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ORIGINAL PAPER

Construction of SH-EP1-α4β2-hAPP695 Cell Line and Effects of Nicotinic Agonists on β-amyloid in the Cells

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Abstract (1) Nicotinic acetylcholine receptors in central nervous system are thought to be new targets for Alzheimer's disease. However, the most involved nicotinic receptor subtype in Alzheimer's disease is unclear. α4β2 receptor is the most widely spread subtype in brain, involving in several important aspects of cognitive and other functions. We constructed cell line by transfecting human amyloid precursor protein (695) gene into SH-EP1 cells which have been transfected with human nicotinic receptor α4 subunit and β2 subunit gene, to observe effects of α4β2 receptors activation on β-amyloid, expecting to provide a new cell line for drug screening and research purpose. (2) Liposome transfection was used to express human amyloid precursor protein (695) gene in SH-EP1-α4β2 cells. Function of the transfected α4β2 receptors was tested by patch clamp. Effects of nicotine and epibatidine (selective α4β2 nicotinic receptor agonist) on β-amyloid were detected by Western blot and ELISA. Effects of nicotine and epibatidine on amyloid precursor protein (695) mRNA level were measured using real-time PCR. (3) Human amyloid precursor protein (695) gene was stably expressed in SH-EP1-α4β2 cells; Nicotine (1 μM) and epibatidine (0.1 μM) decreased intracellular and secreted β-amyloid in the cells; and activation of α4β2 receptors did not affect amyloid precursor protein (695) mRNA level. (4) These results suggest that the constructed cell line, expressing both amyloid precursor protein (695) gene and human nicotinic receptor α4 subunit and β2 subunit gene, might be useful for screening specific nicotinic receptor agonists against Alzheimer's disease. Alteration of Aβ level induced by activation of α4β2 nAChR in our study might occur at a post-translational level.

Keywords Alzheimer's disease · β-Amyloid · Nicotinic acetylcholine receptors · α4β2 subtype · Nicotine · Epibatidine

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that currently affects nearly 2% of the population in industrialized countries, and the incidence of the AD will increase threefold within the next 50 years (Vickers et al. 2000; Mattson 2004). The central tenet of the amyloid hypothesis of AD is that amyloid β-peptide (Aβ) is the major causative agent of the disease process (Selkoe 1994; Dovey et al. 2001; Sommer 2002; Mattson 2004). Amyloid precursor protein (APP) is processed following two different pathways, the so-called amyloidogenic and non-amyloidogenic processing, respectively. When APP undergoes the non-amyloidogenic processing, there is no release of intact Aβ peptide, and the generated stubs do not aggregate and have no amyloidogenic activity. In AD pathology, the production of β-amyloid peptide is the result of APP amyloidogenic processing. This involves the activity first of β- and then of γ-secretase (Perez et al. 1999). When abnormal mutations occur in APP genes, the amyloidogenic processing prevails, which results in increase of Aβ. Aβ aggregate into oligomers, which are toxic to neurons, and induce inflammation, oxidation stress, hyperphosphorylation of tau protein, and tangle of fiber (Dickson et al. 1995). To date, the primary treatments for Alzheimer's disease with proven efficacy have been acetylcholinesterase inhibitors and NMDA receptor antagonist memantine. The acetylcholine inhibitors can prevent the hydrolysis of acetylcholine (ACh) in the synaptic cleft, thereby prolonging its activity. Although these agents have some benefit in alleviating cognitive impairment, they have limited clinical utility because of insufficient efficacy and marginal tolerability. The clinical efficacy of memantine needs to be evaluated further because of limited approval time. Within the last decade, there has been much experimental support for the use of therapeutics that directly target nicotinic acetylcholine receptors (nAChRs) to improve cognitive function and slow neurodegenerative disease progression. These findings have spurred considerable research efforts to develop ligands selective for nAChRs.

There is abundant evidence that nAChR modulators have the potential to alleviate cognitive impairment in demented states. In addition to improving cognitive function, a large body of research implicates a role for nAChRs in neuroprotection, suggesting potential for disease modification. Recent evidence has shown that nAChRs may be one conduit by which Aβ exerts its pathophysiological effect, including downstream impairments on cognition (Oddo and LaFerla 2006). nAChRs may be the new target for drug discovery (Gotti et al. 2006).

nAChRs are ligand-gated ion channels with a pentameric structure formed by a combination of five different subunits (α, β, γ, δ and ε), each encoded by different genes (Gotti et al. 2004; Karlin 2002). The most widely spread subtype in the central nervous system (CNS) is $\alpha4\beta2$ (Wevers and Schroder 1999), which are highly expressed in brain regions that develop AD neuropathology, thus implicating these receptors in the pathogenesis of this dementia. Other data showed that there is a decrease in the number of brain nAChRs, particularly α4-containing receptors, in the absence of a general decrease in the number of neurons (Paterson and Nordberg 2000).

In our study, we constructed a cell line expressing human APP695(hAPP695) in SH-EP1 cells transfected with human nicotinic receptor α4 subunit and β2 subunit gene to observed the effect of $\alpha4\beta2$ nAChR activation on Aβ.

Materials and Methods

hAPP695 Gene Subcloned into pcDNA3.1

Primers with Xba I and Hind III restriction enzyme sites sequence in the ends and Pfu DNA polymerase were used to amplify hAPP695 gene that had been cloned into pXCJL (kindly

provided by Dr. Mucke, USA). The sense primer is 5′-ggctctagaccAcca gctgcccggtttggcactgct-3′, and the antisense primer is 5′-cccaagcttccgctagttctgcatctg ctcaaaga-3′. There is a Kozak translation initiation sequence (ccaccatg) in sense primer. The PCR product was digested by *Xba*I and *Hin*dIII (MBI, Lithuania), then purified by agarose gel electrophoresis.

Human hAPP695 Expressed in SH-EP1-α4βnAChR Cells

*Xba*I and *Hin*dIII restriction enzymes were used to subclone human hAPP695 gene into pcDNA3.1-neomycin. The recombined hAPP695-pcDNA3.1 was transfected with liposome into SH-EP1-α4β2 cells (nAChR α4 unit gene and β2 unit gene had been transfected into SH-EP1 cells) (provided by Dr. Lukas, USA). Briefly, mixture of 0.4 μg hAPP695-pcDNA3.1 and 12 μl Lipofectamine^{TM} (Invitrogen, USA) were added to 10^6 cells which were 50–80% confluent. After incubating at 37°C for 3 h, growth medium containing 2× the normal concentration of serum without removing the transfection mixture was added. Cells were passaged at a 1:10 dilution into selective medium (with neomycin 0.5 mg/ml). Clones of surviving cells were obtained by limiting dilution assay and expended before being screened for hAPP695 expression. Cells were maintained as low passage in medium with 0.25 mg/ml zeocin (selective for α4), 0.4 mg/ml hygromycin (selective for β2) and 0.5 mg/ml neomycin (selective for hAPP695) (Calbiochem, USA) to ensure stable expression of phenotype and passaged once weekly by splitting just-confluent cultures 1/10 to maintain cells in proliferative growth.

Reverse Transcription (RT)-PCR to Confirm Cell Clones Expressing hAPP695

Total RNA was isolated from the cell clones growing at 80% confluence in a 35-mm culture dish using 0.5 ml of TRIzol^{TM} reagent (Invitrogen, USA). Reverse transcription (RT) was carried out using 0.6 μg of total RNA and oligo(dT)20 primer in a 20 μl reaction with ReverTra Ace-α-^{TM}(TOYOBO, Japan). At the end of the RT reaction, reverse transcriptase was deactivated by incubation at 95°C for 5 min. A gradient PCR was performed using 2 μl of cDNA preparation, 1 μl of 10 μM each of 5′ and 3′ gene-specific primers, 0.5 μl of 10 mM dNTP, and 1.5 units of Taq DNA Polymerase in a 20 μl reaction. The primers were designed based on published gene sequences (NCBI: BC065529). The primer sequences and their predicted product sizes are: hAPP695 sense 5′-cactttgtgatcccaccg-3′, hAPP695 antisense 5′-ccaatgattgca ctttgttt-3′ (product size 1500 bp); nAChR, α4 subunit sense 5′-ccatcgctcagctc-attgac-3′, α4 antisense 5′-ctggtcggagggtgacttgc-3′ (product size 1080 bp); nAChR, β2 subunit sense 5′-tattccaatgccgtggtctcc-3′, β2 antisense 5′-tggtcatcgtcctcgctc-3′ (product size 940 bp); Amplification reactions were carried out in a MiniCycler (MJ Researche Inc.) for 15 amplification cycles at 94°C for 80 s, 60°C for 80 s, and 72°C for 150 s, and 16 amplification cycles at 94°C for 80 s, 55°C for 80 s, and 72°C for 150 s, followed by an additional 10-min extension at 72°C. Six microliters of each RT-PCR product was resolved on a 1% agarose gel before ethidium bromide staining was used to visualize bands under ultraviolet illumination.

Western Blot Analysis for Confirmation of hAPP695 Expression

Cells were washed two times with cold phosphate buffered saline (PBS) and lysed by incubating the plates for 5 min on ice with cell cysis buffer containing PMSF. Lysates were clarified by centrifugation and the supernatant stored as aliquots at –70°C. Protein amount was estimated using the BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China). Protein samples were prepared in SDS-PAGE sample buffer, and boiled for 5 min. Ten

micrograms per lane was applied to 10% acrylamide gels and transferred to nitrocellulose membrane for Western blot analysis. Membranes were then blocked by PBS buffer containing 5% skim milk powder for 3 h at room temperature and incubated with primary monoclonal antibody 22C11 (mouse anti-Alzheimer Precursor Protein A4; 1:1,000; Chemicon, USA) for 3 h. After three 5-min washes in PBS containing 0.05% Tween-20, secondary horseradish peroxidase-conjugated, goat anti-mouse IgG antibodies (1:5,000; Proteintech Group Inc. USA) were added to the reaction medium to allow conjugation with hAPP695-primary antibody targets. The horseradish peroxidase-labeled second antibody bands were then visualized by using enhanced chemiluminescence (Qiagen, Holand).

Patch Clamp Whole Cell Current Recordings

Cells plated on poly-lysine-coated 35-mm culture dishes were placed on the stage of an inverted microscope (Olympus, Japan) and continuously superfused with standard external solution (2 ml/min). Glass microelectrodes (3–5 MΩ resistance between pipette and extracellular solutions) were used to form tight seals (>2 GΩ) on the cell surface until suction was applied to convert to conventional whole cell recording. Cells were then voltage clamped at a holding potential of –60 mV, and ion current in response to application of ligand was measured (Axon Instruments 200B amplifier, CA), typically using data filtered at 2 kHz, acquired at 5 kHz, displayed and digitized on-line, and stored on hard media for subsequent off-line analysis. Data acquisition and analyses were done using Pclamp8.0 (Axon Instruments, CA), and results were plotted using Origin 5.0.

Western Blot Analysis for Detection of Aβ

Cells were lysed with cell lysis buffer containing Protease Inhibitor Cocktail Set I (Merck, Germany), and the lysates were clarified by centrifugation and the supernatant containing the solubilized protein was then collected. The proteins were concentrated by vacuum freeze drying before being subjected to SDS-PAGE separation on 16.5% acrylamide–glycerol gels and transferred to PVDF membrane (Millpore, USA) for Western blot analysis. Membranes were then blocked by PBS buffer containing 5% skim milk powder and incubated with primary monoclonal antibody 4G8 (1:1,000; Chemicon, USA) for 3 h. After three 5 min washes in PBS containing 0.05% Tween-20, secondary horseradish peroxidase-conjugated, goat anti-mouse IgG (sc-2006) antibodies (1:3,000; Santa Cruz Biotechnology, USA) were added to the reaction medium to allow conjugation with Aβ-primary antibody targets. The horseradish peroxidase-labeled second antibody bands were then visualized by using enhanced substrate Luminol. Earlier control studies had demonstrated that there was no cross-reactivity on the PVDF membranes between monoclonal antibody and secondary antibody.

Enzyme-linked Immunosorbent Assay for Detection of Aβ

10 cm plates of confluent cells were trypsinized and placed into 50 ml of the appropriate medium. A total of 2 ml of this cell suspension was placed into each of the wells in a six-well plate. The cells were then allowed to grow to confluence. The medium in each well was replaced with 1 ml of the conditioned media for 6 h after which it was collected and analyzed of Aβ by sandwich ELISA kit (Biosource, USA) according to the manufacturer's instructions. The results were read at an optical density of 450 nm using ELISA reader. Measurements were done in triplicate and results were reported as the mean ± SD.

Measurement of hAPP695 mRNA in SH-EP1-α4β2-hAPP695 Cells by Real-time PCR

Total RNA was extracted from SH-EP1-α4β2-hAPP695 cells (allowing samples from several different time points to be processed on the same day) using Trizol™ Reagent. RNA was reverse-transcribed using ReverTra Ace-α-™ and Oligo(dt) primers. SYBR Green I dye was used in this real-time RT-PCR method, which binds to any double stranded DNA produced in the reaction. Each reaction contained 10 μl SYBR Realmaster Mix (Tiangen, China), 1.5 μl forward primer (10 pmol/μl), 1.5 μl reverse primer (10 pmol/μl) and 1 μl template cDNA. Samples were analyzed in triplicate. The PCR conditions were 95°C for 2 min followed by 40 cycles of 94°C for 50 s; 56°C for 30 s; 68°C for 40 s after which a plate read was taken. The reactions were carried out on iCycler (BIO-RAD, USA) and data analyzed using the Opticon Monitor Analysis Software version 2.02. The primers were designed to amplify a 256 bp region of hAPP695 (sense 5′-tctccctgctctacaacgtg-3′; antisense 5′-cagccccaaaagaa gcc-3′). The purified plasmid hAPP695-pcDNA3.1 was used for standard curve.

Data Analysis

Statistical analyses were performed by using a *t*-test for comparison of independent means. Data are shown as means ± SD.

Results

pcDNA3.1-hAPP695 Construction

The double digested PCR product was purified by agarose gel electrophoresis (Fig. 1). Result of sequencing showed that the insert sequence is consistent completely with hAPP695 sequence published in NCBI (BC065529).

Confirmation of SH-EP1-α4β2-hAPP695 Cell Clones

The total RNA was extracted without degradation (Fig. 2a). By RT-PCR, 10 out of 14 analyzed cell clones were confirmed to express hAPP695 (Fig. 2b). No significant hAPP695-specific product was observed in the cells without hAPP695 transfection. 4 out of 10 cell clones were established to express both nAChR α4 and β2 subunit (Fig. 2c). Western blot analyses of the four SH-EP1-α4β2-hAPP695 cell clones (named by 1C8, 1A7, 2D12, 1D4) indicated that 1C8 cell clone expresses hAPP695 significantly (Fig. 2d).

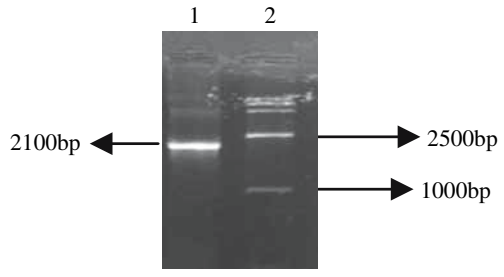

Fig. 1 Electrophoresis of double-digested hAPP695 PCR product. Lane 1: double digested hAPP695 PCR product. Lane 2: marker

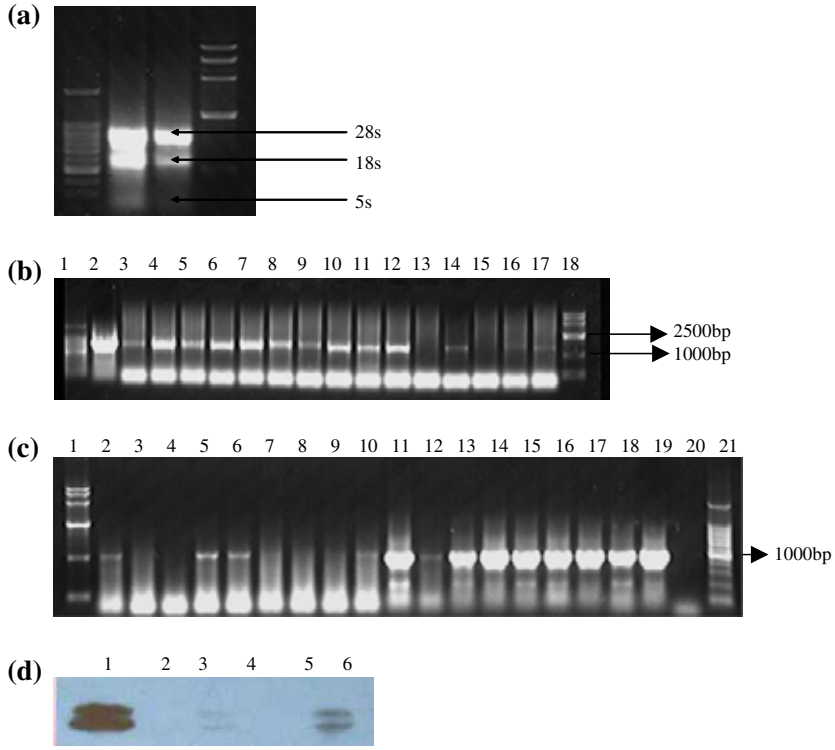

Fig. 2 Confirmation of SH-EP1-$\alpha4\beta2$-hAPP695 cell clones. (**a**) Electrophoresis of total RNA. (**b**) Electrophoresis of RT-PCR products (hAPP695 primer). Lanes 1 and 18: marker; lane 2: positive control (pcDNA3.1(–)-hAPP695 as template); lanes 3–17: RT-PCR products of different clones. (**c**) Electrophoresis of RT-PCR products ($\alpha4$ and $\beta2$ primer). Lanes 1 and 21: marker; lane 20: negative control (PCR without template); lanes 2–10: RT-PCR products of different clones with $\alpha4$ primer; lanes 11–19: RT-PCR products of different clones with $\beta2$ primer. (**d**) Western blotting analysis of the expression products. Lane 1: marker; lane 2: clone 1C8; lane 3: clone 2D12; lane 4: clone 1D4; lane5: negative control (not transfected cell); lane 6: clone 1A7

The whole cell current recording by patch clamp showed that when 100 μM nicotine was applied, the $\alpha4\beta2$ nAChR ion channel in 1C8 cell clone was activated and inward current was induced (Fig. 3). So 1C8 cell clone was considered as the cell model for further experiments.

Effects of Nicotinic Agonists and Antagonist on Aβ in SH-EP1-$\alpha4\beta2$-hAPP695 Cells

Effects of nicotine (1 μM), epibatidine (0.1 μM) on Aβ in SH-EP1-$\alpha4\beta2$-hAPP695 cells were detected by Western blot and ELISA. The data manifested that nicotinic agonists decreased

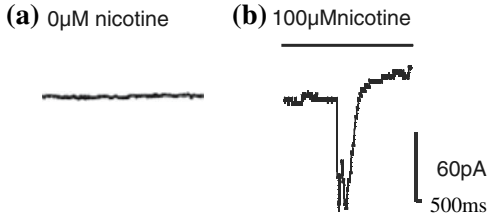

Fig. 3 Effect of nicotine on membrane potential of selected transfected cell clone. (**a**) 0 μM nicotine, there was no effect on membrane potential; (**b**) 100 μM nicotine, $\alpha4\beta2$ nAChR was activated and inward current was induced

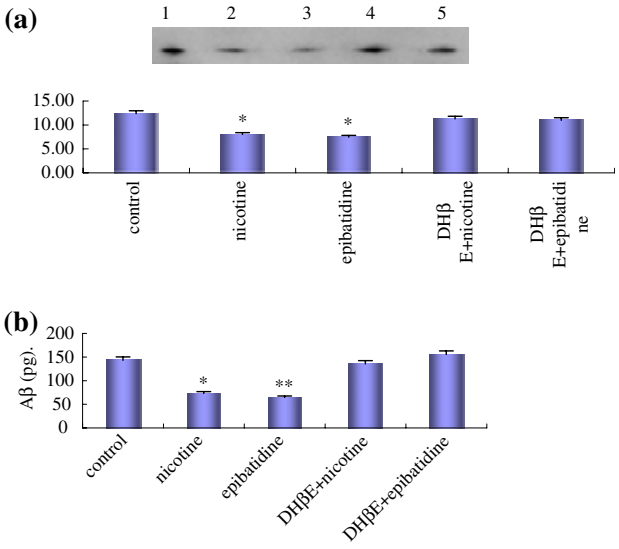

Fig. 4 Effects of nicotinic receptor agonist and antagonist on Aβ in SH-EP1-$\alpha4\beta2$-hAPP695 cells. (**a**) Western blot analysis of intracellular Aβ levels in SH-EP1-$\alpha4\beta2$-hAPP695 cells treated for 6 h. 1: control, 2: nicotine, 3: epibatidine, 4: DHβE + nicotine, 5: DHβE + epibatidine. In each lane, 30.5 μg of protein was applied. Nicotine (1 μM) and epibatidine (0.1 μM) significantly decreased intracellular Aβ in SH-EP1-$\alpha4\beta2$-hAPP695 cells, and DHβE (1 μM) reverseed the decrease. (**b**) ELISA analysis of secreted Aβ levels in SH-EP1-$\alpha4\beta2$-hAPP695 cells after 6 h incubation in the conditioned medium. Nicotine (1 μM) and epibatidine (0.1 μM) significantly decreased secreted Aβ, and DHβE (1 μM) reversed the decrease. Values are means ± SD. $^*P < 0.05$, $^{**}P < 0.01$ vs. control

intracellular and secreted Aβ in SH-EP1 cell model, compared with cells without treatment (control), and dihydro-β-erythroidine (DHβE) (1 μM), a selective $\alpha4\beta2$ nAChR antagonist, reversed the decrease (Fig. 4).

Effects of Agonists on hAPP695 mRNA

There was a single peak in the melt curve (data not shown), and no significant difference existed in hAPP695 mRNA level between groups with nicotine (1 μM) and epibatidine (0.1 μM) treated or not (Fig. 5).

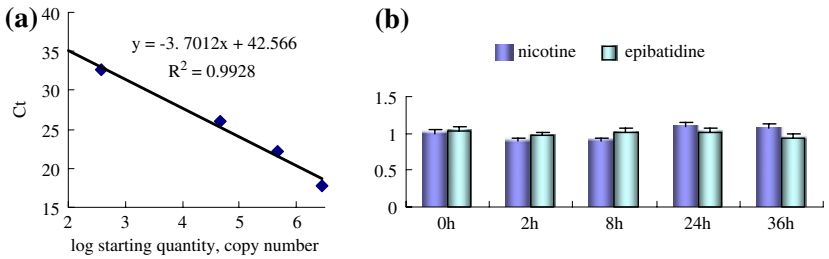

Fig. 5 (**a**) Standard curve of hAPP695 real-time PCR. (**b**) Copy number of hAPP695 mRNA. There were no significant differences in hAPP695 mRNA level between groups with nicotine (1 μM) and epibatidine (0.1 μM) treated for 2, 8, 24, and 36 h and group not treated

Discussion

Nicotinic cholinergic system is involved in several important aspects of cognitive functions, including attention, learning, and memory (Champtiaux et al. 2002). One of the most consistent observations in relation to normal human brain aging is the widespread decline in nicotinic receptors (Zoli et al. 2002). It is reasonable that this reduction can be one of the causes of mild cognitive impairment (MCI) and predispose subjects to neurodegenerative disorders such as AD to which cognitive impairment is associated. Data on nAChR and AD (Oddo and LaFerla 2006; Gotti et al. 2006) showed that specific affection of subtype might be beneficial to learning and memory improvement in AD.

Among the many subtypes present in the brain, the $\alpha4\beta2$ subtypes seem particularly involved in cognitive processes (Tribollet et al. 2004). The $\alpha4\beta2$ subtype is the most widespread receptor subtype in the brain, which is located in the frontal, parietal and temporal cortex, the hippocampus, the basal ganglia and the cerebellum (Perry et al. 2002; Lena et al. 1999; Ferreira et al. 2001). Loss of the $\alpha4\beta2$ nicotinic receptor subtype is found at autopsy in Alzheimer's disease. Using 123I-5IA-85380 single-photon-emission CT(SPECT), O'Brien found changes consistent with significant reductions in the nicotinic $\alpha4\beta2$ receptor in cortical and striatal brain regions (O'Brien et al. 2007). More and more selective $\alpha4\beta2$ receptor agonists with cognition-enhancing and neuroprotective properties are synthesized as potential drugs against AD (Pallavicini et al. 2006; Lippiello et al. 2006; Dunbar et al. 2007).

SH-EP1 cells derived from SK-N-SH (human neuroblastoma), which does not express nAChR originally. In our study, hAPP695 gene was transfected into SH-EP1 cells which have been transfected with human nicotinic receptor $\alpha4$ subunit and $\beta2$ subunit gene to construct a new cell line (SH-EP1-$\alpha4\beta2$-hAPP695) and function of $\alpha4\beta2$ nAChR was tested by patch clamp. Nonspecific and specific $\alpha4\beta2$ nAChR agonists (nicotine and epibatidine, respectively) decreased intracellular and secreted Aβ in SH-EP1-$\alpha4\beta2$-hAPP695. Selective $\alpha4\beta2$ nAChR antagonist DHβE reversed the decrease. This was consistent with reported data (Hellström-Lindahl et al. 2004). In this study, Real-time PCR was applied to detect effects of nicotine and epibatidine on hAPP695 mRNA level in cell model. There was no significant change in hAPP695 mRNA level before and after treatment. Consequently, alteration of Aβ level induced by activation of $\alpha4\beta2$ nAChR in our study might occur in downstream of Aβ processing, including α, β, and γ secretase activities. In summary, the SH-EP1-$\alpha4\beta2$-hAPP695 cell line might be useful for screening nicotinic receptor specific chemicals for Alzheimer's disease and for other research purposes.

As another mainly expressing subtype in brain, $\alpha7$ nAChR is also frequently reported involving in AD. New data shows $\alpha7$ nAChR decreases Aβ through MAPK, NF-κB, and c-myc pathways (Liu et al. 2007). Other findings indicate that $\alpha7$ nAChR may play a significant neuroprotective role by enhancing cleavage of APP by a-secretase, regulating signal transduction, and inhibiting the toxicity of Aβ, which is connected with the pathogenesis of AD (Qi et al. 2007). The effect of $\alpha7$ nAChR specific agents on the Aβ in the SH-EP1-$\alpha7$-hAPP695 cell line is being observed.

A series of reports suggest that the activity of β-secretase (BACE1) is upregulated in sporadic late-onset AD brains (Holsinger et al. 2002; Fukumoto et al. 2002; Yang et al. 2003). Presenilin (PS) provides the active core of the γ-secretase complex. Two mammalian homologs, PS1 and PS2, are found. Mutations in PS1 and PS2 genes account for the familial early-onset form cases. Mutations in PS genes certainly cause an increase in the ratio of Aβ42/Aβ40 species, as determined in mutant cells and transgenic mice (Weihl et al. 1999; Moehlmann et al. 2002; Oakley et al. 2006). The Aβ42 peptide has increased aggregation properties and is

believed to trigger a pathogenic cascade leading to neurodegeneration in AD (Sadowski et al. 2004). To determine which secretase is the target of $\alpha 4\beta 2$ nAChR activation in our study, further experiments are demanded.

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