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Original Article

The expression of the nicotinic acetylcholine receptor α3 subunit in the brains of patients with Alzheimer's disease and its effects on α- and γ-secretases and Notch signal transduction in SH-SY5Y cells

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Abstract: Objective: The aim of this study was to evaluate the correlation between the nicotinic acetylcholine receptor α3 subunit (α3nAChR) and β-amyloid (Aβ) in Alzheimer's disease (AD) patients' brains, α3nAChR on α and γ-secretases in amyloid precursor protein (APP) metabolism, and determine the possible correlation between α3nAChR and the Notch pathway. Methods: In this study, the expression of α3nAChR and Aβ in Alzheimer's disease patients' and normal brains was determined by immunofluorescence, and human neuroblastoma SH-SY5Y cells were treated with α3nAChR siRNA or nicotine to investigate the effects of α3nAChR on the expression of ADAM10 (a component of α-secretase), presenilin 1 (PS1) and nicastrin (NCT) (γ-secretase components), and Notch1 and Hes1 (effectors in the Notch pathway) using quantitative real time PCR and immunoblot. Results: The expression of Aβ in AD patients' brains was high, but the distribution of α3nAChR in AD patients' brains was significantly lower than it was in the normal control group. The results revealed that α3nAChR silencing suppressed ADAM10, PS1, NCT, Notch1, and Hes1 expression in SH-SY5Y cells. Meanwhile, stimulation with nicotine resulted in increased expression levels of α3nAChR, ADAM10, PS1, NCT, Notch1 and Hes1. Conclusion: These results indicated that α3nAChR might work against the production of Aβ in the brains of Alzheimer's patients, and in the amyloidogenic cascade controlling APP metabolism, α3nAChR might enhance the secretion of α- and γ-secretases as well as Notch pathway activation, suggesting that α3nAChR potentially has a critical function in the non-amyloidogenic pathway of APP metabolism in Alzheimer's disease.

Keywords: α3nAChR, nicotine, notch pathway, β-amyloid, amyloid precursor protein, APP metabolism, Alzheimer's disease

Introduction

Alzheimer's disease (AD) represents one of the most common neurodegenerative disorders among the aged. The pathological features of AD include the presence of extracellular senile plaques (SP), intracellular neurofibrillary tangles, and the loss of neurons. SP comprises β-amyloid peptide (Aβ), which has an important function in AD pathogenesis [1]. Meanwhile, Aβ plaques cause cross-sectional synaptic network dysfunction, gradual brain atrophy, and cognitive impairment [2].

Aβ (38 to 43 amino acids) aggregates and accumulates in soluble oligomers, fibrils, and SP. It represents a product of amyloid precursor peptide (APP) via the successive enzymatic actions of β-secretase (BACE1) and γ-secretase. Upon APP cleavage by β-secretase, a C-terminal truncated fragment (C99) is yielded and further cleaved by γ-secretase to finally yield Aβ40 and Aβ42, in a process known as the amyloidogenic pathway. Specifically, Aβ42, which easily aggregates and forms insoluble plaques, is more amyloidogenic than Aβ40. When α-secretase first cleaves APP, C83 and sAPPα are formed

and produce non-toxic fragments, in a process referred to as the non-amyloidogenic pathway. A large number of disintegrin and metalloprotease (ADAM) family members are α-secretases, especially ADAM10 which is considered the most critical enzyme of this group [3]. In addition, γ-secretase is an intramembranous cleaving aspartyl protease composed of presenilin (PS), nicastrin (NCT), PEN-2 and APH-1, which represent indispensable and necessary factors for the activity of this enzyme. PS mediates the intramembrane cleavage of the APP CTF substrate. The potential substrate receptors, APH-1, NCT and PEN-2, constitute subunits essential to stabilize and activate PS. It has been reported that the conditional forebrain inhibition of NCT activity leads to neurodegenerative features resembling neuron loss found in AD, indicating NCT is critical in maintaining neuronal survival [4].

The Notch pathway is a highly conserved signaling pathway, which is fundamental for neuronal development and specification by regulating the transcription of Notch target genes, such as Hes1 and Hes [5]. Notch equally controls neurogenesis, neuritic growth, synaptic plasticity [5], and long term memory [6], both in the developing and adult brain [7]. Aberrant Notch signaling is a possible mechanism for the learning and memory deficits, cognitive impairment and altered neurogenesis associated with AD. Upon ligand binding, the membrane-tethered whole peptide notch receptor is proteolytically cleaved at site 2 (s2) by ADAM, generating Notch extracellular truncation (NEXT). The remaining membrane-associated portion undergoes cleavage by γ-secretase (s3) and generates Notch intracellular domain (NICD), which moves to the cytosol. NICD then translocates from the cytosol to the nucleus to bind with the c-promoter binding factor 1 (CBF-1) family proteins, leading to transcriptional regulation of the Hes gene. This ultimately controls neural stem cell differentiation and brain development.

Nicotinic acetylcholine receptors (nAChRs) belong to the ligand-gated ion channel super-family and participate in many critical physiological reactions. There are 13 subunits of nAChRs, including α2 to 10 and β2 to 4, which form hetero- or homo-pentameric nAChRs. These nAChRs mediate postsynaptic responses, neurotransmitter release and cognitive enhancement, likely via the regulation of multiple Ca^{2+}-dependent reactions. Our previous studies have shown that the downregulation of α7 and α3nAChR subunits decreases αAPP levels, but reducing the amount of BACE1 and upregulating BACE2 inhibits the production of Aβ [8, 9].

RNA interference (RNAi) is a powerful technique and a standard way to silence gene expression by small interference RNAs. Here, we inhibited the RNA expression of α3nAChR by the RNAi technique in human neuroblastoma cells in order to assess the function of α3nAChR in the molecular mechanisms underlying the alterations of the non-amyloidogenic pathway and Notch signal transduction.

Materials and methods

Materials

Goat polyclonal antibodies against α3nAChR (SC1771), rabbit polyclonal antibodies against α3nAChR (GTX105495), mouse polyclonal antibodies against Aβ (Biolegend 803001), horseradish peroxidase conjugated anti-mouse and anti-goat secondary antibodies, fluorescence (FITC)-labeled sheep anti-rabbit IgG, fluorescence (Cy3) labeled sheep against mouse IgG, mouse monoclonal antibodies against NCT (SC136003), ADAM10 (SC28358), PS-1 (SC-365495), Notch1 (SC32745), and Hes1 (SC-166378) (Santa Cruz Biotechnology Inc., USA) were used. The Coomassie Brilliant Blue Protein Assay kit and cDNA synthesis kit were manufactured by Bio-Rad (USA). The ECL Plus reagent was from Amersham Bioscience AB (Sweden). Primers for the α3nAChR, ADAM10, PS1, NCT, Notch1, and Hes1 genes were provided by Shanghai Genecore Bio Technologies, China.

Human brain samples

Post-mortem brain samples from the Dutch Brain Bank (Amsterdam, The Netherlands) are well characterized in terms of specific clinical and neuropathological criteria. According to their medical history, clinical manifestations and laboratory tests, the donor was diagnosed as “probable Alzheimer’s disease” by excluding other possible causes of dementia. The clinical diagnosis was performed according to the National Institute of Neurological and Communication Disorders and Stroke and the As-

sociation of Alzheimer's Disease and Related Diseases (NINCDS-ADRDA) criteria and the severity of dementia assessed according to the Global Deterioration Scale. Each control donor has no known history or symptoms of neurological or psychiatric disorders.

The temporal and frontal cortex hippocampus of 10 patients with AD and 10 controls were studied. The mean age at death for these AD patients was 81.5 ± 7.1 years, and the mean age of these control cases was 79.4 ± 9.2 years; in the AD and controls, the PMI (interval between death and autopsy) was 5.1 ± 1.0 and 8.0 ± 3.4 hours.

Cell culture and treatments

The human neuroblastoma SH-SY5Y cells, purchased from the German Collection of Microorganisms, were maintained in DMEM containing 10% heat-inactivated fetal bovine serum, 100 mg/ml streptomycin and 100 U/ml penicillin, in a humid environment with 5% CO_2 at 37°C. The cells were assessed at no more than 3 passages. Stable transfection was performed with pSilencer™ 3.1-H1 neo negative control or with α3nAChR pSilencer™ 3.1-H1 neo, with 400 ng/ml G418 used or selection.

The analysis of α3nAChR, ADAM10, PS1, NCT, Notch1 and Hes1 mRNA levels using real-time PCR

Total RNA was extracted from the SH-SY5Y cells using TRIzol reagent according to the procedure supplied by the manufacturer. The mRNA amounts of α3nAChR, ADAM10, NCT, PS1, Notch1, Hes1 and β-actin were assessed using real-time PCR as described previously (8). Briefly, 1 μg total RNA was used for cDNA synthesis with a cDNA synthesis kit. The real-time quantitative PCR was performed in a 96-well format on the ABI Step One Plus System. Threshold cycles (CT) were analyzed using SDS 1.4 (Applied Biosystems). PCR was carried out for 25 μl reactions containing the Universal TaqMan 2X PCR SYBR Green Master Mix and ADAM10, PS1, NCT, Notch1, Hes1 or β-actin primers. The primers included: α3nAChR, 5' ACCACCGCAGGATAAAAATCT 3' and 5' CACTTTGGATGGCTTCTTTGA 3'; ADAMI0, 5' GGAAGATGGTGTTGCTGAGAG 3' and 5' ACGCTGGTGTTTTTGGTGTAA 3'; NCT, 5' GGCAATGGTTTGGCTTATGA 3' and 5' TTGATGCTGAAGGTGCTTTG 3'; PS1, 5' ACAATGGTGTGGTTGGTGAAT 3' and 5' ACGAAACAGGCTATGGTTGTG 3'; Notch1, 5' CAACATCCCCTACAAGATCGAG 3' and 5' CACGAAGAACAGAAGCACAAAG 3'; Hes1, 5' CACTGATTTTGGATGCTCTGA 3' and 5' AGGTGCTTCACTGTCATTTCC 3'; β-actin, 5' TGGCACCACACCTTCTACAATG 3' and 5' TCATCTTCTCGCGGTTGGC 3'. The $2^{-\Delta\Delta Ct}$ was used for analysis, with β-actin as a reference control.

The analysis of α3nAChR, ADAM10, PS1, NCT, Notch1, and Hes1 protein levels using immunoblot

The protein amounts of α3nAChR, ADAM10, PS1, NCT, Notch1, Hes1, and β-actin were determined by immunoblot according to a procedure described in a previous study (8). Total protein concentration was determined with the Coomassie Brilliant Blue Protein Assay Kit. Equal amounts of total protein were resolved by 10% SDS-PAGE and electro-transferred onto polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% skim milk for 2 h. This was followed by successive incubations with primary antibodies against α3nAChR, ADAM10, PS1, NCT, Notch1 or Hes1 (1:1000; overnight, 4°C) and HRP-conjugated anti-mouse or anti-goat IgG for 1 h. Finally, the protein bands were visualized with the ECL chemiluminescence detection system. Anti-β-actin (1:20000) primary antibodies were used as a loading control. The relative expression levels of various proteins were calculated by employing a computer-assisted software based on β-actin amounts. The protein amounts were expressed as a percentage of those of the control group.

Statistical analysis

Data are presented as the mean ± SD. Correlation analyses, two-tail Student's *t*-test (group pairs), and one way ANOVA (multiple groups) were employed for analysis with SPSS22.0 (USA). $P < 0.05$ indicated statistical significance.

Results

The expression of α3nAChR and Aβ in the brains of the AD patients and in normal brains

Aβ is expressed mainly in the cytoplasmic and extracellular forms of the hippocampus and cortical neurons (**Figure 1A**) and is abundantly expressed in the brains of the AD patients

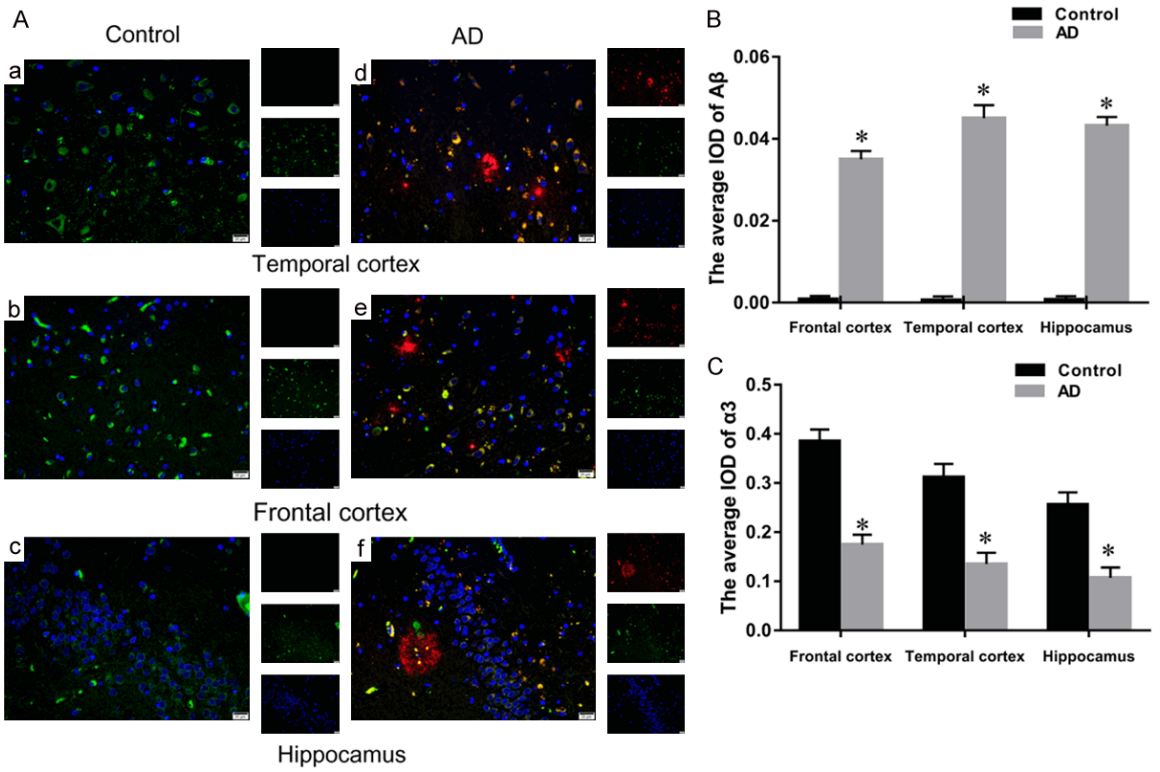

Figure 1. Double immunofluorescence labeling of Aβ and α3nAChR in human brain sections of normal and AD patients (A). The expressions of α3nAChR and Aβ in the brains of the AD patients and in normal brains. Aβ is expressed in the cytoplasms and extracellularly, with high expression in the brain slices of the AD patients (a-c), but almost no expression in normal human brain sections (d-f) is shown by immunofluorescence double labeling (B). α3nAChR is expressed in the cytoplasms and reduced in the brain sections of the AD patients compared with the normal human brain sections by immunofluorescence double labeling (C). The cell nuclei all stained blue (with DAPI), Aβ-positive neurons with red and α3nAChR-positive neurons with green. Scale bar = 10 μm. The data are presented as the mean ± SD (n = 9). *P < 0.05 vs control group.

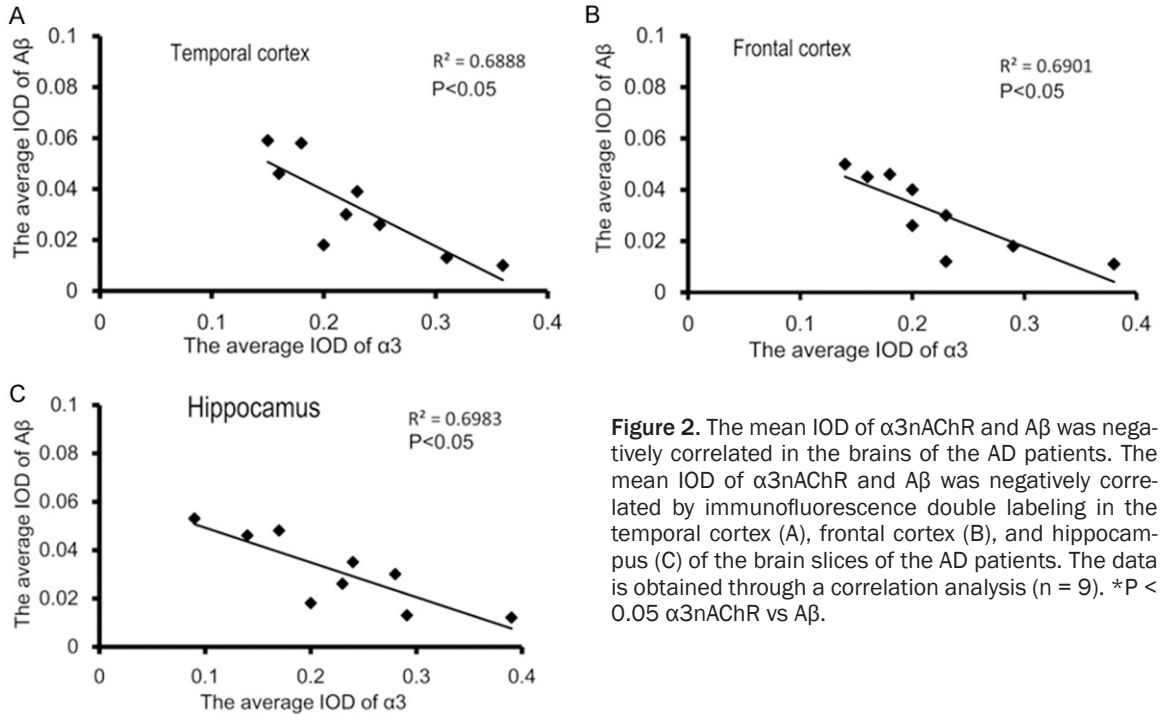

Figure 2. The mean IOD of α3nAChR and Aβ was negatively correlated in the brains of the AD patients. The mean IOD of α3nAChR and Aβ was negatively correlated by immunofluorescence double labeling in the temporal cortex (A), frontal cortex (B), and hippocampus (C) of the brain slices of the AD patients. The data is obtained through a correlation analysis (n = 9). *P < 0.05 α3nAChR vs Aβ.

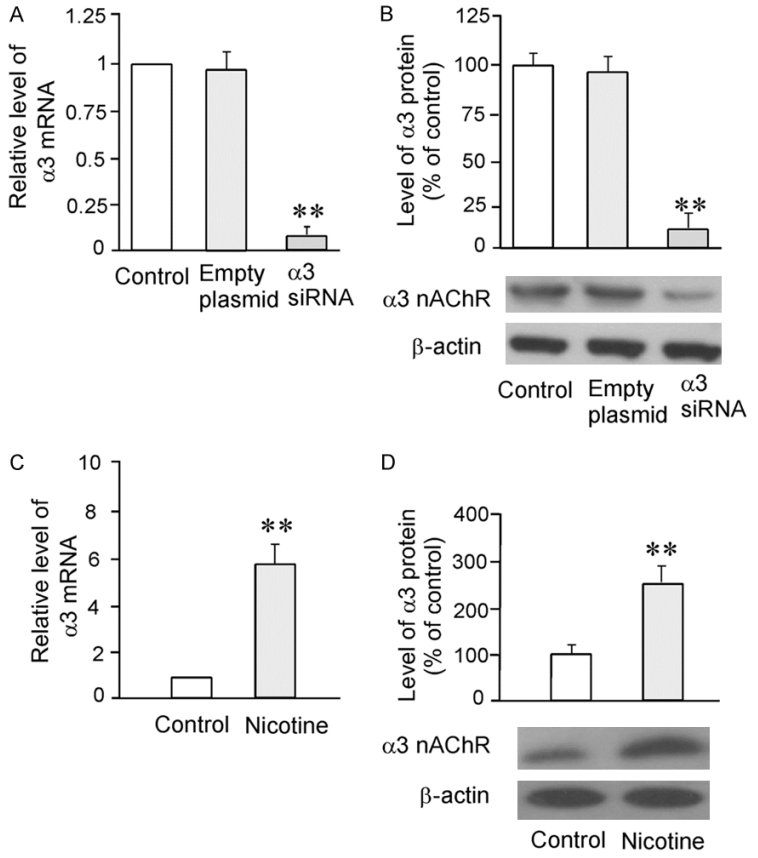

Figure 3. The expression of α3nAChR in SH-SY5Y cells treated with α3 siRNA or nicotine. α3nAChR mRNA (A) and protein (B) amounts in the SH-SY5Y cells incubated with or without α3siRNA, as assessed by real time PCR and Western blotting, respectively. α3nAChR mRNA (C) and protein (D) amounts in SH-SY5Y cells incubated with or without nicotine, as assessed by real time PCR and Western blotting, respectively. The data are presented as the mean ± SD (n = 9). **P < 0.01 vs control group.

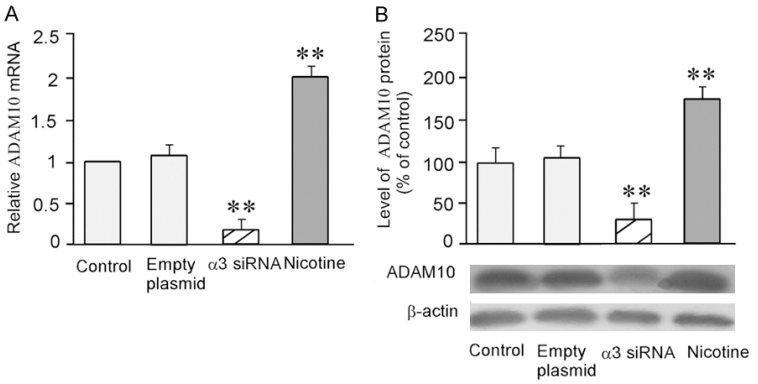

Figure 4. ADAM10 expression in SH-SY5Y cells treated with α3 siRNA or nicotine. ADAM10 mRNA (A) and protein (B) amounts in SH-SY5Y cells administered α3 siRNA or nicotine as determined by real time PCR and Western blotting, respectively. The data are presented as the mean ± SD (n = 9). **P < 0.01 vs control group.

(**Figure 1B**). α3nAChR is mainly expressed in the cytoplasm of the hippocampus and cortical nerves (**Figure 1A**), and its distribution in the brains of the AD patients is significantly less than its distribution in the normal control group (**Figure 1C**).

The mean IOD of α3nAChR and Aβ was negatively correlated in the brains of the AD patients

The mean IOD of α3nAChR and Aβ was negatively correlated using immunofluorescence double labeling in the temporal cortex (**Figure 2A**), frontal cortex (**Figure 2B**), and hippocampus (**Figure 2C**) of the brain slices of the AD patients.

The effects of α3 silencing and nicotine exposure on α3nAChR expression in SH-SY5Y cells

In this study, α3nAChR gene (**Figure 3A**) and protein (**Figure 3B**) expression levels were reduced by 93% and 90%, respectively, in the SH-SY5Y cells after α3 silencing compared with the values of the control cells and the empty plasmid group. In addition, α3 mRNA (**Figure 3C**) and protein (**Figure 3D**) amounts were enhanced 393% and 201%, respectively, in nicotine treated SH-SY5Y cells.

The effects of α3 silencing and nicotine exposure on ADAM10 expression in SH-SY5Y cells

ADAM10 mRNA (**Figure 4A**) and protein (**Figure 4B**) amounts were markedly reduced in the SH-SY5Y cells after α3 silencing compared to the values of the control cells and the empty plasmid group; they were enhanced in cells treated with nicotine (**Figure 4A** and **4B**).

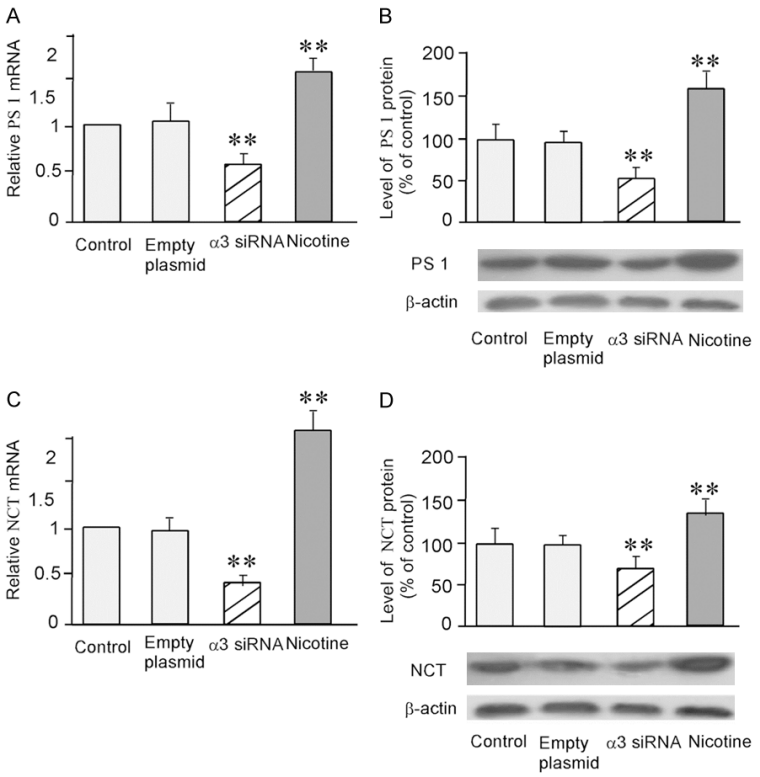

Figure 5. PS1 and NCT levels in SH-SY5Y cells administered α3 siRNA or nicotine. PS1 mRNA (A) and protein (B) amounts in SH-SY5Y cells administered α3 siRNA or nicotine, as assessed by real time PCR and Western blotting, respectively. NCT mRNA (C) and protein (D) amounts in SH-SY5Y cells administered α3 siRNA or nicotine, as determined by real time PCR and Western blotting, respectively. The data are presented as the mean ± SD (n = 9). **P < 0.01 vs control group.

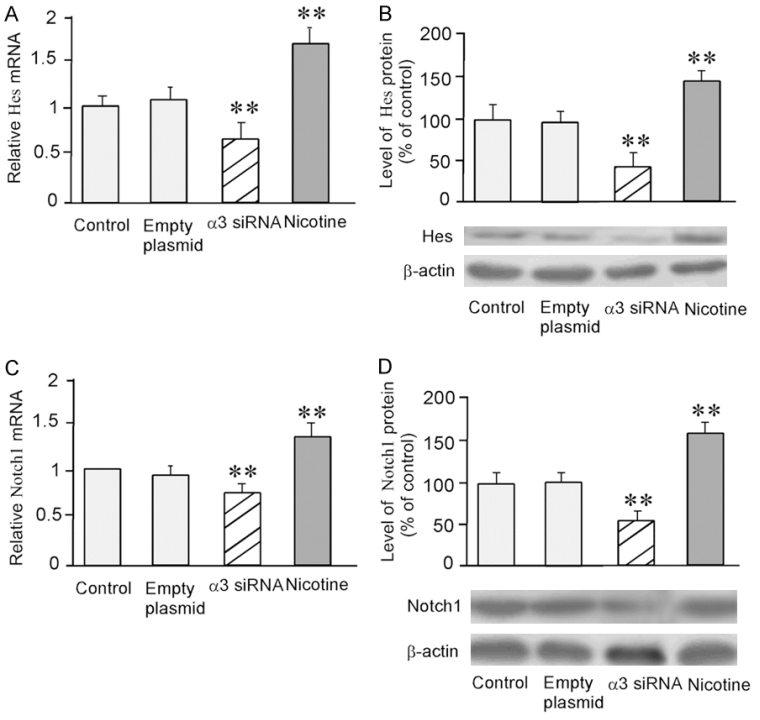

Figure 6. Hes and Notch1 levels in SH-SY5Y cells administered α3siRNA or nicotine. Hes mRNA (A) and protein (B) amounts in SH-SY5Y cells administered α3 siRNA or nicotine, as determined by real time PCR and Western blotting, respectively. Notch1 mRNA (C) and protein (D) amounts in SH-SY5Y cells administered α3 siRNA or nicotine, as assessed by Real time PCR and Western blotting, respectively. The data are presented as the mean ± SD (n = 9). **P < 0.01 vs control group.

The effects of α3 silencing and nicotine exposure on PS1 and NCT expression in SH-SY5Y cells

The PS1 and NCT mRNA (**Figure 5A** and **5C**, respectively) and protein (**Figure 5B** and **5D**, respectively) amounts were decreased in the SH-SY5Y cells after α3 silencing compared with the values of the control cells and the empty plasmid group, and enhanced in the cells treated with nicotine.

The effects of α3 silencing and nicotine exposure on Hes1 and Notch1 expressions in the SH-SY5Y cells

The Hes1 and Notch1 mRNA (**Figure 6A** and **6C**, respectively) and protein (**Figure 6B** and **6D**, respectively) amounts were decreased in the SH-SY5Y cells after α3 silencing compared with the values of the control cells and the empty plasmid group, and enhanced in cells treated with nicotine.

Discussion

The loss of nAChRs has been reported in multiple cerebral areas in AD by receptor binding experiments and is positively correlated with senile plaque formation in the temporal lobe

of the brain. One of the early signs of AD demonstrated by positron emission tomography (PET) is the lack of such nAChRs in the AD brain, which has a close relationship with cognitive dysfunction in AD. Our previous studies also found that Aβ can inhibit nicotine receptor gene expression in cultured cells in vitro [10, 11], and region-specific decreases of α3, α4, and α7nAChR were reported in the AD brain [12].

SH-SY5Y cells contain α3, α5, α7, β2, and β4 nAChR subunits. Upon chronic exposure to the nicotine of the SH-SY5Y cells, the α3nAChR amounts were increased by 500-600%, but α7nAChR increased by only 30%; this indicated that nicotine mainly stimulates α3nAChR production in SH-SY5Y cells. Although a multitude of studies have focused on α7nAChR, which was thought to be the most important neuroprotective factor in the pathogenesis of AD, α3nAChR also plays an important role in controlling the activities of nicotine and acetylcholine in precise brain regions. Apart from its effects on the central nervous system, α3nAChR is also essential in the peripheral neuronal tissue in mammals, acting on sympathetic neurons, parasympathetic ganglia [13] and sensory neurons. We reported in a previous investigation that by inhibiting α3nAChR expression in SH-SY5Y cells with RNAi, the levels of αAPPs are decreased, but Aβ amounts are enhanced by increasing the levels of BACE1 and reducing the BACE2 amounts, indicating that α3nAChR may have a neuroprotective function in the pathogenesis of AD [9].

Aβ is derived from the amyloid precursor protein (APP) digested by α-, β-, or γ-secretase; in the amyloidogenic pathway, successive APP cleavage by β-secretase and γ-secretase yields Aβ. In contrast, APP digestion by α- and γ-secretases in the non-amyloidogenic pathway results in the reduced formation of Aβ. α-secretase is an important factor in preventing the generation of Aβ, which appears to be a metalloprotease of the ADAM family. ADAM10 is a membrane protein whose active site is located within the ectodomain [14]. A key substrate of ADAM10 is APP for which it acts as a constitutive α-secretase and prevents the formation of Aβ in AD. Another major substrate of ADAM10 is the Notch receptor; indeed, ADAM10 mediates notch signaling during cell differentiation and development [15]. Meanwhile, γ-secretase comprises four integral membrane proteins essential for its effects. PS1 and its homolog PS2 mediate the intramembrane cleavage of APP and act as catalytic subunits [16]. NCT binds to the substrate, while APH-1 and PEN-2 help stabilize and activate γ-secretase [17]. PS is essential for γ-secretase activity, but the change of PS content is affected by many factors, such as the feedback regulation of the Notch signaling pathway.

It was reported that NCT and the heterodimers of PS specifically bind γ-secretase inhibitors and are associated with γ-secretase activity, which indicates that NCT is also an active member affecting the activity of γ-secretase. Downregulating NCT reduces the expression levels of APH-1 and PEN-24, while increasing C83 APP CTF amounts [18].

The expression of Aβ in the brains of patients with Alzheimer's disease was higher than it was in the normal control group, according to an immunofluorescence detection of the brain slices of AD patients and the normal human brain slices, and the expression of α3nAChR in the brain slices of AD patients was decreased. The immunofluorescence double label showed that the expression of Aβ was decreased in the region with more α3 expression. The correlation between α3nAChR and Aβ in the temporal lobe, frontal lobe and hippocampus of AD patients showed a negative correlation between α3nAChR and Aβ in the brain.

The levels of β-secretase and Aβ are increased in SH-SY5Y cells upon α3nAChR silencing. The present results indicated that the α-secretase ADAM10 as well as the γ-secretase related genes PS and NCT were downregulated in SH-SY5Y cells upon α3 knockdown. Interestingly, all ADAM10, PS, and NCT showed increased amounts in cells treated with nicotine. These results further emphasize that increased levels of α3nAChR could induce the non-amyloidogenic pathway, thereby reducing the generation of Aβ, which suggests the neuroprotective effect of α3nAChR in AD.

The notch pathway is important in cell proliferation, differentiation, and apoptosis. When a notch ligand binds to the extracellular domain of a Notch receptor, the Notch signaling pathway is activated [19]. Notch receptors are then cleaved by an ADAM family member (ADAM17

or ADAM10) and the γ-secretase complex [20]. After cleavage, NICD is released and translocated into the nucleus [21]. NICD binds to the CBF-1/suppressor of hairless/Lag1 (CSL) and leads to the transcriptional activation of the Hes and Hey related genes. The Notch-1 pathway is critical in regulating adult neurogenesis in the hippocampal tissue [22, 23]. A study showed that treatment of neural progenitor cells with DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), a γ-secretase inhibitor, induces the inhibition of the Notch pathway and leads to reduced proliferation and increased neuronal differentiation [24]. The current results showed that the levels of Notch1 and its target gene Hes 1 were decreased in cells upon α3 silencing. Meanwhile, Notch1 and Hes1 amounts were increased in cells treated with nicotine. These results showed that changes in the Notch signal transduction pathway may be related to α- and γ-secretase regulation by α3nAChR.

In summary, the expression of Aβ in the brains of AD patients was significantly higher than it was in the normal control group. The expression of α3nAChR in the brains of AD patients was significantly lower than it was in the normal control group. The expressions of α3nAChR and Aβ were negatively correlated in the brains of AD patients. a3nAChR silencing by siRNA downregulated ADAM10, PS, NCT, Notch1, and Hes1 at the mRNA and protein levels, which may be associated with an enhanced production of Aβ. Meanwhile, these factors showed elevated amounts in non-transfected SH-SY5Y cells administered nicotine. Changes of the non-amyloidogenic pathway in APP metabolism as well as the corresponding notch signal transduction regulated by α3nAChR suggest that the receptor likely plays a critical neuroprotective role in AD pathogenesis.

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Disclosure of conflict of interest

None.

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