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Overexpression of SNX3 Decreases Amyloid- β Peptide Production by Reducing Internalization of Amyloid Precursor Protein

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Abstract

Background—Sorting nexins (SNXs) have diverse functions in protein sorting and membrane trafficking. Recently, single-nucleotide polymorphisms in SNX3 were found to be associated with Alzheimer disease. However, it remains unknown whether SNX3 participates in amyloid (A) β peptide production.

Objective—To examine the role of SNX3 in A β production and APP processing.

Methods—The effect of increased expression of SNX3 was studied in HEK293T cells. A β peptides were measured by immunoassay. Protein-protein association was analyzed by a bimolecular fluorescence complementation (BiFC) assay. APP uptake was measured with an α -bungarotoxin-binding assay, and flow cytometry was used to measure cell surface APP levels.

Results—We found that overexpression of SNX3 in HEK293T cells decreases the levels of secreted A β and soluble N-terminal APP fragments (sAPP β). The reduction correlated with a decreased association of APP with BACE1, as revealed by BiFC. This effect may, in part, be explained by a reduced internalization of APP; SNX3 overexpression reduced APP internalization as determined by an α -bungarotoxin-binding assay, and caused increased APP levels on the cell surface, as shown by flow cytometry. In addition, SNX3 overexpression increased the cellular levels of full-length APP.

Conclusion—These results provide evidence that SNX3 regulates A β production by influencing the internalization of APP.

Keywords

Alzheimer disease; Amyloid peptide; Amyloid precursor protein; Secretase; Sorting nexin

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Statement of Ethics

The study complied with the ethical guidelines of the Karolinska Institutet.

Disclosure Statement

The authors declare that there are no conflicts of interest.

Introduction

Alzheimer disease (AD) is a complex and progressive neurodegenerative disease. Although the molecular mechanisms are not fully understood, aberrant production of neurotoxic amyloid (A) β peptides is thought to be one of the key initiators in the pathogenesis of AD [1]. A β peptides are derived from sequential cleavages of the amyloid precursor protein (APP) in what is described as the amyloidogenic pathway. In this pathway, APP is first cleaved by β -secretase to produce a soluble N-terminal APP fragment (sAPP β), and a membrane anchored C-terminal fragment (CTF β), which is subsequently cleaved by γ -secretase to generate A β . Alternatively, in the non-amyloidogenic pathway, APP can be cleaved by α -secretase; this precludes A β formation [2, 3].

It is thought that the α -secretase cleavage of APP primarily occurs on the cell surface, whereas β -secretase cleavage mainly takes place in endosomes [4]. Alterations in APP trafficking between the cell surface and different intracellular organelles and, consequently, in its localization, may influence whether α - or β -secretase-mediated cleavage occurs. Indeed, increased APP levels on the cell surface or decreased APP internalization reduces A β release [5, 6], but accelerated endocytosis enhances β -cleavage of APP [7]. Several lines of evidence indicate that dysfunction of the endocytic pathway is linked to AD. For instance, early endosomes are enlarged in sporadic AD [4, 8], and the levels of proteins involved in endosomal trafficking are altered in AD brains [9]. It has also been shown that perturbation of endosomal proteins can influence APP processing or A β production [10, 11]. Moreover, genome-wide association studies (GWAS) have shown that variability in endosomal trafficking genes contributes to the risk of developing AD [12].

Sorting nexins (SNXs) are a family of evolutionarily conserved proteins characterized by a phox-homology domain [13, 14]. SNXs have diverse cellular functions, many of which relate to protein trafficking and sorting [15]. An association between SNXs and APP processing was first supported by the observation that SNX17 regulates APP trafficking [16]. Subsequently, several studies have linked other SNXs with different aspects of APP processing. For instance, SNX15 regulates the cell surface recycling of APP [17], SNX33 promotes α -secretase cleavage of APP [18], SNX27 influences A β generation by controlling the assembly of γ -secretase [19], and SNX6 and SNX12 regulate the transport of BACE1, the main β -secretase [20, 21].

SNX3 was first identified in a search for protein homologs to the original family member SNX1 [22]. SNX3 preferentially binds to phosphatidylinositol 3-phosphate (PtdIns(3)P) [23–25], and it is enriched in endosomes [23]. Several studies have shown that SNX3 plays critical roles in intracellular trafficking, such as protein trafficking from early endosome to recycling endosome [23], and transport to the *trans*-Golgi network [26], and to lysosomes [23]. It may also participate in endocytosis, as it can regulate cell surface protein levels [23, 27–29]. SNX3 mRNA is expressed in several brain regions, such as the cerebral cortex and hippocampus [30], two regions that are vulnerable in AD. Moreover, SNX3 was found to be enriched in the postsynaptic density fraction [31], and it has been implicated in neurite outgrowth [25], a process that is compromised in AD [32]. Additionally, it was recently found that single nucleotide polymorphisms (SNPs) in SNX3 are associated with AD [33].

However, whether SNX3 directly influences APP processing and A β production remains unknown. In this study, we provide a first test of this possibility using the cell line HEK293T as a model. Our results indicate that overexpression of SNX3 alters A β production by influencing APP internalization.

Materials and Methods

Antibodies and Reagents

The following antibodies and reagents were used: Alexa Fluor[®] 555-conjugated α -bungarotoxin (B35451, Life Technologies), Hoechst 33342 (H3570, Life Technologies), anti-FLAG (F7425, Sigma-Aldrich), anti-SNX3 (sc-10619, Santa Cruz), anti-SNX3 (sc-376667, Santa Cruz), c-Myc antibody (9E10, Santa Cruz), anti-GAPDH (Synaptic System), anti-BACE1 (D10E5, Cell Signaling), anti-APP (22C11, Millipore), anti-APP (Y188, Epitomics), and the corresponding secondary antibodies HRP-conjugated goat anti-rabbit IgG (AP307P, Millipore), HRP-conjugated goat anti-mouse IgG (31430, Thermo Scientific), HRP-conjugated rabbit anti-goat IgG (Invitrogen), Alexa Fluor 555-conjugated goat anti-mouse IgG (A21424, Life Technologies).

cDNA Constructs and Cell Culture

cDNA constructs were generated according to standard cloning procedures, and all PCR reactions were performed using Q5[®] Hot Start High-Fidelity DNA polymerase (New England Biolabs). The SNX3 (isoform a) coding sequence was amplified by PCR using the primers SNX3_forward and SNX3_reverse (Table 1). The PCR product was cloned into the *EcoRI* and *KpnI* restriction sites of the p3 $\times$ FLAG-CMV-10 (Sigma) and pEGFP-C1 (Clontech) (kindly provided by Professor Marianne Farnebo, Karolinska Institutet, Sweden). Similarly, SNX29 construct p3 $\times$ FLAG-CMV-10_SNX29 was cloned with *BglII* and *XbaI* as the digestion sites. N-terminally Myc-tagged full-length APP construct pcDNA3.1_Myc-APP (APP695 isoform) [34] was generously provided by Professor Stefan Kins (University of Kaiserslautern, Germany). An α -bungarotoxin binding site (BBS) [35] tagged construct (BBS-APP) was made by replacing Myc to BBS by site-directed mutagenesis. APP_VN (APP695 isoform), APPstop40_VN (aa 1–636, APP695 numbering), BACE1_VC, and mCherry were generously provided by Professor Subhojit Roy [36] (University of California, San Diego, USA). Human wild-type BACE1 construct was a kind gift from Dr. Shaobo Jin [37] (Karolinska Institutet, Sweden). The reading frames of all cloned constructs were confirmed by DNA sequencing.

HEK293T cells were cultured in complete medium of DMEM-GlutaMAX supplemented with 10% FBS, and SH-SY5Y cells stably overexpressing APP695 [38] (kindly provided by Professor Eirikur Benedikz, Syddansk Universitet, Denmark) were cultured in DMEM/F-12, GlutaMAX supplemented with 10% FBS. Cells were maintained at 37 °C in 5% CO₂ humidified incubators.

Transfection, A β , and sAPP β Assay

A total of 5×10^5 HEK293T cells were plated into each well of a 6-well plate 1 day before transfection. Cotransfection of empty vector p3 $\times$ FLAG-CMV-10, SNX3, or SNX29 together

with Myc-APP was performed with Lipofectamine[®] 3000 reagent (Invitrogen) according to the manufacturer's instructions; 2.5 µg of DNA in total and Lipofectamine complex was then prepared in Opti-MEM, and added directly to the cell culture. The cells were maintained for a total of 48 h after transfection. For Aβ and sAPPβ measurement, cells were maintained in Opti-MEM medium for 24 h before harvest. The conditioned medium was centrifuged for 10 min at 700 g to remove cellular debris and collected for further measurement. The corresponding cells were harvested for the Western blot analysis.

Aβ levels in the conditioned medium were measured using a V-PLEX Aβ peptide panel 1 (4G8) kit (K15199E, Meso Scale Discovery [MSD]). Briefly, the MSD plate, precoated with capture antibodies, was blocked in Diluent 35 for 1 h at room temperature. After blocking, the wells were rinsed 3 times with wash buffer. The detection antibody (4G8) solution was then added to the wells, followed by adding the samples and calibrators. Incubation was carried out with shaking for 2 h at room temperature. After washing 3 times, 2× Read Buffer T was added to each well. The electrochemiluminescence signals of each well were subsequently read on a SECTOR imager 2400 instrument.

sAPPβ levels in the conditioned medium were analyzed using an MSD kit (K15120E) according to the supplier's instructions. The plate was incubated first with 3% Blocker A solution, subsequently with samples or calibrators, and then with detection antibody solution. All incubations were performed at room temperature with shaking for 1 h, with 3 washes in Tris wash buffer after 2 incubations. After another 3 washes, 1× Read Buffer T was added to each well and incubated for 10 min. The electrochemiluminescence signals of each well were detected.

Bimolecular Fluorescence Complementation

Cells were plated on a poly-D-lysine-coated Falcon[®] 96-well imaging microplate (353219, Corning) at a density of 8,000 cells/well. The following day, 0.1 µg plasmid DNA in total was transfected for each well. APP_VN and BACE1_VC were used to examine the association between APP and BACE1. mCherry was included to indicate the positively transfected cells. The above plasmids were cotransfected with empty vector p3×FLAG-CMV-10 or SNX3. As a negative control of bimolecular fluorescence complementation (BiFC), APPstop40_VN was used instead of APP_VN for transfection. After 4 h of incubation, the transfection medium was changed to prewarmed complete medium. Two days after transfection, the cells were washed twice with DPBS and fixed in 4% paraformaldehyde, 0.1 M phosphate buffer pH 7.4 with 4% sucrose for 20 min. After washing in DPBS twice, the cells were stained with Hoechst 33342 in DPBS for 20 min, and subsequently imaged and scored on an ImageXpress micro high-content imaging system (Molecular Devices). This assay was carried out in triplicate for each condition, and cells from 9 different sites of each well were imaged and scored. The APP/BACE1 association was defined as the average number of Venus-positive cells to the average number of mCherry-positive cells from 27 (9 × 3) sites. Thereafter, all experimental conditions were normalized to the empty vector control group to calculate the relative level of BiFC.

APP Internalization Assay

The internalization assay was performed as previously described [5, 18] with some minor changes. Briefly, cells were plated on poly-D-lysine-coated cover slips at a density of 0.25×10^5 to 0.5×10^5 cells/well of a 24-well plate. BBS-APP was transfected alone, or cotransfected with pEGFP-C1 or pEGFP-C1_SNX3. Two days after transfection, cells were washed twice with ice-cold DPBS, and incubated at 4 ° C for 20 min with Alexa Fluor 555-conjugated α -bungarotoxin at a final concentration of 3 μ g/mL in DPBS. Unbound Alexa Fluor-555 conjugated α -bungarotoxin was removed by washing twice in ice-cold DPBS. Prewarmed culture medium was added to each well, and cells were then kept in a 37 ° C incubator for various time periods. After washing twice in ice-cold DPBS, cells were fixed in 4% paraformaldehyde, 0.1 M phosphate buffer pH 7.4 with 4% sucrose for 20 min, and mounted in ProLong[®] Gold antifade mountant (Life Technologies). Hoechst 33342 was applied before mounting. Then z-stack images were acquired using a Zeiss LSM 510 Meta confocal microscope equipped with a 63 $\times$ /1.4 NA oil objective. For image analysis, the middle 3 or 4 scans from each z-stack file of APP labeling were examined with Image J. Throughout the analysis, the same thresholding percentage (1%), and filtering option (median) were applied. Particles with the same size range and circularity (0.2–1.0) were quantified.

Flow Cytometry

Cotransfection of empty vector p3 $\times$ FLAG-CMV-10, or SNX3 and Myc-APP was performed as described above. After 4 h of incubation, the transfection medium was replaced with prewarmed DMEM-GlutaMAX supplemented with 10% FBS. Two days after transfection, cells were washed twice with DPBS and dissociated in DPBS with 2 mM EDTA at 37 ° C. After the addition of complete medium, cells were detached and centrifuged at 700 *g* for 5 min. Indirect labeling of cell surface APP was carried out by incubating cells with c-Myc antibody on ice for 30 min, followed by incubation with Alexa Fluor-555 conjugated, goat anti-mouse IgG secondary antibody on ice for 30 min. Nontransfected HEK293T cells were stained as a negative staining control. Cells were analyzed by LSRFortessa (Becton Dickinson) using FlowJo software (Tree Star).

Western Blot

Cells were washed twice in cold DPBS, and lysed on ice for 30 min in RIPA lysis buffer (50 mM Tris-Base pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor cocktail (Sigma, P8340) and 1 mM PMSF. Cell extracts were centrifuged at 14 000 *g* for 15 min at 4 ° C, and equal amounts of supernatant were subjected to SDS-PAGE on NuPAGE[®] Bis-Tris gels. Proteins on the gels were then transferred onto PVDF membranes. After blocking in 5% nonfat milk/TBST (Tris-buffered saline and 0.1% Tween 20, pH 7.5), membranes were washed in TBST 3 times and probed with primary antibodies overnight at 4 ° C. Then membranes were washed in TBST again and subsequently incubated with secondary antibodies for 1.5 h at room temperature. After several wash steps, membranes were developed with SuperSignal[®] West Pico Chemiluminescent Substrate (Thermo Scientific). Images were taken and protein bands were quantified using ImageLab software (Bio-Rad). In some cases, when quantification of

protein bands was not needed, the exposed membrane was further stripped, and subsequently probed for another detection.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5. Two-tailed unpaired *t* test, one-sample *t* test, and one-way ANOVA followed by the Bonferroni post hoc test for multiple comparisons were used as specified. Data values are presented as mean $\pm$ SEM.

Results

Overexpression of SNX3 Reduces A β 40, A β 42, and sAPP β Production

To examine whether A β 40 and A β 42 formation depends on SNX3, we overexpressed either p3 $\times$ FLAG-CMV-10 (empty vector), or SNX3 together with APP in HEK293T cells and measured secreted A β levels in the culture medium. To examine the expression of SNX3 in the cell model, we performed Western blot to detect endogenous and overexpressed SNX3. As shown in Figure 1a, in the cells transfected with empty vector, a band at around 20 kDa corresponding to endogenous SNX3 was detected with an anti-SNX3 antibody whereas in the SNX3-overexpressing cells, there was an additional band at around 28 kDa corresponding to the FLAG tagged SNX3. Quantification showed that FLAG-SNX3 was expressed at a level of about 23 times more than that of endogenous SNX3. In addition, some weaker bands at a lower molecular weight were also detected in the SNX3-overexpressing cells, possibly cleaved fragments of SNX3. By performing immunocytochemistry, we also examined whether the high expression of SNX3 would alter its distribution. HEK293T cells were transfected with either empty vector or FLAG-SNX3, and pEGFP-C1 was included to indicate positively transfected cells. Consistent with previous studies [23], endogenous SNX3 in the empty vector-transfected cells mainly showed a vesicular distribution. Overexpressed SNX3, with a stronger signal, showed a similar vesicular localization, suggesting that overexpression did not have a major influence on the cellular distribution of SNX3 (Fig. 1b).

The levels of A β 40 and A β 42 were measured using a specific MSD multiplex system. We found that SNX3 overexpression significantly reduced the levels of both A β 40 and A β 42 (Fig. 1c, d). We also examined the effect of SNX3 on the N-terminal β -cleavage product, sAPP β . SNX3 overexpression decreased sAPP β levels (Fig. 1e), indicating that SNX3 affects β -cleavage of APP. In control experiments, we tested the effect of a reference protein, SNX29, which has a different domain organization from that of SNX3. A β 40, A β 42, and sAPP β (Fig. 1c–e) were not altered by the overexpression of SNX29, suggesting a specificity in the effect of SNX3 on secreted APP β -cleavage products.

We performed similar experiments in the human neuroblastoma cell line SH-SY5Y stably expressing APP695 (SH-SY5Y_APP). SNX3 could be moderately overexpressed in the SH-SY5Y_APP cells (Fig. 1f). However, the increase was only about 3.6 times that of endogenous SNX3 (Fig. 1g), presumably due to a low transfection efficiency of SNX3 in SH-SY5Y_APP cells. The A β 40 levels in the SNX3-overexpressing cells showed a trend of decreasing compared to that in the empty vector-expressing cells (0.89 ± 0.28 times), but

this failed to reach significance ($p > 0.7$; Fig. 1h). These results give a hint that overexpression of SNX3 may potentially reduce A β production, also in a neuronal-like cell-line. However, the relative difficulty in overexpressing SNX3 in these cells made experimental analysis difficult. Therefore, we continued the investigation by using HEK293T cells as a model.

Since BACE1 is the principal β -secretase, and β - cleavage of APP is the rate-limiting step in A β production [39], we sought to test whether SNX3 can influence the BACE1 levels. Western blot was performed to examine the levels of endogenous BACE1 in HEK293T cells co-transfected with either empty vector or SNX3 together with APP. Quantification revealed no significant difference in the levels of mature or immature BACE1 between empty vector-expressing and SNX3-overexpressing cells (Fig. 1i–k).

Overexpression of SNX3 Reduces the APP/BACE1 Association in Intact Cells

Since the association between APP and its secretases is an important prerequisite for APP cleavage, we next tested whether SNX3 can interfere with the association between APP and BACE1. To this end, we employed a BiFC assay [36]. In this assay, APP is tagged with the nonfluorescent N-terminal fragment of the Venus protein (VN), while BACE1 is tagged with the nonfluorescent C-terminal fragment of the Venus protein (VC) (Fig. 2a). When APP and BACE1 are in proximity, VN and VC form a functional Venus protein that produces a fluorescent signal.

We first performed an experiment to verify the expression and cleavage of the APP_VN construct. A deletion mutant, APPstop40_VN (residues 1–636), which contains the β -cleavage site and membrane-anchoring region but lacks the C terminus thought to mediate the APP/BACE1 association [40], was also examined. As shown in Figure 2b, there was a prominent APP signal in APP_VN- and APPstop40_VN-expressing cells, suggesting that these 2 constructs were properly expressed. APP_VN, but not APPstop40_VN, was cleaved to produce CTFs when BACE1_VC was coexpressed (Fig. 2b, c), indicating that APP_VN could be processed by BACE1_VC, but that APPstop40_VN could not be cleaved presumably due to an inefficient association with BACE1_VC. When APP_VN was singly expressed, tagged CTFs were also observed (Fig. 2c), suggesting that APP_VN could mimic endogenous APP to be cleaved by native BACE1. Thus, this system may also reflect the native APP/BACE1 association.

To test the specificity of the BiFC assay, we expressed individual constructs of APP_VN or BACE1_VC alone. Neither of them gave rise to any significant fluorescence (data not shown). In addition, APPstop40_VN was co-expressed with BACE1_VC. As shown in Figure 2d (negative control), co-expression of APPstop40_VN and BACE1_VC did not give rise to any significant BiFC signal. Consistent with previous data [36], coexpression of the empty vector, APP_VN, and BACE1_VC produced a prominent BiFC signal (Fig. 2d, empty vector). To validate the system with a relevant protein, wild-type BACE1 was used. We found that overexpression of BACE1 can alter the APP/BACE1 BiFC signal (data not shown). These results demonstrate that the BiFC system can be used to effectively monitor the APP/BACE1 association.

As shown in Figure 2(d, e), overexpression of SNX3 caused a small but significant reduction in the association between APP_VN and BACE1_VC. It is possible that the degree of reduction in intracellular organelles that are optimal for β -cleavage (e.g., endosomes) was even larger, but was obscured by increased APP levels at other locations. In any event, these results suggest that overexpression of SNX3 decreases the association between APP and BACE1, which can explain its effect on the β -cleavage of APP.

SNX3 Overexpression Inhibits APP Internalization and Increases APP Levels at the Cell Surface

Many lines of evidence indicate that APP encounters BACE1 in endosomes following its internalization [41, 42]. Thus, it seems possible that the reduced APP/BACE1 association, and subsequent β -cleavage, could be due to the inhibition of APP endocytosis by SNX3. Hence, we tested whether SNX3 affects the internalization of APP by using a well-established internalization assay [35]. In this assay, APP was N-terminally tagged with a BBS. Internalization of APP was investigated by monitoring cell surface BBS-APP labeled by incubation with α -bungarotoxin conjugated with Alexa Fluor 555. To characterize this assay, we first examined 3 different incubation periods at 37°C, i.e., at 0, 10, and 30 min [5]. At the time point 0 min, all fluorescence occurred on the cell surface (Fig. 3a, top panel). After 10 min of incubation, a large fraction of APP had been internalized, as shown by the distribution of fluorescence in punctate structures (Fig. 3a, middle panel). Almost all of the cell surface APP had been internalized by 30 min (Fig. 3a, bottom panel). Based on these observations, we focused the continued analysis on the time points 0 min and 10 min.

In control cells cotransfected with pEGFP-C1 (empty vector), BBS-APP displayed an internalization pattern similar to that described above: the fluorescence occurred on the cell surface at 0 min, and was largely internalized after 10 min of incubation (Fig. 3b). Quantitative analysis of the number of fluorescent vesicular structures showed that there was a significantly higher number of vesicular structures at 10 min in control cells expressing empty vector (Fig. 3d). In cells overexpressing SNX3, the fluorescence distribution at 0 min was similar to that in the control cells (Fig. 3c, d). However, internalization of APP was significantly reduced in cells overexpressing SNX3 compared to in control cells (Fig. 3c, d).

The above results suggest that SNX3 reduces the internalization of APP from the cell surface. If so, it would be expected that SNX3-overexpressing cells exhibit higher levels of APP on the cell surface. To test this possibility, cell surface levels of APP were examined by flow cytometry. We found that SNX3 overexpression indeed resulted in elevated cell surface levels of APP as indicated by the geometric mean fluorescence intensity (MFI) of total cells (Fig. 3e, f). The MFI was also increased after increased expression of SNX3 (data not shown). Moreover, SNX3 overexpression increased the percentage of surface APP-labeled cells (Fig. 3g). Taken together, the data from the internalization assay and flow cytometry indicate that SNX3 overexpression prevents APP from entering cells, which would explain the reduction of β -cleavage of APP.

As overexpression of SNX3 decreased the β -cleavage of APP, it is possible that it also influences the steady-state levels of full-length APP. To test this, we measured the APP levels in cell lysates after the overexpression of SNX3. Indeed, SNX3 overexpression

significantly increased the APP levels revealed by anti-Myc antibody (Fig. 4a, b). Moreover, mature (but not immature) APP (both revealed by the APP 22C11 antibody) was elevated by SNX3 overexpression (Fig. 4c–e).

Discussion

Although many studies have focused on APP trafficking and processing, the details of this process are not yet fully understood. Emerging evidence suggests that SNXs play critical roles in APP processing. Here, we focused on SNX3, inspired by the finding that SNPs occur in the *SNX3* gene in AD patients [33]. We employed an overexpression approach, which has been used in several previous studies to investigate SNX functions [17, 23, 27]. It can be assumed that overexpression of a protein such as SNX3 alters the function of the pathway in which it normally functions, but overexpression may potentially cause nonspecific effects. Hence, the fact that overexpressed SNX3 showed a specific localization similar to that of endogenous SNX3, and that overexpression of the control protein SNX29 had no effect on A β production is of critical importance to interpret our data.

We found that overexpression of SNX3 reduced A β 40 and A β 42. It also reduced sAPP β , suggesting that it affects BACE1 cleavage of APP. The notion that SNX3 affects BACE1 cleavage was supported by the experiments with BiFC. This technique, which provides a sensitive measure of the association between 2 proteins [36], showed that the APP/BACE1 association was reduced by SNX3 over-expression. Moreover, the processing of overexpressed APP in this system may reflect the fate of endogenous APP, as partly demonstrated by the result that APP_VN was cleaved by native BACE1.

There are several possible explanations for the observed reduction of the APP/BACE1 association, including direct interference with the binding of the two proteins, or impaired trafficking of APP secretases or APP. Although we did not examine the first two possibilities, our results suggest that, at least, the trafficking of APP is altered. It is widely believed that a key step in amyloidogenic APP processing involves the internalization of APP, to bring it into endosomes where it encounters BACE1 [36]. Two observations support the possibility that overexpression of SNX3 acted by impairing the uptake of APP. First, internalization of APP tagged with BBS was strongly reduced by SNX3. Second, as shown by the flow cytometry experiments, SNX3 increased the APP levels at the cell surface.

Taken together, our findings suggest that the involvement of SNX3 is critical for APP internalization. This coincides with previous studies that have suggested that SNX3 is involved in the endocytosis of cargo molecules such as transferrin [29], the epithelial Na⁺ channel [27], and the reductive iron transporter [28]. At present, the precise role of SNX3 in endocytosis is unclear and will require further studies. It is interesting to note, however, that SNX3 can interact with the heavy chain of clathrin [26, 43], suggesting that it may interfere directly with the clathrin machinery. A previous study showed that SNX12 regulates A β production by affecting the internalization of BACE1 [21]. It is thus possible that SNX3 and SNX12, which are close homologs, jointly control APP processing via complementary mechanisms.

Our study was primarily based on nonneural HEK293T cells. This raises the question of whether the results are applicable to neurons. We did observe a trend of A β reduction after overexpression of SNX3 in the neuronal-like SH-SY5Y_APP cell line. We speculate that the lack of a significant effect was due to the fact that we were only able to induce a low degree of overexpression (3.6 vs. 23 times in HEK293T cells), and that a significant effect would be seen if a comparable degree of overexpression had been induced. Thus, the easily transfectable HEK293T cells were useful for a first analysis of the role of SNX3, and have provided valuable insights for the analysis to be continued. To verify its role in neurons, it will be important to transfer our findings to other cell types. It would be of considerable interest to examine whether SNX3 influences APP processing in cells such as primary neurons. While our study provided the first insight into the role of SNX3 in APP internalization, future studies addressing its possible role in other aspects of APP processing will also be of great interest.

In conclusion, our results demonstrate that SNX3 regulates A β production by influencing the internalization of APP, implicating SNX3 as a putative target in AD treatment research.

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References

1. Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med*. 2016; 8:595–608. [PubMed: 27025652]
2. O'Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer's disease. *Annu Rev Neurosci*. 2011; 34:185–204. [PubMed: 21456963]
3. Haass C, Kaether C, Thinakaran G, Sisodia S. Trafficking and proteolytic processing of APP. *Cold Spring Harb Perspect Med*. 2012; 2:a006270. [PubMed: 22553493]
4. Nixon RA. Endosome function and dysfunction in Alzheimer's disease and other neurodegenerative diseases. *Neurobiol Aging*. 2005; 26:373–382. [PubMed: 15639316]
5. Carey RM, Balcz BA, Lopez-Coviella I, Slack BE. Inhibition of dynamin-dependent endocytosis increases shedding of the amyloid precursor protein ectodomain and reduces generation of amyloid β protein. *BMC Cell Biol*. 2005; 6:30. [PubMed: 16095541]
6. Koo EH, Squazzo SL. Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *J Biol Chem*. 1994; 269:17386–17389. [PubMed: 8021238]
7. Grbovic OM, Mathews PM, Jiang Y, Schmidt SD, Dinakar R, Summers-Terio NB, Ceresa BP, Nixon RA, Cataldo AM. Rab5-stimulated up-regulation of the endocytic pathway increases intracellular beta-cleaved amyloid precursor protein carboxyl-terminal fragment levels and Abeta production. *J Biol Chem*. 2003; 278:31261–31268. [PubMed: 12761223]
8. Cataldo AM, Peterhoff CM, Troncosco JC, Gomez-Isla T, Hyman BT, Nixon RA. Endocytic pathway abnormalities precede amyloid beta deposition in sporadic Alzheimer's disease and Down syndrome – differential effects of APOE genotype and presenilin mutations. *Am J Pathol*. 2000; 157:277–286. [PubMed: 10880397]
9. Ginsberg SD, Mufson EJ, Alldred MJ, Counts SE, Wu J, Nixon RA, Che S. Upregulation of select rab GTPases in cholinergic basal fore-brain neurons in mild cognitive impairment and Alzheimer's disease. *J Chem Neuroanat*. 2011; 42:102–110. [PubMed: 21669283]
10. Udayar V, Buggia-Prevot V, Guerreiro RL, Siegel G, Rambabu N, Soohoo AL, Ponnusamy M, Siegenthaler B, Bali J, Simons M, Ries J, Puthenveedu MA, Hardy J, Thinakaran G, Rajendran L.

- Aesg: A paired RNAi and Rab-GAP overexpression screen identifies Rab11 as a regulator of β -amyloid production. *Cell Rep.* 2013; 5:1536–1551. [PubMed: 24373285]
11. Jiang S, Li Y, Zhang X, Bu G, Xu H, Zhang YW. Trafficking regulation of proteins in Alzheimer's disease. *Mol Neurodegener.* 2014; 9:6. [PubMed: 24410826]
 12. Hardy J, Bogdanovic N, Winblad B, Portelius E, Andreassen N, Cedazo-Minguez A, Zetterberg H. Pathways to Alzheimer's disease. *J Intern Med.* 2014; 275:296–303. [PubMed: 24749173]
 13. Seet LF, Hong W. The Phox (PX) domain proteins and membrane traffic. *Biochim Biophys Acta.* 2006; 1761:878–896. [PubMed: 16782399]
 14. Cullen PJ. Endosomal sorting and signalling: an emerging role for sorting nexins. *Nat Rev Mol Cell Biol.* 2008; 9:574–582. [PubMed: 18523436]
 15. Worby CA, Dixon JE. Sorting out the cellular functions of sorting nexins. *Nat Rev Mol Cell Biol.* 2002; 3:919–931. [PubMed: 12461558]
 16. Lee J, Retamal C, Cuitino L, Caruano-Yzermans A, Shin JE, van Kerkhof P, Marzolo MP, Bu G. Adaptor protein sorting nexin 17 regulates amyloid precursor protein trafficking and processing in the early endosomes. *J Biol Chem.* 2008; 283:11501–11508. [PubMed: 18276590]
 17. Feng T, Niu M, Ji C, Gao Y, Wen J, Bu G, Xu H, Zhang YW. SNX15 regulates cell surface recycling of APP and A β generation. *Mol Neurobiol.* 2016; 53:3690–3701. [PubMed: 26115702]
 18. Schobel S, Neumann S, Hertweck M, Dislich B, Kuhn PH, Kremmer E, Seed B, Baumeister R, Haass C, Lichtenthaler SF. A novel sorting nexin modulates endocytic trafficking and alpha-secretase cleavage of the amyloid precursor protein. *J Biol Chem.* 2008; 283:14257–14268. [PubMed: 18353773]
 19. Wang X, Huang T, Zhao Y, Zheng Q, Thompson RC, Bu G, Zhang YW, Hong W, Xu H. Sorting nexin 27 regulates A β production through modulating gamma-secretase activity. *Cell Rep.* 2014; 9:1023–1033. [PubMed: 25437557]
 20. Okada H, Zhang W, Peterhoff C, Hwang JC, Nixon RA, Ryu SH, Kim TW. Proteomic identification of sorting nexin 6 as a negative regulator of BACE1-mediated APP processing. *FASEB J.* 2010; 24:2783–2794. [PubMed: 20354142]
 21. Zhao Y, Wang Y, Yang J, Wang X, Zhao Y, Zhang X, Zhang YW. Sorting nexin 12 interacts with BACE1 and regulates BACE1-mediated APP processing. *Mol Neurodegener.* 2012; 7:30. [PubMed: 22709416]
 22. Haft CR, de la Luz Sierra M, Barr VA, Haft DH, Taylor SI. Identification of a family of sorting nexin molecules and characterization of their association with receptors. *Mol Cell Biol.* 1998; 18:7278–7287. [PubMed: 9819414]
 23. Xu Y, Hortsman H, Seet L, Wong SH, Hong W. SNX3 regulates endosomal function through its PX-domain-mediated interaction with PtdIns(3)P. *Nat Cell Biol.* 2001; 3:658–666. [PubMed: 11433298]
 24. Lim KP, Hong W. Human Nischarin/imidazoline receptor antisera-selected protein is targeted to the endosomes by a combined action of a PX domain and a coiled-coil region. *J Biol Chem.* 2004; 279:54770–54782. [PubMed: 15475348]
 25. Mizutani R, Yamauchi J, Kusakawa S, Nakamura K, Sanbe A, Torii T, Miyamoto Y, Tanoue A. Sorting nexin 3, a protein upregulated by lithium, contains a novel phosphatidylinositol-binding sequence and mediates neurite outgrowth in N1E-115 cells. *Cell Signal.* 2009; 21:1586–1594. [PubMed: 19576982]
 26. Harterink M, Port F, Lorenowicz MJ, Mc-Gough IJ, Silhankova M, Betist MC, van Weering JR, van Heesbeen RG, Middelkoop TC, Basler K, Cullen PJ, Korswagen HC. A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion. *Nat Cell Biol.* 2011; 13:914–923. [PubMed: 21725319]
 27. Boulkroun S, Ruffieux-Daidie D, Vitagliano JJ, Poirot O, Charles RP, Lagnaz D, Firsov D, Kellenberger S, Staub O. Vasopressin-inducible ubiquitin-specific protease 10 increases ENaC cell surface expression by deubiquitylating and stabilizing sorting nexin 3. *Am J Physiol Renal Physiol.* 2008; 295:F889–900. [PubMed: 18632802]
 28. Strohlic TI, Setty TG, Sitaram A, Burd CG. Grd19/Snx3p functions as a cargo-specific adapter for retromer-dependent endocytic recycling. *J Cell Biol.* 2007; 177:115–125. [PubMed: 17420293]

29. Chen C, Garcia-Santos D, Ishikawa Y, Seguin A, Li L, Fegan KH, Hildick-Smith GJ, Shah DI, Cooney JD, Chen W, King MJ, Yien YY, Schultz IJ, Anderson H, Dalton AJ, Freedman ML, Kingsley PD, Palis J, Hattangadi SM, Lodish HF, Ward DM, Kaplan J, Maeda T, Ponka P, Paw BH. Snx3 regulates recycling of the transferrin receptor and iron assimilation. *Cell Metab*. 2013; 17:343–352. [PubMed: 23416069]
30. Mizutani R, Nakamura K, Yokoyama S, Sanbe A, Kusakawa S, Miyamoto Y, Torii T, Asahara H, Okado H, Yamauchi J, Tanoue A. Developmental expression of sorting nexin 3 in the mouse central nervous system. *Gene Expr Patterns*. 2011; 11:33–40. [PubMed: 20817026]
31. Li KW, Hornshaw MP, Van Der Schors RC, Watson R, Tate S, Casetta B, Jimenez CR, Gouwenberg Y, Gundelfinger ED, Smalla KH, Smit AB. Proteomics analysis of rat brain postsynaptic density. Implications of the diverse protein functional groups for the integration of synaptic physiology. *J Biol Chem*. 2004; 279:987–1002. [PubMed: 14532281]
32. Dowjat WK, Wisniewski T, Efthimiopoulos S, Wisniewski HM. Inhibition of neurite outgrowth by familial Alzheimer's disease-linked presenilin-1 mutations. *Neurosci Lett*. 1999; 267:141–144. [PubMed: 10400232]
33. Vardarajan BN, Bruesegem SY, Harbour ME, Inzelberg R, Friedland R, St George-Hyslop P, Seaman MN, Farrer LA. Identification of Alzheimer disease-associated variants in genes that regulate retromer function. *Neurobiol Aging*. 2012; 33:2231.e2215–e2231.e2230.
34. Baumkotter F, Schmidt N, Vargas C, Schilling S, Weber R, Wagner K, Fiedler S, Klug W, Radzimanowski J, Nickolaus S, Keller S, Eggert S, Wild K, Kins S. Amyloid precursor protein dimerization and synaptogenic function depend on copper binding to the growth factor-like domain. *J Neurosci*. 2014; 34:11159–11172. [PubMed: 25122912]
35. Sekine-Aizawa Y, Haganir RL. Imaging of receptor trafficking by using alpha-bungarotoxin-binding-site-tagged receptors. *Proc Natl Acad Sci USA*. 2004; 101:17114–17119. [PubMed: 15563595]
36. Das U, Wang L, Ganguly A, Saikia JM, Wagner SL, Koo EH, Roy S. Visualizing APP and BACE-1 approximation in neurons yields insight into the amyloidogenic pathway. *Nat Neurosci*. 2016; 19:55–64. [PubMed: 26642089]
37. Jin S, Agerman K, Kolmodin K, Gustafsson E, Dahlqvist C, Jureus A, Liu G, Falting J, Berg S, Lundkvist J, Lendahl U. Evidence for dimeric BACE-mediated APP processing. *Biochem Biophys Res Commun*. 2010; 393:21–27. [PubMed: 20097169]
38. Agholme L, Hallbeck M, Benedikz E, Marcusson J, Kagedal K. Amyloid-beta secretion, generation, and lysosomal sequestration in response to proteasome inhibition: involvement of autophagy. *J Alzheimers Dis*. 2012; 31:343–358. [PubMed: 22555375]
39. Cole SL, Vassar R. The role of amyloid precursor protein processing by BACE1, the β -secretase, in Alzheimer disease pathophysiology. *J Biol Chem*. 2008; 283:29621–29625. [PubMed: 18650431]
40. Citron M, Teplow DB, Selkoe DJ. Generation of amyloid beta protein from its precursor is sequence specific. *Neuron*. 1995; 14:661–670. [PubMed: 7695913]
41. Cirrito JR, Kang JE, Lee J, Stewart FR, Verges DK, Silverio LM, Bu G, Mennerick S, Holtzman DM. Endocytosis is required for synaptic activity-dependent release of amyloid-beta in vivo. *Neuron*. 2008; 58:42–51. [PubMed: 18400162]
42. Das U, Scott DA, Ganguly A, Koo EH, Tang Y, Roy S. Activity-induced convergence of APP and BACE-1 in acidic microdomains via an endocytosis-dependent pathway. *Neuron*. 2013; 79:447–460. [PubMed: 23931995]
43. Skanland SS, Walchli S, Brech A, Sandvig K. SNX4 in complex with clathrin and dynein: implications for endosome movement. *PLoS One*. 2009; 4:e5935. [PubMed: 19529763]

Fig. 1. Overexpression of SNX3 reduces the production of Aβ peptides. For the Western blot and MSD measurement, HEK293T cells were cotransfected with empty vector p3×FLAG-CMV-10, SNX3, or SNX29 together with Myc-APP. **a** The expression of endogenous and overexpressed SNX3 was confirmed by Western blot with anti-SNX3 (sc-10619). The blot shown is representative of 4 independent experiments. **b** The distribution of endogenous and overexpressed SNX3 was examined by immunocytochemistry with anti-SNX3 (sc-376667). Representative images from 2 independent experiments are shown. To avoid saturation, the

confocal image of SNX3 labeling in the cells overexpressing SNX3 was scanned with a lower detection gain than that in the empty vector-transfected cells. Scale bar, 10 μ m. Levels of secreted A β 40 (**c**), A β 42 (**d**), and sAPP β (**e**) were measured in the conditioned medium of cells overexpressing SNX3 or SNX29, and normalized to the levels in the “empty vector” control group. **f** SH-SY5Y_APP cells were transfected with empty vector or SNX3. Levels of SNX3 in cell lysates were detected by Western blot with anti-SNX3 (sc-10619). **g** Quantification of SNX3. **h** Levels of secreted A β 40 in the conditioned medium. Mature and immature BACE1 levels were examined by Western blot (**i**), and quantifications of mature (**j**) and immature BACE1 (**k**) are shown. SNX3 was detected with anti-SNX3 (sc-10619) antibody. One-sample *t* test was performed from at least 4 independent experiments for all the statistical analyses. ns, not significant. **p* < 0.05, ***p* < 0.01.

Fig. 2.

Overexpression of SNX3 decreases the association between APP and BACE1 in intact cells.

a Schematic diagram of BiFC constructs. **b, c** Western blot was performed to confirm the expression and cleavage of APP constructs. Lane 1: nontransfected HEK293T cells. Lane 2–5: HEK293T cells transfected with different constructs as indicated in the figures. Full-length APP was detected with antibody 22C11 (**b**), and APP CTFs were detected with antibody Y188 (**c**). Representative blots from 2 independent experiments are shown. **d** Representative images of cells labeled with nuclei staining (blue), expressing mCherry (red)

and emitting BiFC signals (green) in each experimental condition. Scale bar, 100 μm . **e** Quantification of BiFC signals from 4 independent experiments. The APP/BACE1 association in “empty vector” control cells was normalized to 1. One-sample *t* test was performed. **p* < 0.05, ****p* < 0.001.

Fig. 3.

Overexpression of SNX3 inhibits APP internalization and increases APP levels on the cell surface. **a** Characterization of APP internalization. HEK293T cells were transfected with BBS-APP, and cell surface BBS-APP was labeled by incubation with Alexa Fluor 555-conjugated α -bungarotoxin at 4 ° C for 20 min. Then cells were incubated at 37 ° C for different time periods (0, 10, and 30 min). Representative images of APP labeling (red) and nuclei staining (blue) from each time point are shown. Scale bar, 10 μ m. **b, c** HEK293T cells were cotransfected with empty vector (pEGFP-C1) or pEGFP-C1_SNX3 and BBS-APP, and

internalization of cell surface APP was examined. Scale bar, 10 μm . **d** APP fluorescent vesicular structures with the same size range and circularity were quantified. More than 20 cells from 3 independent experiments were imaged and analyzed for every time point of each condition. One-way ANOVA followed by Bonferroni's post hoc test was used for the statistical analysis. **e–g** Cell surface levels of APP were determined by flow cytometry. **e** Representative overlay plot of cell surface labeling of APP. **f** The geometric mean fluorescence intensity (MFI) of cell surface APP was quantified. **g** Quantification of the percentages of surface APP-labeled cells. Four independent experiments were performed. Two-tailed unpaired *t* test was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4. Overexpression of SNX3 increases the level of full-length APP. **a–e** HEK293T cells were cotransfected with Myc-APP and either empty vector or FLAG-SNX3. Western blot was performed to examine the level of full-length APP in cell lysates, with GAPDH used as a loading control. **a, b** APP was detected with anti-Myc antibody. **c–e** Mature and immature APP was revealed by anti-APP (22C11) antibody. Anti-SNX3 (sc-10619) antibody was used to detect SNX3. Quantitative analysis of the APP levels normalized to that in control cells expressing empty vector. At least 3 independent experiments were performed. One-sample *t* test was performed. * $p < 0.05$.

Table 1

List of primers used in this study

Primers	Sequence (5'–3')
SNX3_forward	CAGGAATTCGGCGGAGACCGTGGCTGACA
SNX3_reverse	CTAGGTACCAAATTCAGGCATGTCTTAT
SNX29_forward	AGGAGATCTTAGCGGATCACAGAACAAATG
SNX29_reverse	TGCTCTAGAGATCACCAGGTGGACGTG
BBS-APP_forward	CCTGGAGCCCTACCCTGACACTGATGGTAATGCTGGC
BBS-APP_reverse	GAGCTCTCGTAGTATCTCCAGGGTACCTCCAGCGCCCCG