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Up-regulation of nicotinic acetylcholine receptors by central-type acetylcholinesterase inhibitors in rat cortical neurons

Toshiaki Kume [a], Mitsuhiro Sugimoto [a], Yuki Takada [a], Toshiaki Yamaguchi [a], Atsushi Yonezawa [a], Hiroshi Katsuki [a], Hachiro Sugimoto [b], Akinori Akaike [a,*]

[a] Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan
[b] Department of Neuroscience for Drug Discovery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan

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Abstract

We previously reported that donepezil, a central-type acetylcholinesterase inhibitor, showed neuroprotective action via α4-and α7-nicotinic acetylcholine receptors against glutamate neurotoxicity in rat cortical culture. The present study was performed to investigate whether the neuroprotective action of acetylcholinesterase inhibitors is accompanied by the alteration of expression and function of nicotinic receptors. Four days treatment with acetylcholinesterase inhibitors (10 μM) enhanced the nicotine-induced increase of the intracellular calcium concentration. Immunoblot analysis revealed that donepezil increased both α4 and α7 subunit proteins. Donepezil and galanthamine increased the number of cells expressing α4- and α7-nicotinic receptors in immunocytochemical analysis. We examined whether up-regulation of nicotinic receptors affected the neuroprotective action of acetylcholinesterase inhibitors. Under up-regulating conditions, donepezil and galanthamine exerted neuroprotective action at lower concentrations. These results suggest that donepezil and galanthamine up-regulate nicotinic receptors in cortical neurons, and that the up-regulation of nicotinic receptors may make cortical neurons more sensitive to the neuroprotective action of donepezil and galanthamine.

Keywords: Acetylcholinesterase inhibitor; Nicotinic acetylcholine receptor; Up-regulation; Neuroprotection; Alzheimer's disease

1. Introduction

Alzheimer's disease is a progressive neurodegenerative dementia characterized by the impairment of memory and cognition. The main pathological findings in Alzheimer's disease are atrophy of the brain, amyloid deposition and neurofibrillary degeneration (Yankner, 2000; Selkoe, 1991). Approximately 80% of the central nervous system (CNS) cholinergic neurons are located in the nucleus basalis of Meynert, the structure providing major cholinergic pathways to the cortex and they are known to undergo selective and severe degeneration in Alzheimer's disease (Whitehouse et al., 1982). The neurobiochemical evidence additionally indicates decrease of choline acetyltransferase and the reduction of neuronal nicotinic acetylcholine receptors (Davies and Maloney, 1976; Guan et al., 2000; Court et al., 2001). Today, central-type acetylcholinesterase inhibitors, such as donepezil, galanthamine and tacrine, have been approved for Alzheimer's disease therapy because these findings suggest that the activation of cholinergic systems is effective in improving the symptoms of Alzheimer's disease (Wynn and Cummings, 2004).

Nicotinic receptors are members of the superfamily of ligand-gated cation-selective ion channels, widely expressed in the CNS. These receptors are composed of an assembly between eight alpha (α2–α9) and three beta (β2–β4) subunits as pentameric structures in plasma membranes. The two major classes of nicotinic receptors identified in the CNS are α4 (α4β2)-and α7-nicotinic receptors (Gotti et al., 1997; Weiland et al., 2000). In contrast to the muscle-type nicotinic receptors, neuronal nicotinic receptors are highly permeable to Ca^{2+} and rectify at negative voltages: the high Ca^{2+} permeability influences intracellular processes and it is proposed that rectification

* Corresponding author. Tel.: +81 75 753 4550; fax: +81 75 753 4579.
E-mail address: aakaike@pharm.kyoto-u.ac.jp (A. Akaike).

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acts as a coincidence detector (Fucile, 2002; Dajas-Bailador and Wonnacott, 2004). Homomeric α7 nicotinic receptors have a higher fractional Ca^{2+} current than heteromeric α4 nicotinic receptors, indicating that the subunit composition of nicotinic receptors influences their intrinsic Ca^{2+} permeability (Fucile, 2002). These actions of nicotinic receptors are considered to play an important role in the neuroprotection and enhancement of learning and memory (Levin, 2002; Dajas-Bailador and Wonnacott, 2004).

Chronic treatment with nicotine or other nicotinic receptor agonists induces the up-regulation of nicotinic receptors (Whiteaker et al., 1998). Moreover, chronic nicotine exposure can be associated with a long-lasting desensitization of nicotinic receptors and it has been proposed that chronic nicotine exposure results in the up-regulation of nicotinic receptors because nicotine produces desensitized nicotinic receptors, such that it acts as a functional antagonist (Schwartz and Kellar, 1985; Wonnacott, 1990). It was reported, however, that the concentration that induces nicotinic receptor desensitization is not sufficient for the up-regulation of nicotinic receptors to occur and the detailed mechanism is unclear (Rowell and Li, 1997; Fenster et al., 1999). Nicotinic receptors exist in two interconvertible states, one with a high affinity for ligand and a high conductance and the other with low affinity and low conductance. It has been also hypothesized that chronic treatment of nicotine stabilizes a larger fraction of receptors in the high affinity state (Romanelli et al., 1988; Buisson and Bertrand, 2002).

We have previously reported that donepezil protected cultured cortical neurons against glutamate neurotoxicity via α4- and α7-nicotinic receptors (Takada et al., 2003). The present study was carried out to determine whether the neuroprotective action of acetylcholinesterase inhibitors such as donepezil, galanthamine and tacrine, is accompanied by the alteration of the expression and function of nicotinic receptors. We investigated the effects of chronic acetylcholinesterase inhibitor treatments on the change of the functional properties and expression levels of protein and mRNA of nicotinic receptors.

2. Materials and methods

2.1. Materials

Eagle's minimal essential medium (MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum and horse serum were purchased from JRH Biosciences (Lenexa, KS, USA). Drugs and sources were as follows: polyethyleneimine solution, cytosine β-d-arabinofuranoside hydrochloride, galanthamine-HBr and tacrine were from Sigma-Aldrich (Saint Louis, MO, USA); l-glutamic acid monosodium salt was from Nacalai Tesque (Kyoto, Japan); Trypan blue was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); Fura 2-acetoxymethylester was from Dojindo Laboratories (Kumamoto, Japan); and donepezil hydrochloride [(±)-2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one monohydrochloride; E2020] was supplied by Eisai Co. Ltd.(Tsukuba-shi, Japan).

2.2. Cell cultures

Primary cultures were obtained from the cerebral cortex of fetal Wistar rats (17–19 days of gestation) according to the procedures described previously (Kume et al., 1997). Briefly, single cells dissociated from the whole cerebral cortex of fetal rats were plated on 0.1% polyethyleneimine-coated plastic or glass coverslips placed in Falcon 60-, 35-mm dishes, 12- or 24-well plates ($1.8–4.8 \times 10^5$ cells/cm^2). Cultures were incubated in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum (1–7 days after plating) or 10% heat-inactivated horse serum (8–13 days after plating), glutamine (2 mM), glucose (total 11 mM), $NaHCO_3$ (24 mM), and HEPES (10 mM). Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Six days after plating, non-neuronal cells were removed by adding cytosine arabinoside (10 μM). Only mature cultures (10–13 days in vitro) were used for experiments.

2.3. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultures using ISOGEN as recommended by the manufacturer (Nippon Gene, Tokyo, Japan). Two μg of total RNA were reverse-transcribed at 37 °C for 2 h in a 50 μl reaction mixture containing SuperScript II RnaseH-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and hexanucleotide random primers, and then cDNAs were obtained after the reaction Ribonuclease H (Invitrogen) for 30 min. The following sets of primers were used (from 5′ to 3′, position 1 being the first base of the start codon). α4 Forward: (−29)TGCTAGCAGCCACATAGAGA, α4 Reverse: (414) AACTTCATGGTACAGTTCTG; α7 Forward: (88) ACAAGGAGCTGGTCAAGAAC, α7 Reverse: (346) AAAGCGCTCATCAGCACTGT. The amplification protocol involved denaturation at 94 °C for 1 min, primer annealing at 51 °C (α4 subunit) or 59 °C (α7 subunit) for 1 min and extension at 72 °C for 1 min. This cycle was repeated 35 (α4 subunit) or 30 (α7 subunit) times. PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with 0.1% ethidium bromide. The bands were quantified using the Chemi Imager software.

2.4. Immunoblotting

Cells were fixed on the 12th day of culture to determine the protein levels of nicotinic receptors (α4 and α7 subunit) by Western blotting. Cells were washed twice with cold Tris-buffered saline, harvested using a cell scraper, and lysed in buffer containing Tris (20 mM), β-glycerophosphate (25 mM), EGTA (2 mM), Triton X-100 (1%), phenylmethylsulfonyl fluoride (1 mM), aprotinin (1%), dithiothreitol (2 mM), and vanadate (1 mM) on ice. Lysates were sonicated and centrifuged at 15,000 rpm at 4 °C for 30 min. The protein was denatured by boiling for 5 min. An aliquot (approx. 30 μg of protein) of the supernatant was loaded onto a sodium dodecyl sulfate polyacrylamide gel, separated electrophoretically, and transferred to a

polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The polyvinylidene difluoride membrane was incubated with 10 mM Tris-buffered saline containing 0.1% Tween 20 and 5% dehydrated skim milk to block non-specific protein binding. The membrane was then incubated with primary antibodies (anti-nicotinic receptors, α4 and α7 subunit, antibodies (Covance Research Products, Berkeley, CA, USA) (1:1000)) and with secondary antibody. Subsequently, the membrane-bound antibodies were treated with avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories Inc., Burlingame, CA, USA) and detected with an enhanced chemiluminescence detection system (ECL, Amersham Biosciences, Buckinghamshire, UK) and exposed to Fuji X-ray film. The bands were quantified using the NIH image software.

2.5. Immunocytochemistry

Cells were fixed in 0.1 M phosphate buffer containing 4% paraformaldehyde for 30 min, and processed for nicotinic receptor immunocytochemistry. After rinsing with phosphate-buffered saline (PBS), fixed cells were exposed to 0.2% Triton X-100 in PBS for 15 min. Cells were subsequently incubated for 2 h at room temperature with rat anti-nicotinic receptor, α4 subunit, antibody (1:800, Sigma, Saint Louis, MO, USA) and mouse anti-nicotinic receptor, α7 subunit, antibody (1:800, Sigma) rabbit anti-microtubule-associated protein 2 (MAP2) (1:1000, Chemicon International Inc., Temecula, CA, USA), a neuron specific maker protein. Alexa Fluor 488-labeled goat anti-rat IgG (1:1000, Molecular Probes, Eugene, OR, USA), Alexa Fluor 488-labeled goat anti-mouse IgG (1:1000, Molecular Probes) and Alexa Fluor 568-labeled goat anti-rabbit IgG (1:1000, Molecular Probes) were used as secondary antibodies. Immunofluoroescence was visualized with a fluorescence microscope (Nikon Eclipse TE 300 with a CCD spot camera) or a Nikon Diaphot 200 microscope equipped with a laser scanning confocal imaging system (MRC-1024, Bio-Rad Laboratories). The captured images were analyzed using the NIH image software.

2.6. Intracellular Ca^{2+} imaging

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured with a Ca^{2+} -sensitive fluorescent dye, fura 2-acetoxymethylester, on a fluorescence imaging system (ARGUS/HiSCA, Hamamatsu Photonics K. K., Shizuoka, Japan), according to methods described previously (Shirakawa et al., 2002). Cortical neurons cultured on glass coverslips were incubated in Krebs-Ringer buffer (137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 25 mM d-(+)-glucose, 10 mM HEPES, pH 7.4) containing 5 μM fura 2-acetoxymethylester and 0.01% cremophor EL for 1 h at 37 °C and then rinsed with the buffer. A coverslip was mounted on a recording chamber placed under a fluorescent microscope. The cells were alternatively illuminated with lights (wavelengths of 340 and 380 nm) at an interval of 2 s, and the emission was measured at 500 nm. Fluorescence imaging was performed for 3 min at room temperature. The peak amplitude of the fluorescence ratio (340/380 nm) immediately after nicotine application was adopted as an index of the nicotine-induced increase in $[Ca^{2+}]_i$.

2.7. Measurement of neurotoxicity

Neurotoxicity induced by glutamate was quantitatively assessed by examining cultures under Hoffman modulation microscopy according to the methods described previously (Kume et al., 1997; Takada et al., 2003). All experiments were performed in Eagle's MEM at 37 °C. Cells were in Trypan blue solution for 10 min at room temperature, fixed with isotonic formalin (pH 7.0, 2–4 °C), and rinsed with physiological saline. Cell viability was assessed by counting the Trypan blue-stained cells and non-stained cells. In each experiment, the cells on five coverslips were counted to obtain the means±S.E.M. of cell viability.

2.8. Statistics

Data were expressed as means±S.E.M. The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's two-tailed test using the InStat (Graph Pad Software, San

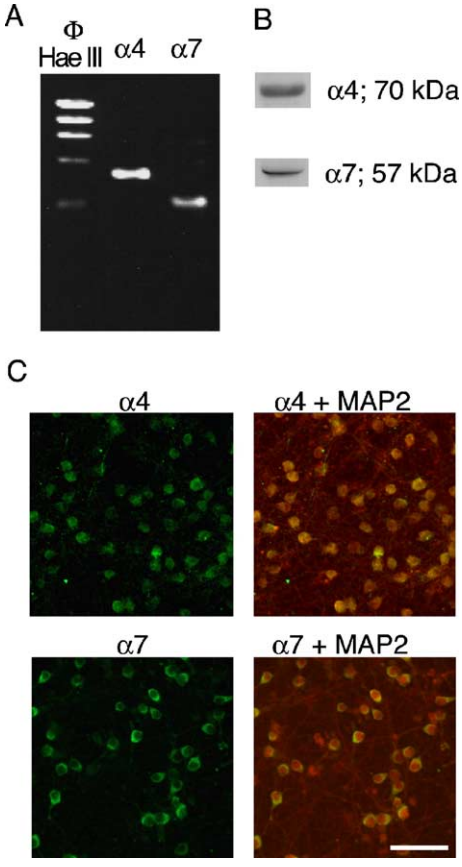

Fig. 1. Expression of α4 and α7 receptor subunits in rat cortical neurons. A, detection of nicotinic receptor subunit mRNAs by RT-PCR. B, C, detection of nicotinic receptor subunit proteins by Western blotting and immunocytochemistry. Scale bars=50 μm.

Diego, USA). Statistical significance was defined as a probability value of less than 5%.

3. Results

3.1. Expression of α4 and α7 nicotinic receptor subunits in rat cortical neurons

It has been reported that the major subtypes of nicotinic receptors in the cortex were α4- and α7-nicotinic receptors (Gotti et al., 1997). To confirm the expression of α4 and α7 subunits in our primary cortical cultures, RT-PCR, immunoblotting and immunocytochemical analyses were carried out using primers and antibodies specific for each neuronal nicotinic receptor subunits. The mRNA and protein expression of α4 and α7 subunits were detected (Fig. 1).

3.2. Effects of acetylcholinesterase inhibitors on the nicotine-induced increase of the intracellular calcium concentration

To examine the effects of acetylcholinesterase inhibitors on the functional change of nicotinic receptors, we measured intracellular calcium concentration ($[Ca^{2+}]_i$) induced by nicotine after 4 days treatment with acetylcholinesterase inhibitors. Nicotine (10 μM) induced transient increase in $[Ca^{2+}]_i$ via nicotinic acetylcholine receptors in cortical neurons (Fig. 2A) and donepezil (10 μM) treatment for 4 days enhanced the nicotine-induced increase in $[Ca^{2+}]_i$ (Fig. 2). Treatment of galanthamine or tacrine (0.1–10 μM, respectively) for 4 days also significantly enhanced the nicotine-induced increase in $[Ca^{2+}]_i$ (Fig. 2B).

3.3. Effects of acetylcholinesterase inhibitors on the expression of α4 and α7 nicotinic receptor subunits

Because it was revealed that 4 days treatment with central-type acetylcholinesterase inhibitors potentiated the nicotine-induced increase in $[Ca^{2+}]_i$, the protein levels of α4 and α7 nicotinic receptor subunits were analyzed by immunoblotting. Cultures were treated with donepezil, galanthamine or tacrine (0.1–10 μM, respectively) for 1 or 4 days. The α4 and α7 nicotinic receptor subunit protein levels were increased significantly after 4 days treatment with donepezil (10 μM) (Figs. 3 and 4A, B). In the case of galanthamine, the α7 nicotinic receptor subunit protein level showed a tendency to increase for 4 days treatment and the α4 nicotinic receptor subunit protein had no alteration (Fig. 4C, D). On the other hand, no significant change of the α4 and α7 nicotinic receptor subunit protein levels were detected with tacrine (Fig. 4E, F).

In order to investigate the alteration in the proportion of nicotinic receptors expressing neurons, immunocytochemistry was carried out. Fig. 5A showed images typical of the α4 or α7 nicotinic receptor subunit- and MAP2-positive cells. Donepezil (10 μM) treatment for 4 days increased the proportion of α4- and α7-nicotinic receptor subunit-positive neurons. Similarly, galanthamine or tacrine (10 μM) was administrated for 1 or 4 days and the nicotinic receptor subunit-positive neurons were

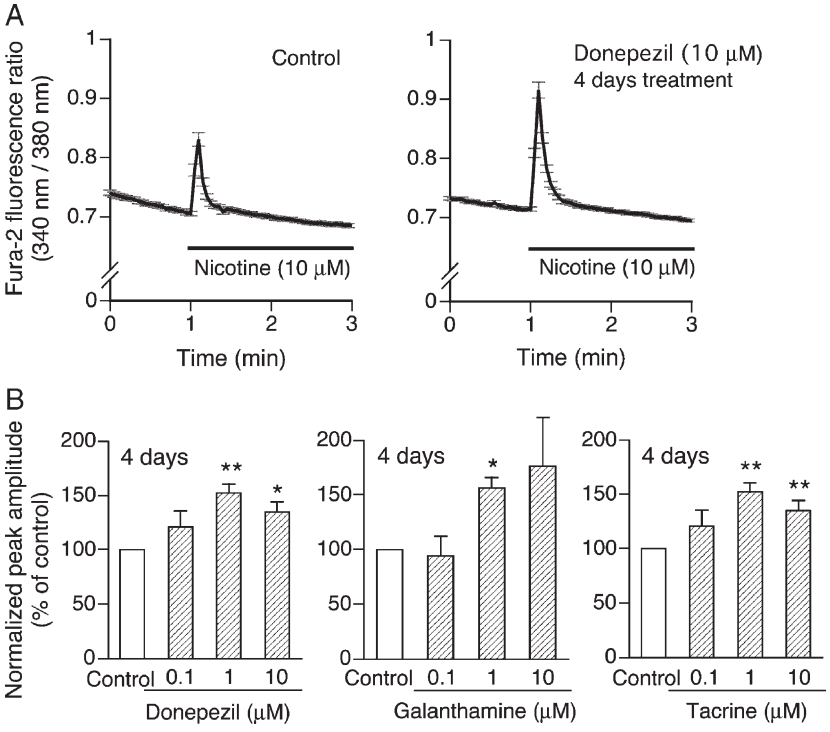

Fig. 2. Effects of acetylcholinesterase inhibitors on nicotine-induced increases in $[Ca^{2+}]_i$. A, representative charts of changes in fura-2 fluorescence ratio. Donepezil (10 μM) was pretreated for 4 days then washed out 3 times for 2 h. Donepezil was not present in the extracellular fluid and nicotine at a final concentration of 10 μM was added to the extracellular fluid at 1 min. B, donepezil, galanthamine or tacrine (0.1–10 μM, respectively) were pretreated for 4 days and nicotine at a final concentration of 10 μM was added to the extracellular fluid at 1 min. The peak amplitude was estimated immediately after the addition of nicotine. * $P<0.05$, ** $P<0.01$ compared with control.

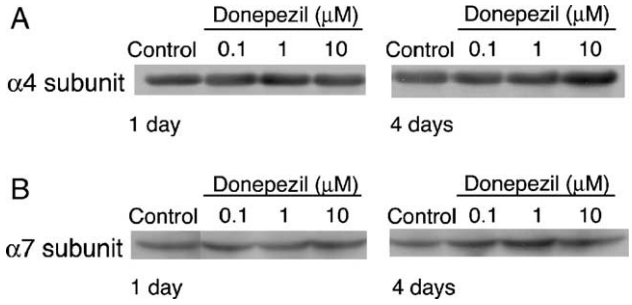

Fig. 3. Representative data of expression of α4 (A) and α7 (B) receptor subunit proteins by donepezil treatment for 1 or 4 days detected by western blotting analysis. Cultures were treated with donepezil (0.1–10 μM) for 1 or 4 days. The bands of 70 and 57 kDa were identified as α4 and α7 receptor subunit proteins, respectively.

counted (Fig. 5B). The proportions of α4 and α7 nicotinic receptor subunit expressing neurons were significantly increased by treatment with donepezil or galanthamine for 4 days, but no change was induced by tacrine (Fig. 5B). These results suggested that treatment with donepezil and galanthamine for 4 days induced the up-regulation of α4- and α7-nicotinic receptors at least in the case of immunocytochemical analysis.

To clarify whether the alteration in expression of mRNA for nicotinic receptors was involved in the change of their protein levels, we examined the mRNA levels with RT-PCR analysis. Cultures were treated with acetylcholinesterase inhibitors (0.1–10 μM, respectively) for 1 or 4 days and then the mRNA was extracted and RT-PCR performed. Donepezil had no effect on α4 and α7 nicotinic receptor subunit mRNA levels in each treatment (Fig. 6A, B). Galanthamine and tacrine also had no influence (data not shown).

3.4. Effects of up-regulation of nicotinic receptors by acetylcholinesterase inhibitors on neuroprotective action

Next, we examined the influence of the neuroprotective action of acetylcholinesterase inhibitors against glutamate neurotoxicity in up-regulated conditions of nicotinic receptors after treatment with three acetylcholinesterase inhibitors (10 μM) for 4 days. We previously reported that the exposure of cortical neurons to glutamate markedly reduced the viability and donepezil protected cortical neurons against glutamate neurotoxicity via α4- and α7-nicotinic receptors in a concentration dependent manner (Takada et al., 2003). Trypan blue exclusion revealed that the viability was decreased by treatment with glutamate (1 mM) for 10 min followed by incubation with glutamate-free medium for 1 h, whereas most of the neurons without glutamate treatment had non-stained cell bodies (Fig. 7A, B). Treatment with donepezil (10 nM) for 24 h before glutamate exposure shows no protective effect against glutamate neurotoxicity (Fig. 7C). However, 4 days pretreatment with donepezil (10 μM) prior to 24 h treatment 10 nM donepezil followed by glutamate

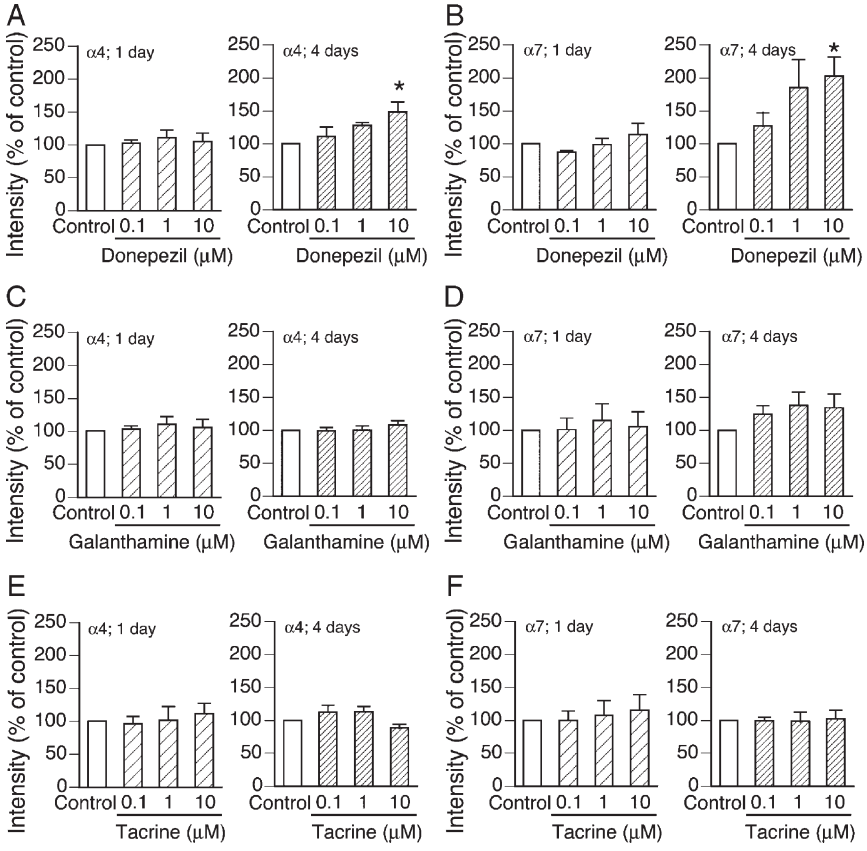

Fig. 4. Effects of acetylcholinesterase inhibitors on expression of α4 (A, C, E) and α7 (B, D, F) receptor subunit proteins by western blotting analysis. Cultures were treated with donepezil, galanthamine or tacrine (0.1–10 μM, respectively) for 1 or 4 days. The results are expressed as the percentage of detected signals obtained from control taken as 100%. * $P<0.05$ compared with control.

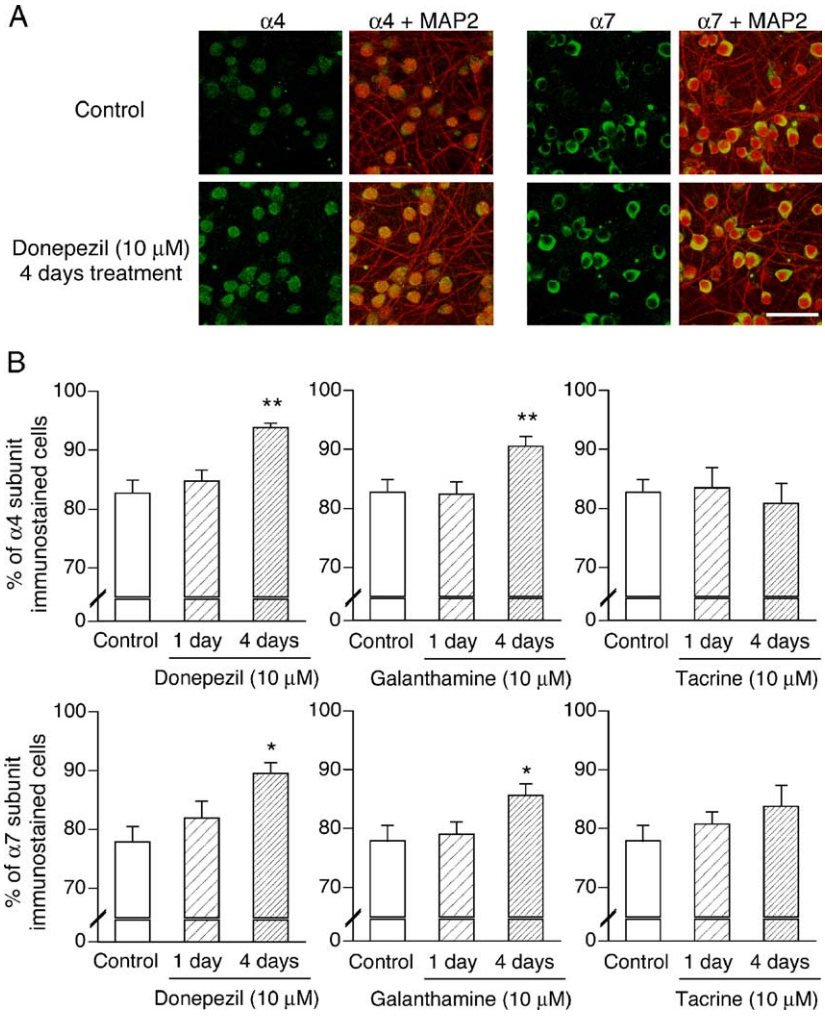

Fig. 5. Effects of acetylcholinesterase inhibitors on the expression of α4 and α7 receptor subunits in rat cortical neurons. Cultures were treated with donepezil, galanthamine or tacrine (10 μM, respectively) for 1 or 4 days. A, typical images of α4 and α7 nicotinic acetylcholine receptor subunits-and MAP2-immunocytochemistry. B, the change of nicotinic subunit expressing cells (% of nicotinic receptors-positive cells to MAP2-positive cells) after acetylcholinesterase inhibitors treatment. * $P<0.05$, ** $P<0.01$ compared with control. Scale bar=30 μm.

exerted significant neuroprotective effect against glutamate neurotoxicity (Fig. 7D, E). No neuroprotective effect was observed even in the up-regulated conditions when the cultures were incubated without donepezil for 24 h prior to glutamate treatment. As well as donepezil, 4 days treatment with galanthamine (10 μM) prior to 1 nM galanthamine significantly inhibited glutamate neurotoxicity (Fig. 7E). On the other hand, tacrine had no neuroprotective effect on glutamate in each treatment (Fig. 7E).

4. Discussion

In the present study, the effects of central-type acetylcholinesterase inhibitors on the function and expression of nicotinic

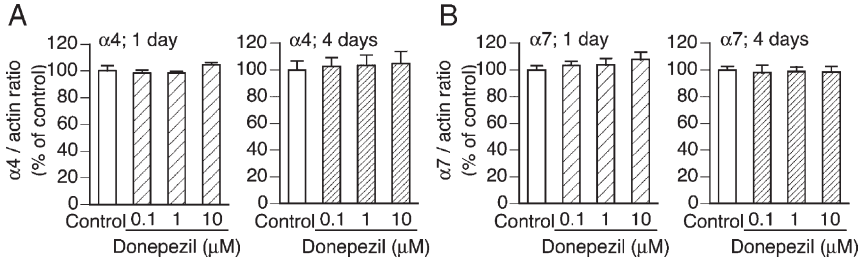

Fig. 6. Effects of donepezil on the expression of α4 (A) and α7 (B) receptor subunit mRNAs. Cultures were treated with donepezil (0.1–10 μM) for 1 or 4 days. The results are expressed as the percentage of detected signals obtained from each sample divided by the mean of the control, taken as 100%. Each signal was corrected using that of the corresponding β-actin value.

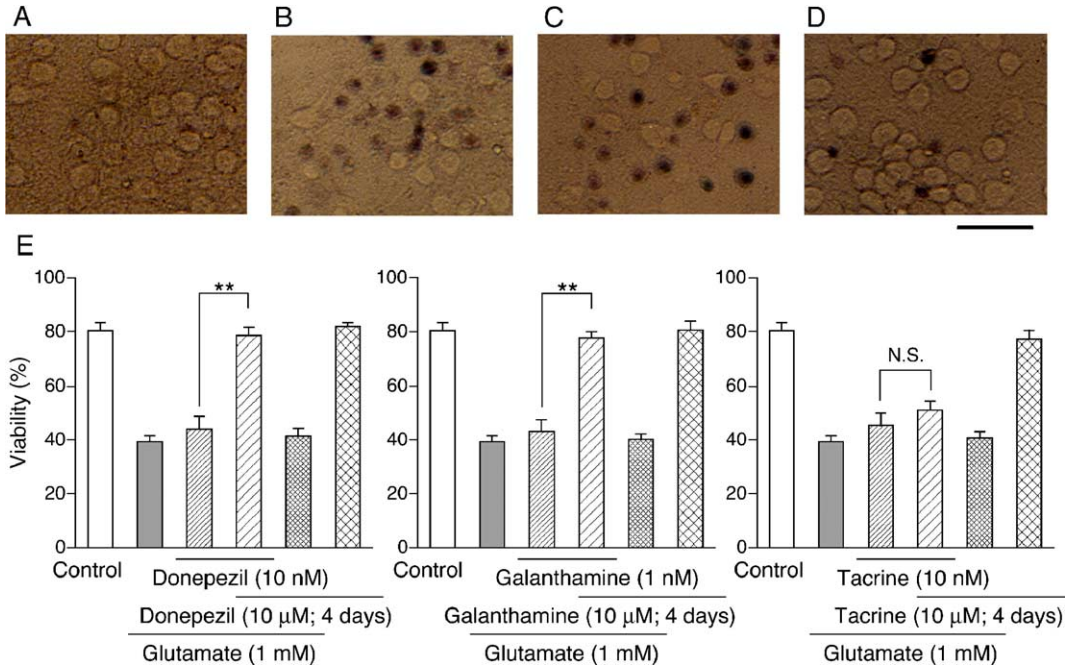

Fig. 7. Effects of 4 days of treatment with acetylcholinesterase inhibitors on glutamate neurotoxicity. Cultures were exposed to donepezil (10 nM), galanthamine (1 nM) or tacrine (10 nM) for 24 h after the 4 day treatments of donepezil, galanthamine or tacrine (10 μM, respectively). They were then exposed to glutamate (1 mM) for 10 min followed by incubation with glutamate-free medium for 1 h. Culture fields were photographed after Trypan blue staining, followed by formalin fixation. A indicates the cells with sham treatment (control). B shows the cells treated with glutamate. C shows the cells exposed to donepezil (10 nM) for 24 h prior to glutamate treatment. D shows the cells pretreated for 4 days with donepezil (10 μM) prior to the 24 h 10 nM donepezil treatment followed by glutamate. E is the viability of the cortical neurons treated with acetylcholinesterase inhibitors on glutamate-induced neurotoxicity. ** $P < 0.01$, N.S.: not significant. Scale bar = 50 μm.

receptors were examined in rat primary cortical neurons. Donepezil and galanthamine induced the up-regulation of nicotinic receptors without alteration in the mRNA level, and tacrine induced the functional up-regulation of nicotinic receptors without change in the protein and mRNA level (Table 1). In addition, donepezil or galanthamine exerted a neuroprotective action at lower concentrations in up-regulated conditions of nicotinic receptors after 4 days treatment. On the other hand, tacrine had no influence on the neuroprotective effect after 4 days treatment.

Donepezil induced the up-regulation of both α4- and α7-nicotinic receptors, which concomitantly caused an increase in the protein level, while their mRNAs had no change. These results suggested that donepezil modulated the up-regulation of nicotinic receptors at posttranscriptional levels. It was reported that nicotine induced the up-regulation of α4-nicotinic receptors in M10 cells and the up-regulation resulted from a decrease in the rate of receptor turnover; in short, nicotinic receptors were removed from the surface and degraded more slowly (Peng et al., 1994). Thus, donepezil may suppress the metabolism and degradation from plasma membrane modulating receptor turnover similar to nicotine.

Table 1
Effects of acetylcholinesterase inhibitors on α4- and α7-nicotinic acetylcholine receptors

	Donepezil	Galanthamine		Tacrine
Nicotine-induced increase in $[Ca^{2+}]_i$	↑	↑		↑
Protien level	↑	α_4 →	α_7 (↑)	→
% of nAChR expression cells	↑	↑		→
mRNA level	→	→		→

↑: Significant increase. (↑): Tendency to increase. →: No change.

Galanthamine showed different effects between receptor subtypes in this study. The α7 subunit tended to increase the protein level after 4 days treatment with galanthamine, but the α4 subunit was unchanged. In this case, galanthamine may also induce the up-regulation of nicotinic receptors in a similar way to nicotine. In addition, galanthamine is a potent allosteric potentiating ligand (APL) of nicotinic acetylcholine receptors; while the AChE activity is relatively weak compared to donepezil and tacrine (Dajas-Bailador et al., 2003; Samochocki et al., 2003; Ogura et al., 2000; Tang, 1996). APLs are thought to bind to a distinct site in the extracellular N-terminal domain or the nicotinic receptors and potentiate agonist responses (Schrattenholz et al., 1996). As previously reported, galanthamine acts as on an APL on both α4- and α7-nicotinic receptors (Samochocki et al., 2003). Taking together these results and the APL action, we suggested that galanthamine may directly modulate to α4- and α7-nicotinic receptors and facilitate receptor function.

Tacrine enhanced the nicotine-induced increase in $[Ca^{2+}]_i$ without effects on the mRNA and protein levels of nicotinic receptors. In some cases, it was reported that nicotinic receptors exchanged low-affinity nicotinic receptors for high-affinity nicotinic receptors on plasma membrane (Romanelli et al., 1988; Buisson and Bertrand, 2002). Although the detail of the mechanisms is unclear, tacrine may induce functional up-regulation in this way.

It was also reported that the function of nicotinic receptors were regulated by phosphorylation via cAMP (Margiotta et al., 1987; Vijayaraghavan et al., 1990). 8-Bromo-cAMP, a membrane-permeable cAMP analog, phosphorylated α-type nicotinic receptor subunits and enhanced the ACh response. In addition, nicotine enhanced the phosphorylation of α4-nicotinic receptors by cAMP-dependent protein kinase (Hsu et al., 1997). Nicotine activates nicotinic receptors and generates several signals. In this case, we considered that nicotine phosphorylated nicotinic receptors by regulating several signals and inducing the cAMP-related mechanism after stimulation of nicotinic receptors and enhanced the function of nicotinic receptors. On the other hand, it has been reported that acetylcholinesterase inhibitors exerted neuroprotective action via nicotinic receptors to the same extent as nicotine (Kaneko et al., 1997; Takada et al., 2003). Together, acetylcholinesterase inhibitors-, as well as the nicotine-induced up-regulation of nicotinic receptors, may be involved in cAMP.

We previously reported that the neuroprotective effect of donepezil via α4- and α7-nicotinic receptors was in a concentration dependent manner against glutamate neurotoxicity (Takada et al., 2003). Galanthamine and tacrine also had concentration-dependent neuroprotective effect (data not shown). To determine whether the up-regulation of nicotinic receptors affects the neuroprotective action of acetylcholinesterase inhibitors, we examined the neuroprotective action of acetylcholinesterase inhibitors against glutamate neurotoxicity in up-regulated conditions of nicotinic receptors. Donepezil and galanthamine exerted their neuroprotective action at a lower concentration after 4 days treatment with donepezil or galanthamine. These results suggested that the up-regulation of nicotinic receptors by donepezil or galanthamine contributed to enhancing the sensitivity of nicotinic signals via nicotinic receptors and potentiating the neuroprotective action of donepezil or galanthamine. Nicotine protects against several models of neuronal death in vitro and in vivo via nicotinic receptors (Kaneko et al., 1997; Hejmadi et al., 2003; Ryan et al., 2001). It was also reported that nicotine protects against amyloid-β protein-induced neurotoxicity by α7-nicotinic receptor activation, followed by the Ca^{2+}-dependent phosphatidylinositol 3-kinase pathway, which activates Akt and up-regulates Bcl-2 (Kihara et al., 2001). Therefore, the enhancement of nicotinic receptor signals may also contribute to the neuroprotective action.

Although the pathogenesis of Alzheimer's disease remains unknown, amyloid deposition is present in the brain and it has been suggested that amyloid-β protein plays an important role in the neurodegeneration accompanying the progress of the disease (Selkoe, 2002). Recent studies have shown that naturally secreted oligomers of amyloid-β protein potently inhibit the hippocampal long-term potentiation in vivo (Walsh et al., 2002). On the other hand, the amyloid-β protein levels in the brain of smoking Alzheimer's disease patients were significantly decreased compared to non-smokers with Alzheimer's disease in the temporal cortex and hippocampus (Hellstrom-Lindahl et al., 2004). In other words, it was suggested that nicotinic receptor stimulation reduces the amyloid-β protein level and contributes to suppress the progression of the state of Alzheimer's disease. In addition, deficits of nicotinic receptors have been seen in the postmortem brain of patients with Alzheimer's disease. The protein level of the α4 nicotinic receptor subunit was reduced significantly in the temporal cortex of the patients with Alzheimer's disease and that of the α7 nicotinic receptor subunit was decreased in temporal cortex without reducing the mRNA of nicotinic receptors respectively (Guan et al., 2000; Hellstrom-Lindahl et al., 1999). Accordingly, several studies indicate a significant correlation between Alzheimer's disease pathology and nicotinic receptor loss (Court et al., 2001). Therefore, the suppression of nicotinic receptors loss or the increment of nicotinic receptors should improve the symptoms of Alzheimer's disease. In this study, we indicated that donepezil and galanthamine up-regulated the nicotinic receptors. Donepezil and galanthamine may contribute to the improvement in the symptoms of Alzheimer's disease by up-regulating or inhibiting the loss of nicotinic receptors.

In conclusion, it was shown that donepezil and galanthamine induced the up-regulation of α4- and α7-nicotinic receptors without mRNA modulation, and tacrine induced the functional up-regulation of nicotinic receptors without protein synthesis. In addition, donepezil and galanthamine enhanced the neuroprotective effect against glutamate neurotoxicity after 4 days of treatment. The present findings suggest that donepezil and galanthamine induce the up-regulation of nicotinic receptors and this up-regulation makes the nicotinic receptors more sensitive to the neuroprotective action of donepezil and galanthamine.

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