transported through the Golgi apparatus. The major population of secreted Aβ in neurons is generated within the trans-Golgi network (TGN), an organelle where the majority of APP resides at steady state (Hartmann *et al.* 1997; Xu *et al.* 1997; Greenfield *et al.* 1999). The neurotoxic Aβ peptides have been shown to accelerate the formation of neurofibrillary tangles of tau, another hallmark of AD, and to trigger a cascade of pathogenic events such as calcium influx involving excitoactivation of glutamate/NMDA receptors and dystrophy of neuritis, culminating in neuronal apoptosis/death (Gotz *et al.* 2001; Lewis *et al.* 2001; Bossy-Wetzel *et al.* 2004; Han *et al.* 2005).

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Abbreviations used: AD, Alzheimer's disease; AICD, APP intracellular domain; APP, β-amyloid precursor protein; Aβ, amyloid-β peptide; CNG, calnuc-GFP; Co-IP, co-immunoprecipitation; CTF, C-terminal fragment; ER, endoplasmic reticulum; GFP, green fluorescence protein; GST, glutathione S-transferase; sAPP, soluble APP ectodomain; TGN, trans-Golgi network; wt, wild type.

Recently, the pathophysiological functions of APP CTFs (including AICD) have attracted much scrutiny. These functions include impairing neuronal activities pertinent to memory and learning through blocking LTP; triggering inflammation through MARK, NF-kappaB, and inducible nitric oxide synthase; disturbing calcium homeostasis; and modulating transcription of genes including glycogen synthase kinase-3β and APP (Kim *et al.* 2003; von Rotz *et al.* 2004; Chang and Suh 2005). Several APP-associated proteins such as Fe65, X11α, histone acetyltransferase Tip60, CP2, Shc, Numb and 14-3-3γ have been recently identified to bind to APP CTF/AICD, translocating AICD into nucleus and thus affecting gene expression (Cao and Sudhof 2001; Chang and Suh 2005; Muresan and Muresan 2005; Sumioka *et al.* 2005). Therefore, identifying novel APP-binding partners is important for our understanding of cellular mechanisms and pathways underlying APP functions.

In addition to the accumulation/aggregation of Aβ in the brain, many other factors, such as an imbalanced Ca^{2+} homeostasis, have also been suggested to contribute to AD pathology (LaFerla 2002; Mattson 2004; Braunewell 2005). The major intracellular compartment for Ca^{2+} storage is the ER, which has been studied extensively. The ER Ca^{2+} pool is known to be maintained by organelle-associated Ca^{2+} ATPase (Ca^{2+} pumps) and a bunch of luminal Ca^{2+}-binding proteins (Bastianutto *et al.* 1995; Meldolesi and Pozzan 1998). Recently, the Golgi complex has also been identified as a Ca^{2+}-enriched compartment, however, information regarding the mechanisms for Ca^{2+} uptake, storage, and release from the Golgi remain to be elucidated (Chandra *et al.* 1991; Grohovaz *et al.* 1996; Pezzati *et al.* 1997). Moreover, the Golgi complex is well known to be involved in post-translational modification of newly synthesized proteins and serves as the main sorting station for protein and vesicular traffic (Farquhar and Palade 1998).

Calnuc (nucleobindin) is an EF-hand Ca^{2+}-binding protein (Miura *et al.* 1992; Wendel *et al.* 1995). It has been found that calnuc is a major Ca^{2+}-binding protein in the Golgi which plays an important role in establishing an agonist-releasable Ca^{2+} store in the Golgi lumen (Lin *et al.* 1998, 1999). In addition, calnuc has been shown to be a minor constituent of bone extracellular matrix and may play a role in mineralization (Petersson *et al.* 2004; Somogyi *et al.* 2004). There are two pools of calnuc: one associated with the luminal surface of Golgi membranes and the other in the cytosol. Cytosolic calnuc interacts with Gαi subunits (Lin *et al.* 2000; Weiss *et al.* 2001), whereas membrane-associated calnuc can be anchored on the Golgi luminal membrane for up to 24 h, and subsequently secreted along the constitutive-like pathway (Lavoie *et al.* 2002). In the present study, we explore potential functions of calnuc in relation to AD pathogenesis by investigating its interaction with APP and effects on APP biogenesis/metabolism.

Experimental procedures

Materials

Polyclonal antibody against full-length, recombinant calnuc (F-5059) was generated and affinity purified as previously described for immunoblotting and immunoprecipitation (Lin *et al.* 1998). Affinity purified rabbit anti-calnuc IgG raised against the C-terminal 14 amino acids of rat/mouse calnuc (EQPPVLPQLDSQHL) or human calnuc (LLERLPEVEVPQHL) was obtained from AVIVA System Biology (San Diego, CA, USA) and used for immunofluorescence and immunohistochemistry assays. Chicken anti-calnuc antibody was from Genway Biotech (San Diego, CA, USA). Polyclonal antibody 369 against the C-terminal region of APP was developed in our lab (Buxbaum *et al.* 1990; Xu *et al.* 1997). Monoclonal antibodies 4G8 and 6E10 against Aβ were from Signet Laboratories (Dedham, MA, USA). Mouse anti-α-tubulin antibody was from Sigma (St. Louis, MO, USA). Highly cross-adsorbed Alexa Fluor® 488 or 594-conjugated $F(ab')_2$ fragments of goat anti-chicken, goat anti-mouse or goat anti-rabbit IgG (H + L) were from Molecular Probes (Eugene, OR, USA). Affinity purified goat anti-mouse and goat anti-rabbit IgG (H + L) conjugated to horseradish peroxidase were from Bio-Rad (Hercules, CA, USA). FITC conjugated anti-IgY was obtained from Chemicon (Temecula, CA, USA). Green fluorescent protein (GFP) cDNA was kindly provided by Dr Roger Tsien (University of California, San Diego, CA, USA). TrueBlot IP beads and antibody were purchased from eBiosciences (San Diego, CA, USA). Supersignal chemiluminescent reagent was purchased from Pierce (Rockford, IL, USA).

Cell cultures

N2a cells were maintained in cell culture medium containing 50% DME high glucose medium (Irvine Scientific, Santa Ana, CA, USA) and 50% OptiMEM medium supplemented with 5% FBS (Invitrogen, Carlsbad, CA, USA). For N2a cells stably expressing human APP (N2a-APP), the cell culture medium contains G418 (0.2 mg/mL) (Calbiochem, San Diego, CA, USA). For N2a-APP cells stably expressing calnuc-GFP or GFP, additional Zeocin (0.2 mg/mL) (Invitrogen, Carlsbad, CA, USA) was added. All media contained 100 U/mL of penicillin G and 100 μg/mL of streptomycin sulfate. Cells were used as 80% confluent monolayers for transfection.

Establishment of stable cell lines co-expressing β-amyloid precursor protein and calnuc-green fluorescent protein or green fluorescent protein using flow cytometry

The protocol is similar to that previously published (Lin *et al.* 1999). Briefly, Calnuc-GFP or GFP cDNA subcloned in the pcDNA3.1/Zeo (Invitrogen, Carlsbad, CA, USA) were transfected into previously established N2a-APP cells. Following selection with both G418 (0.2 mg/mL) and Zeocin (0.2 mg/mL) for 1 week, cells were subjected to FACS sorter (Ex/Em: 488/530 ± 15) (FACStar Plus®; Becton Dickinson, Franklin Lakes, NJ, USA). The top 0.1% of the positive cells was collected and maintained in culture medium. Selection by sorting was repeated thrice until 100% of the cells expressed calnuc-GFP or GFP (data not shown).

Subcellular fractionation

Membrane (100 000 *g* pellets) and cytosolic fractions (100 000 *g* supernatants) were prepared by ultracentrifugation of post-nuclear

supernatants from cells and analyzed by immunoblotting and ECL (Lin *et al.* 1998).

Precipitation of [^{35}S]-labeled β-amyloid precursor protein using glutathione S-transferase-calnuc

Pull-down protocol was similar to that published previously (Lin *et al.* 1998). Briefly, APP cDNA was subcloned into pcDNA3.1/Zeo vector at Hind III/Xba I sites. *In vitro* transcription/translation of APP from the T7 promoter was performed using the TNT-coupled reticulocyte lysate system (Promega, Madison, WI, USA) in the presence of [^{35}S]-methionine (*in vivo* cell labeling grade; Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) according to the manufacturer's instructions. Purified GST-calnuc fusion protein or GST control (6 μg) was immobilized on glutathione-Sepharose beads and incubated with 15 000 cpm [^{35}S]-labeled, *in vitro*-translated APP in binding buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% NP-40, 1 mmol/L DTT, and protease inhibitors). The mixture was incubated by rotating for 2 h at 4°C. The beads were washed thrice in the same binding buffer, resuspended in 25 μL of Laemmli buffer, boiled for 5 min, and then the proteins were loaded on 10% SDS gels and exposed for autoradiography.

Co-immunoprecipitation

Co-immunoprecipitation of APP with anti-calnuc antibody or vice versa was similar to that previously described (Lin *et al.* 2000). Briefly, cells (1×10^7 cells/100-mm dish) were lysed in 300 μL co-immunoprecipitation (coIP) buffer (0.5% Triton X-100/in Tris-buffered saline, pH 7.4) containing either 2 mmol/L Ca^{2+} or 5 mmol/L EDTA in the presence of protease inhibitors for 30 min at 4°C. After centrifugation (16 000 *g* for 15 min), the lysates were pre-cleared with pre-immune serum coupled to Protein A-Sepharose CL-4B and subsequently were incubated with pre-immune serum, anti-calnuc antibody, or anti-APP antibody 369, with further, additional TrueBlot IP beads (eBiosciences, San Diego, CA, USA), following the manufacturer's protocol. Beads were washed four times (5 min each) at 4°C with coIP buffer. Eluted proteins were separated by SDS-PAGE, followed by immunoblotting with corresponding antibodies plus TrueBlot HRP-IgG, and enhanced chemiluminescence (ECL) analysis (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

Immunofluorescence

The protocol is essentially the same as that previously published by Lin *et al.* 1999 and Zhang *et al.* 2005; For immunofluorescence in cell cultures, parental N2a cells were fixed in 2% paraformaldehyde in phosphate buffer and permeabilized as previously described (Lin *et al.* 1998; Lavoie *et al.* 2002). They were then incubated with polyclonal anti-calnuc antibody and monoclonal anti-APP antibody 6E10 at 4°C for 3 h, followed by incubation with corresponding Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse conjugates. Specimens were analyzed with an Applied Precision DeltaVision imaging system (Issaquah, WA, USA) coupled to a Zeiss S100 fluorescence microscope (Carl Zeiss; Thornwood, NY, USA). Cross-sectional images of cells were obtained with 150-nm step width to optimize reconstruction of the center plane image. De-convolution was done on a Silicon Graphics Octane® visual workstation (SGI, Mountain View, CA, USA) equipped with Delta Vision reconstruction software. For immunofluorescence in mouse brains, fresh sections from C57Bl/6 mouse at 3 months of age were utilized. After permeabilization, the sections were incubated with chicken anti-calnuc antibody and polyclonal anti-APP antibody 369 at 4°C overnight, followed by incubation with corresponding Alexa fluor 488 goat anti-chicken and Alexa fluor 594 goat anti-rabbit conjugates. Samples were examined by confocal microscopy (LSM510, Zeiss, Thornwood, NY, USA) (Han *et al.* 2005).

Yeast-two-hybrid

Three APP cDNA fragments covering the ectodomain region (amino acids 18–627), the transmembrane region (amino acids 597–639), and the intracellular domain (amino acids 646–695), respectively, were generated by PCR with appropriate primers (sequences available on request). These fragments were subcloned in pGBKT7 (bait) vector (BD Biosciences, San Jose, CA, USA). Calnuc/pACT2 AD (prey) was constructed as described (Lin *et al.* 1998). All constructs were verified by automated DNA sequencing (Center for AIDS Research, University of California, San Diego, USA). For one-to-one interaction, APP/pGBKT7 DNA and calnuc/pACT2 DNA were co-transformed into competent yeast cells (strain AH109). Transformed yeast cells were selected on QDO selection plates which lack histidine, leucine, tryptophan and adenine.

siRNA transfection

The experiment was performed on a 6-well culture plate. Two siRNA oligos designed to knock out calnuc were purchased from Dharmacon (Chicago, IL, USA). For each well, cells were transfected with 4 μL of equally mixed siRNA 1 (70 nmol) and siRNA 2 (70 nmol) using 12 μL of lipofectamine (Invitrogen, Carlsbad, CA, USA) for 72 h. Cells were subsequently subjected to further analysis.

Pulse-chase of β-amyloid precursor protein

To assay APP metabolism, N2a-APP cells expressing calnuc-GFP or GFP alone were pulse-labeled with [^{35}S]methionine (500 μCi/mL) for 5 min at 37°C and collected for analysis. In some experiments, cells were first labeled for 15 min and then washed with phosphate-buffered saline and chased for indicated time. Cell lysates were immunoprecipitated with anti-APP antibody 369, followed by SDS-PAGE analysis and autoradiography (Cai *et al.* 2003).

Quantitative real-time polymerase chain reaction

To study whether calnuc affects the transcription of *APP* gene, we transiently transfected parental N2a cells with calnuc-GFP or GFP vector. After 2 days, cells were subjected to cell sorting. The top 5% of the positive cells was collected and maintained in media for 2 days. Total RNA was then extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). SuperScript First-Strand kit (Invitrogen, Carlsbad, CA, USA) was used to synthesize first strand cDNA from the samples with an equal amount of RNA according to the manufacturer's instruction. Equal amounts of synthesized cDNAs were amplified using IQ™ SYBR green supermix and ICycler from Bio-Rad (Hercules, CA, USA); and the data were analyzed using Bio-Rad MyIQ 2.0. Primers used for *APP* amplification were APP-5, 5′ AAGCACCGAGAGAGAATGT 3′ and APP-3, 5′ AATGCTTTAGGGTGTGCTG 3′. Primers used for

β-actin amplification were actin-5, 5′ AGCCATGTACGTAGCCATCC 3′ and actin-3, 5′ CTCTCAGCTGTGGTGGTGAA 3′. The level of *APP* mRNA was normalized to that of *β-actin*. Three independent experiments were performed and statistical analysis was carried out using Student's *t*-test.

Immnohistochemistry

Post-mortem brain materials from AD patients and healthy controls were obtained from University of California, San Diego. Three definite AD cases and three matched controls were used. The scores of mini mental state examination (MMSE) for the three AD patients were all less than 10 and the scores of MMSE for non-AD controls were 28–30. C57Bl/6 mouse brain sections were taken from 6-month-old animals. Paraffin-embedded sections of human brain frontal cortex regions and whole mouse brains were de-paraffinized, pre-treated with 3% H_2O_2, and incubated with polyclonal anti-calnuc antibody overnight at 4°C. Later, sections were incubated with biotinylated horse anti-rabbit antibody for 1 h at room temperature (25°C) and then incubated in ABC-Elite (HRP) reagent (Vector Laboratories, Burlingame, CA, USA) for another 1 h. Reactions were visualized by developing in DAB substrates (Vector Laboratories, Burlingame, CA, USA). All samples were visualized under light microscope. For quantification purposes, calnuc-positive neurons and the total neurons (counting based on cell morphology) were counted from five random fields in each case (three AD and three controls) and averaged. The ratio of calnuc-positive neurons over the total neurons in AD samples was calculated in each case and compared with that of age-matched non-AD controls.

Results

Calnuc binds to β-amyloid precursor protein and the interaction is likely Ca^{2+}-sensitive

It has been reported that there are two pools of calnuc which are cytosolic and membrane-associated. We first studied the distribution pattern of calnuc in neuroblastoma N2a cells. Post-nuclear supernatants of cell lysates were centrifuged to separate membrane and cytosolic fractions. Similar to what was reported previously in other cell types (Lin *et al.* 1998, 1999), we found that endogenous calnuc in N2a cells (Fig. 1a) distributed both on the membrane (P) and in cytosolic fraction (S). But the level of calnuc in the membrane was much higher (about 3.5-folds) than that in the cytosol. Furthermore, comparing with those in N2a cells, the distribution pattern and level of calnuc were not significantly changed in N2a cells stably over-expressing human APP (N2a-APP) (Fig. 1a), suggesting that over-expression of APP does not affect the distribution/level of calnuc.

To study whether calnuc interacts with APP, we incubated [^{35}S]-labeled, *in vitro*-translated APP with immobilized GST-calnuc fusion protein or GST control, in the presence of 2 mmol/L calcium or 5 mmol/L EDTA. As shown in Fig. 1b, we found that APP binds to GST-calnuc but not GST; and immobilized GST-calnuc pulled down slightly more APP in the presence of EDTA. To confirm the binding of calnuc to

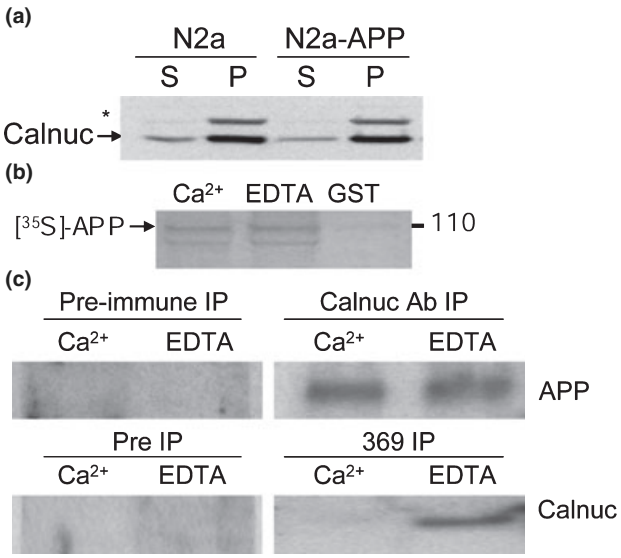

Fig. 1 Calnuc interacts with β-amyloid precursor protein (APP) (a). Calnuc is endogenously expressed in N2a cells. Post-nuclear supernatants prepared from parental N2a cells or N2a cells stably expressing human APP (N2a-APP) were centrifuged to separate cytosolic (S) and membrane (P) fractions, followed by Western analysis using polyclonal anti-calnuc antibody to detect endogenous calnuc. Asterisk indicates non-specific band. (b). Binding of immobilized GST-calnuc fusion proteins with [^{35}S]-labeled, *in vitro*-translated APP in the presence of added Ca^{2+} or EDTA. (c) Co-IP of calnuc and APP in N2a cells. Cell lysates were immunoprecipitated with anti-calnuc antibody or anti-APP antibody 369 in the presence of either 2 mmol/L Ca^{2+} or 5 mmol/L EDTA, followed by immunoblotting using 369 (upper panels) or anti-calnuc antibody (lower panels), respectively. Lysates immunoprecipitated with pre-immune serum were used as the negative control.

APP *in vivo*, co-IP was performed. In N2a cells, anti-calnuc antibody co-immunoprecipitated APP in the presence of calcium and EDTA; the binding affinity is slightly higher in the presence of EDTA (Fig. 1c, upper panels). However, anti-APP antibody 369 co-immunoprecipitated calnuc in the presence of EDTA but failed to co-immunoprecipitate calnuc in the presence of Ca^{2+} (Fig. 1c, lower panels). These results confirm the interaction between APP and calnuc, and suggest that the interaction is likely sensitive to Ca^{2+}. The potential involvement of calcium in modulating APP-calnuc interaction requires further investigation.

Furthermore, we performed yeast-two-hybrid assay to map the calnuc binding sites on APP. Constructs containing different APP domains (as baits) were individually co-transformed with calnuc construct (as prey) into competent yeast cells which were then grown on selection plates. Only yeast cells expressing both calnuc and APP fragments that interact with calnuc would survive and grow on selection plates. Results from co-transformation of calnuc cDNA and different APP fragments into yeast showed that only the intracellular domain (amino acids 646–695) but not the

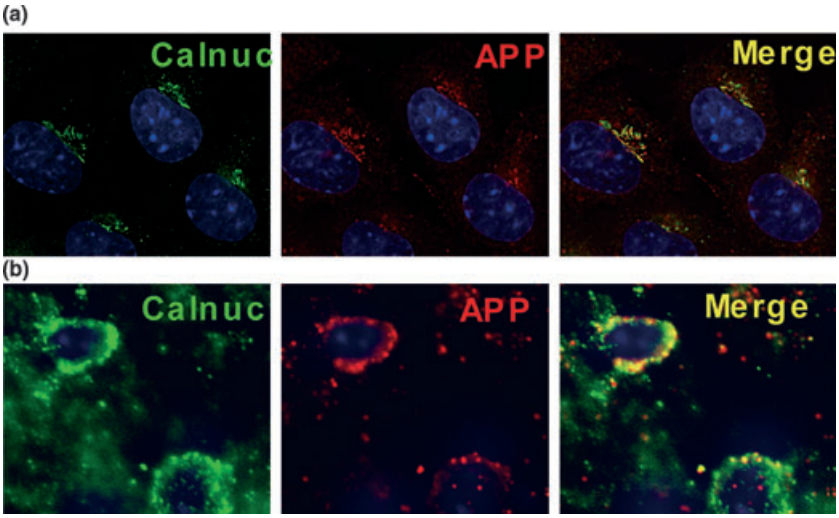

Fig. 2 Calnuc co-localizes with β-amyloid precursor protein (APP) *in vivo*. Fixed N2a cells (a) and fresh brain sections from mice at 3 month of age (b) were permeabilized, incubated with anti-calnuc and anti-APP antibodies, and followed by incubation with corresponding Alexa Fluor-conjugated antibodies. Cell nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI). Samples were examined using a de-convolution or confocal microscopy.

extracellular (amino acids 18–627) or transmembrane (amino acids 597–639) domains of APP interact with calnuc (data not shown).

Calnuc co-localizes with β-amyloid precursor protein *in vivo*

We next examined the binding of calnuc to APP by de-convoluting immunofluorescence. De-convolved images indicated that endogenous calnuc (green) significantly co-localizes with APP (red) in the Golgi of N2a cells (Fig. 2a). More importantly, immunofluorescence of calnuc (green) and APP (red) in mouse brain sections demonstrated that the two proteins also colocalize in the Golgi in mouse brains (Fig. 2b), supporting the physiological relevance of such an interaction.

Calnuc regulates β-amyloid precursor protein level through affecting its synthesis

Having identified the interaction of calnuc with APP, we further investigated whether calnuc may affect APP metabolism. We observed that the endogenous level of APP was dramatically reduced when Calnuc-GFP was transiently transfected into parental N2a cells (Fig. 3, left panel). On the other hand, when endogenous calnuc was down-regulated by siRNA, APP level was increased (Fig. 3, right panel).

To further study the effects of calnuc on the proteolytic processing of APP, we constructed N2a-APP cells that stably express calnuc-GFP or GFP (as control). As shown in Fig. 4, the APP level was significantly reduced upon calnuc over-expression, as expected. The levels of APP proteolytic products, CTFs, Aβ and sAPPα, were also reduced to various extents. Moreover, we performed the pulse-chase experiment to investigate APP metabolism in the presence of calnuc over-expression. The results showed that after 5 min of pulse-labeling, the level of nascent APP in N2a-APP cells expressing calnuc-GFP was significantly lower than that in GFP control cells (Fig. 5a). However, after 15 min of labeling and having chased for different times, there is little difference between the catabolism pattern of APP in cells expressing calnuc-GFP and cells expressing GFP alone,

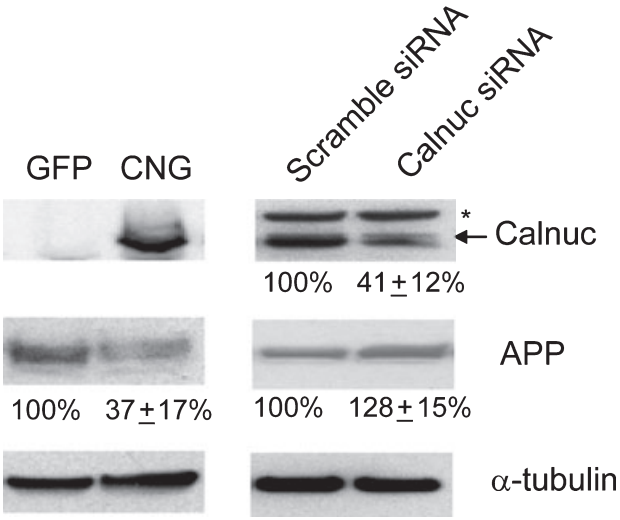

Fig. 3 Over-expression of calnuc reduces cellular levels of APP. Calnuc-GFP (CNG) or GFP constructs were stably transfected into parental non-transfected N2a cells. Calnuc siRNA or scramble siRNA were transiently transfected into N2a cells. Cells were harvested after 72 h. The amounts of calnuc and APP were analyzed by immunoblotting, using anti-calnuc antibody and anti-APP antibody 369, respectively. α-tubulin was immunoblotted using commercial antibody (Sigma) as loading controls. Data represent mean ± SD of protein level change relative to that of control, based on densitometry analysis and are weighted on α-tubulin levels (from three separate experiments). $p < 0.05$, calnuc siRNA vs scramble siRNA, and calnuc overexpression versus vector transfection. Asterisk indicates non-specific band.

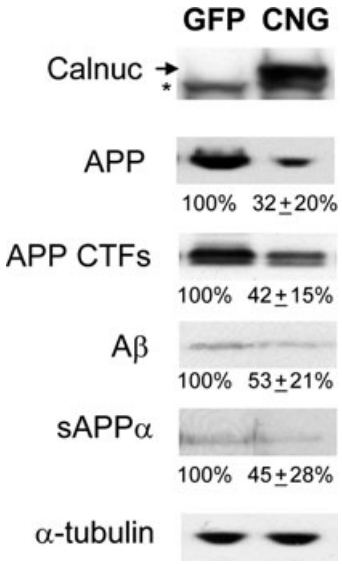

Fig. 4 Over-expression of calnuc reduces the level of APP as well as its proteolytic metabolites. N2a-APP cells stably expressing calnuc-GFP (CNG) or GFP were lysed. Equal amounts of lysate proteins were run on SDS-PAGE gel and analyzed by immunoblotting. Anti-calnuc antibody, 369 and anti-α-tubulin antibody were used to recognize calnuc, APP/APP-CTFs and α-tubulin, respectively. Soluble APPα from conditioned media was compared by direct western blot. Secreted Aβ from conditioned media was immunoprecipitated by 4G8 antibody and then immunoblotted with 6E10 antibody. Data represent mean ± SD of protein level change relative to that of control, based on densitometry analysis and are weighted on α-tubulin levels (from three separate experiments). $p < 0.02$, calnuc overexpression versus vector transfection. Asterisk indicates non-specific band.

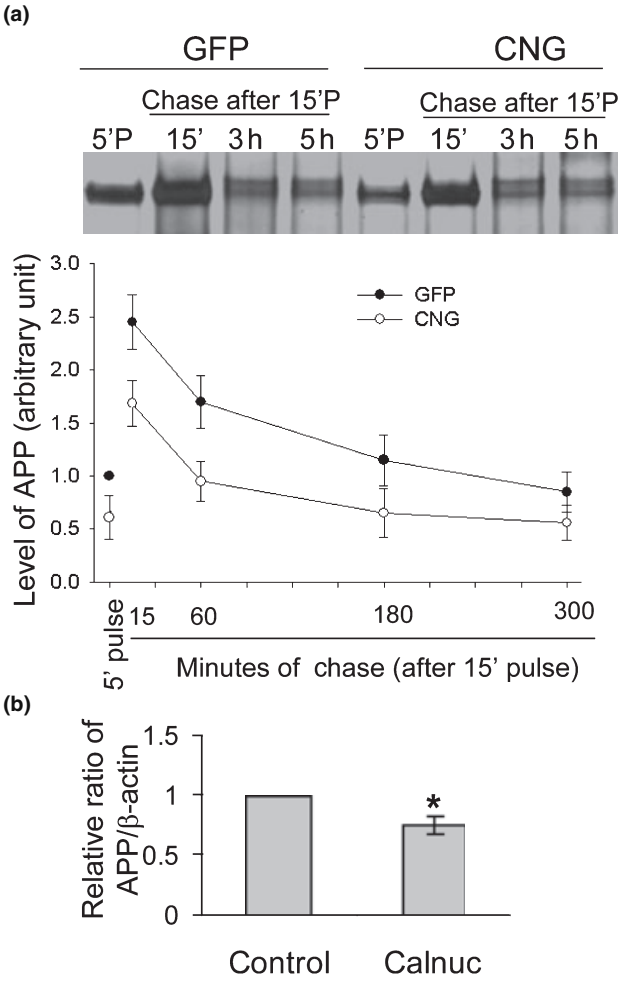

Fig. 5 Calnuc inhibits biosynthesis of β-amyloid precursor protein. (a) N2a cells stably expressing calnuc-GFP (CNG) or GFP were pulse-labeled with [^{35}S]methionine for 5 min and immunoprecipitated with anti-APP antibody 369. In some experiments, cells were pulse-labeled for 15 min and then chased for various time periods. Cell lysates were immunoprecipitated with 369, followed by SDS-PAGE analysis and autoradiography to detect labeled APP. (b) N2a cells were transiently transfected with calnuc-GFP or GFP vector. After enrichment of transfection-positive cells by flow cytometry for GFP fluorescence, the total RNA was extracted from the cells, and reverse-transcribed for real-time-PCR. The level of *APP* mRNA was normalized to that of *β-actin* and compared with that of controls (defined as one arbitrary unit). *: $p < 0.01$, $n = 3$.

suggesting that calnuc affects the APP level mainly at its synthesis stage.

Over-expressing calnuc reduces *APP* mRNA level

To investigate whether calnuc affects APP biogenesis through regulating its transcription, we transiently transfected N2a cells with calnuc-GFP or GFP vector. After enrichment of transfection-positive cells by flow cytometry for GFP fluorescence, the total RNA was extracted from the cells and reverse-transcribed for quantitative real-time-PCR. Our results show that over-expression of calnuc reduces the *APP* mRNA level by approximately 25% (Fig. 5b), which can be attributed, at least in part, to the observed reduction of APP protein levels.

The level of calnuc is reduced in Alzheimer's disease brains

Calnuc is involved in calcium homeostasis which is crucial to AD pathogenesis. The preceding results have demonstrated a potential role for calnuc in modulating APP metabolism which is also a key to AD pathogenesis. Calnuc has been shown to bind to necdin, a growth suppressor expressed predominantly in post-mitotic neurons, in a yeast-two-hybrid assay. Another Ca^{2+}-binding protein NEFA, with a similar structure to calnuc, also binds to necdin in neurons (Taniguchi *et al.* 2000). However, the existence and distribution of calnuc in human brains as well as whether the level of calnuc changes in AD brains have not been previously explored. Hence we first examined whether calnuc is expressed in the brains. As shown in Fig. 6a, we found that calnuc is indeed abundantly expressed in mouse brains, especially in the cortical neurons and

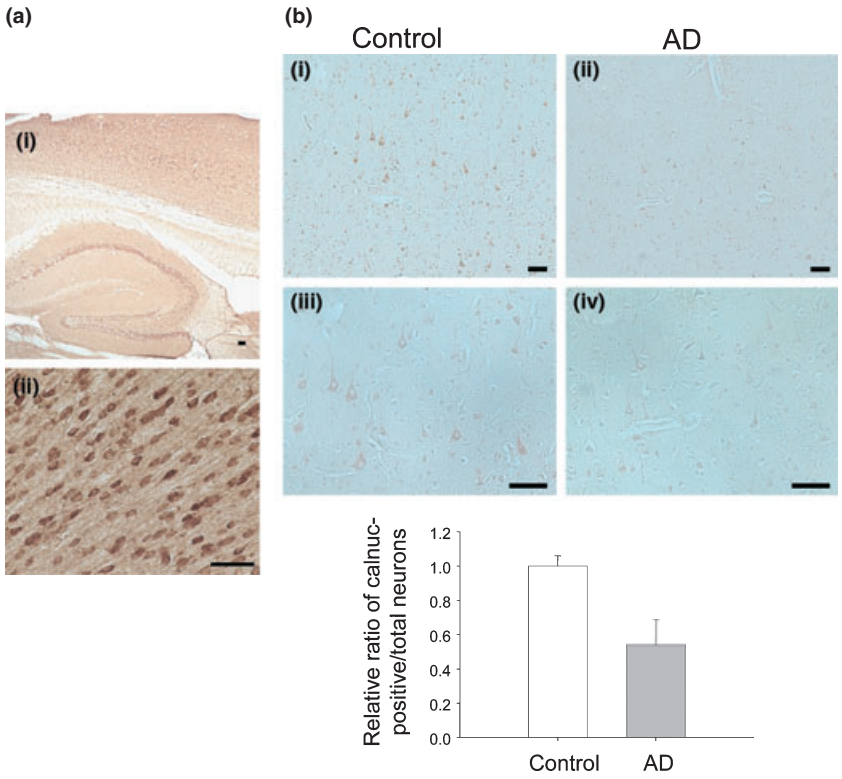

Fig. 6 Calnuc is expressed in brain neurons and the level of calnuc is reduced in Alzheimer's disease(AD) brains. (a) Immunostaining of calnuc in mouse brain sections from 6-month-old animals. Paraffin-embedded brain sections were de-paraffinized, sequentially incubated with polyclonal anti-calnuc antibody, with biotinylated horse anti-rabbit antibody, and with ABC-Elite (HRP) reagent. Reactions were visualized by developing in DAB substrates. (i) A low magnification display of cortex region and hippocampus region. (ii) A high magnification demonstration of calnuc-stained hippocampal neurons. Scale bar: 50 μm. (b) Immunostaining and quantification of calnuc in frontal cortex neurons from AD patients (ii and iv) and healthy controls (i and iii). (i and ii) show low magnification of calnuc staining. (iii and iv) represent higher magnification. The ratios of calnuc-positive neurons over total neurons in AD samples were calculated and compared to that of age-matched non-AD controls (defined as one arbitrary unit). *: $p < 0.02$ by Student's *t*-test, $n = 3$. Scale bar 100 μm.

hippocampal neurons. Similarly, we found that calnuc is also expressed in human brain neurons (Fig. 6b). Strikingly, by comparing the level of calnuc in the frontal cortex, one of the most afflicted brain regions in AD, between AD patients and age-matched non-AD controls, we found that calnuc is markedly reduced in AD brains (Fig. 6b).

Discussion

AD is the most common form of senile dementia diseases and characterized by the presence of extracellular Aβ plaques and intraneuronal hyperphosphorylated tau tangles, as well as a massive loss of neurons in the late stages of the disease. Aβ, which is known to trigger a cascade of pathogenic events including tau phosphorylation and neuronal excitotoxicity, is proteolytically derived from APP (Greenfield *et al.* 2000; Selkoe 2001). However, the physiological functions of APP remain largely elusive, although several studies have suggested that APP may play a role in transmembrane signal transduction, calcium metabolism, neurite outgrowth, neuronal protein trafficking through the axon, etc (Mucke *et al.* 1996; Mattson 1997; Neve *et al.* 2000; Koo 2002; Han *et al.* 2005; Zheng and Koo 2006). Thus, studies on APP functions and APP processing, including identifying and characterizing new factors that bind APP and affect APP metabolism, will have a great impact on AD therapeutics.

Calnuc is an EF-hand Ca^{2+}-binding protein that distributes in both cytosolic and membrane fractions (Miura *et al.* 1992; Wendel *et al.* 1995). The membrane pool of calnuc is tightly associated with the luminal surface of Golgi membranes and calnuc has been shown as a major Ca^{2+}-binding protein in the Golgi (Lin *et al.* 1998, 1999). Over-expression of calnuc in the Golgi led to an increase in Ca^{2+} storage, suggesting that calnuc is directly involved in Ca^{2+} homeostasis in the Golgi (Lin *et al.* 1999). Calnuc was found to interact with several different subfamilies of Gα, including Gαi and Gαs (Mochizuki *et al.* 1995; Lin *et al.* 1998, 2000; Weiss *et al.* 2001). Calnuc binds to the C-terminal region of Gαi3, and the interaction is regulated by Ca^{2+} and Mg^{2+} (Lin *et al.* 2000; Weiss *et al.* 2001). As the Golgi is a major site for

APP processing/Aβ generation (Hartmann *et al.* 1997; Xu *et al.* 1997; Greenfield *et al.* 1999), it is possible that calnuc may modulate APP processing/trafficking in the Golgi through interaction with APP (and altering the ionic conditions of the Golgi milieu). In the present study, we found that calnuc can interact with APP in a calcium-sensitive manner. Similar to the binding of calnuc on Gαi3 (Lin *et al.* 2000), we also found that calnuc interacts with APP through binding to the intracellular domain of APP (AICD). Because APP is a type 1 transmembrane protein with its C-terminus facing the cytosol, it is conceivable that the section of calnuc that binds to APP is from the cytosolic pool of calnuc. Previous studies (Lin *et al.* 2000; Weiss *et al.* 2001) have also shown that cytosolic calnuc binds to Gαi3 on the Golgi *in vivo*, providing further supporting evidence.

Along with Aβ generation by presenilin/γ-secretase cleavage, AICD is released from the membrane (Cao and Sudhof 2001; von Rotz *et al.* 2004). It has been shown that AICD can bind to a series of factors such as Fe65, histone acetyltransferase Tip60, X11α, CP2, Shc, etc. (Cao and Sudhof 2001; Chang and Suh 2005; Muresan and Muresan 2005; Sumioka *et al.* 2005). One well-studied case is the binding of AICD to the adaptor Fe65, upon which the AICD-Fe65 complex translocates into the nucleus and binds to the histone acetyltransferase Tip60 to form an AICD-Fe65-Tip60 complex (Cao and Sudhof 2001). This complex can regulate gene expression of a series of proteins, including APP and glycogen synthase kinase-3β, a major kinase for tau hyperphosphorylation (Kim *et al.* 2003; von Rotz *et al.* 2004). Our results demonstrate that over-expressing calnuc dramatically reduced the level of APP through regulating its biosynthesis (Fig. 5). Thus, it is possible that extensive binding of calnuc to APP/AICD may affect the stability of AICD, and that a feedback regulatory loop may exist between APP and calnuc. On the other hand, we noticed that calnuc over-expression causes a reduction of *APP* mRNA by only 25% (Fig. 5b) instead of 60% which is the level of reduction of APP protein (Fig. 3). Although it is possible that the 25% reduction in APP mRNA may account for a larger percentage of reduction at the protein level, we cannot rule out the possibility that calnuc may also modulate the translational events involved in APP biosynthesis. In parallel to reduced APP level, the levels of APP metabolism products, soluble APPα, Aβ, and APP CTFs were also reduced (Fig. 4). Interestingly, we found that the level of calnuc is significantly reduced in AD brains. Because the level of APP was reversely correlated to the calnuc level, it is very possible that a reduced calnuc level may increase the level of APP and hence the Aβ/AICD in the AD brains, accelerating disease development – a possible reflection of Down's syndrome.

Ca^{2+} plays critical roles in numerous physiological functions including learning and memory and is involved in neuron survival and death. Recent studies have linked Ca^{2+} disturbance to AD (LaFerla 2002; Mattson 2004; Braunewell 2005). Aβ has been shown to affect Ca^{2+} influx probably by inducing oxidative stress, forming channels in membranes, and/or activating cell surface receptors coupled to Ca^{2+} influx. Dysregulated Ca^{2+} signaling in turn contributes to synaptic dysfunction, excitotoxicity, apoptosis, etc. (Gabuzda *et al.* 1994; Le *et al.* 2001; Mattson and Chan 2003). In addition, another hallmark of AD, the neurofibrillary tangles, has been shown to increase Ca^{2+} levels as well as Ca^{2+}-dependent proteases in neurons (Murray *et al.* 1992; Nixon *et al.* 1994; Grynspan *et al.* 1997), whereas elevated intracellular Ca^{2+} levels may induce cytoskeleton changes similar to those found in neurofibrillary tangles (Mattson 1990). Furthermore, FAD mutations in APP and PS1 have also been found to destabilize Ca^{2+} homeostasis (Eckert *et al.* 2001; LaFerla 2002). These studies suggested that dysregulated calcium homeostasis is an important aspect of AD pathogenesis. Our observation that the interaction between calnuc and APP is likely Ca^{2+}-sensitive (Figs 1b and c) also indicated that altered Ca^{2+} homeostasis may possibly affect calnuc-APP interaction, disturbing APP metabolism.

Together, our results showed a novel function of calnuc in modulating the levels of APP and its metabolites. Given the importance of calcium homeostasis in the patho- and physiological processes, the involvement of calnuc in AD pathogenesis is manifested by our observations: (i) the co-localization of calnuc and APP in the Golgi, a major site for APP processing/Aβ generation; (ii) the direct binding of calnuc to APP/AICD, which may modulate a series of gene expression including *APP*; (iii) the possible Ca^{2+}-sensitivity feature of calnuc-APP interaction; and (iv) the reduced calnuc levels in AD brains.

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