Donepezil Improves Cognitive Function in Mice by Increasing the Production of Insulin-Like Growth Factor-I in the Hippocampus

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ABSTRACT

Insulin-like growth factor-I (IGF-I) exerts beneficial effects on cognitive function. The selective acetylcholinesterase inhibitor donepezil increases serum IGF-I levels in elderly subjects. Because stimulation of sensory neurons induces IGF-I production by releasing calcitonin gene-related peptide (CGRP) in the mouse brain, we hypothesized that donepezil increases IGF-I production by sensory neuron stimulation to improve the cognitive function in mice. Donepezil, but not tacrine, increased the CGRP release from dorsal root ganglion neurons isolated from wild-type (WT) mice. Pretreatment with the protein kinase A inhibitor KT5720 [(9S,10S,12R)-2,3,9,10,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3,4-i][1,6]-benzo-diazocine-10-carboxylic acid hexyl ester] reversed the effects induced by donepezil. Increase in tissue levels of CGRP, IGF-I, and IGF-I mRNA in the hippocampus was observed at 4 weeks after oral administration of donepezil in WT mice. In these animals, c-fos expression in spinal dorsal horns, parabrachial nuclei, the solitary tract nucleus, and the hippocampus was increased. Enhancement in angiogenesis and neurogenesis was observed in the dentate gyrus of the hippocampus of WT mice after donepezil administration. Improvement of spatial learning was observed in WT mice after donepezil administration. Oral administration of tacrine for 4 weeks produced none of the aforementioned effects induced by donepezil in WT mice. However, none of the effects observed in WT mice was seen after donepezil administration in CGRP-knockout mice and WT mice subjected to functional denervation. These observations suggest that donepezil may improve cognitive function in mice by increasing the hippocampal production of IGF-I through sensory neuron stimulation. These effects of donepezil may not be dependent on its acetylcholinesterase inhibitory activity.

Activation of the central cholinergic system has been shown to induce hippocampal neurogenesis, thereby contributing to improvement of cognitive function (Kotani et al., 2006). In this context, the selective acetylcholinesterase inhibitor donepezil has been reported to improve the cognitive impairment in Alzheimer’s disease (Winblad et al., 2006).

Insulin-like growth factor-I (IGF-I) is a basic peptide composed of 70 amino acids, with ubiquitous distribution in various tissues and cells (Okajima and Harada, 2008). It mediates the growth-promoting actions of growth hormone (GH) and plays an important role in postnatal and adolescent growth (Okajima and Harada, 2008). IGF-I has been shown to enhance excitatory synaptic transmission in the CA1 region of the hippocampus (Ramsey et al., 2005) and to improve spatial learning by inducing neurogenesis in the hippocampus (Aberg et al., 2000). The impaired spatial learning in mice with low serum levels of IGF-I is reversed by exogenous administration of IGF-I (Trejo et al., 2008). A close correlation has been shown between the plasma IGF-I levels, and cognitive function has been shown in older individuals (Landi et al., 2007). These observations strongly suggest that IGF-I may improve cognitive function by increasing the plasticity and promoting neurogenesis in the hippocampus.

ABBREVIATIONS: IGF-I, insulin-like growth factor-I; GH, growth hormone; VEGF, vascular endothelial growth factor; CGRP, calcitonin gene-related peptide; TRPV1, transient receptor potential vanilloid 1; WT, wild type; CPZ, capsazepine; BrdU, 5-bromo-2-deoxyuridine; GH, growth hormone; VEGF, vascular endothelial growth factor; CGRP, calcitonin gene-related peptide; TRPV1, transient receptor potential vanilloid 1; WT, wild type; CPZ, capsazepine; BrdU, 5-bromo-2-deoxyuridine; PKA, protein kinase A; PCR, polymerase chain reaction; DRG, dorsal root ganglion; PBS, phosphate-buffered saline; GFAP, glial fibrillary acidic protein; NTS, solitary tract nucleus; DG, dentate gyrus; PBN, parabrachial nuclei; KT5720, (9S,10S,12R)-2,3,9,10,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3,4-i][1,6]-benzo-diazocine-10-carboxylic acid hexyl ester; CT, calcitonin.
The angiogenesis factor vascular endothelial growth factor (VEGF) has an important role in the coupling between angiogenesis and neurogenesis in the brain (Greenberg and Jin, 2005). IGF-I promotes angiogenesis via a VEGF-dependent mechanism in the brain (Lopez-Lopez et al., 2004). IGF-I may promote hippocampal neurogenesis by promoting angiogenesis because vascular elements are thought to be an essential feature of the stem-cell niche in the hippocampus (Palmer et al., 2000).

Capsaicin-sensitive sensory neurons are nociceptive neurons found in many tissues: within the lining epithelia, around blood vessels, and in the nonvascular smooth muscle and myocardium of the atria (Okajima and Harada, 2006). These sensory neurons release calcitonin gene-related peptide (CGRP) after stimulation with a wide variety of noxious physical and chemical stimuli via activation of the transient receptor potential vanilloid 1 (TRPV1) expressed on them (Okajima and Harada, 2006), thereby exerting sensory-efferent functions. CGRP, a 37-amino acid neuropeptide, is produced by alternative splicing of the calcitonin (CT) gene (Okajima and Harada, 2006). It is widely distributed in the central and peripheral nervous systems and has been considered to possess diverse functions (Okajima and Harada, 2006). We reported that CGRP rapidly increases IGF-I production via increasing its transcription in various tissues including the brain of mice administered capsaicin (Harada et al., 2007).

The hippocampus has been shown to receive sensory input from the parabrachial nuclei, the site of termination of the spinothalamic tracts (Suzuki et al., 2002). Nonprincipal neurons in the mouse hippocampus have been shown to be immunoreactive for CGRP (Sakurai and Kosaka, 2007). CGRP receptors are expressed in astrocytes, and CGRP increases intracellular cAMP levels in these cells (Moreno et al., 2002). Because IGF-I is synthesized in astrocytes in the hippocampus (Ye et al., 2004) and cAMP has an important role in the CGRP-induced increase in IGF-I production (Vignery and McCarthy, 1996), stimulation of sensory neurons may increase IGF-I production in astrocytes via increasing CGRP levels in the mouse hippocampus.

Serum levels of IGF-I have been shown to be reduced in patients with Alzheimer's disease, and a significant positive correlation has been shown to exist between serum IGF-I levels and Mini Mental State Examination scores in all of the subjects (Tei et al., 2008). Down-regulation of the IGF-I axis in elderly males is significantly reversed by donepezil (Obermayr et al., 2005).

Based on these observations, donepezil may have a stimulatory effect on sensory neurons in addition to an inhibitory effect on acetylcholinesterase, thereby promoting angiogenesis and neurogenesis through an increase in IGF-I production in the hippocampus. To examine this possibility, we analyzed the effects of two selective acetylcholinesterase inhibitors (donepezil and tacrine) on hippocampal IGF-I production and cognitive function in wild-type (WT) mice, CGRP-knockout \( \text{CGRP}(-/-) \) mice, and WT mice with functional sensory denervation caused by neonatal administration of capsaicin.

## Materials and Methods

**Reagents.** Donepezil hydrochloride \((\pm)-2-[(1\text{-benzylpiperidin-4-yl})\text{methyl}]\text{-5,6-dimethoxy-indan-1-one monohydrochloride} \) was kindly supplied by Eisai Co. Ltd. (Tokyo, Japan) (Fig. 1) (Sugimoto et al., 2002). Tacrine \((9\text{-amino-1,2,3,4-tetrahydroacridine hydrochloride}) \) (Fig. 1) (Sugimoto et al., 2002), capsazepine (CPZ), an inhibitor of TRPV1 activation (Okajima and Harada, 2006), capsaicin, and 5-bromo-2'-deoxyuridine (BrdU) were purchased from Sigma-Aldrich (St. Louis, MO). KT5720, an inhibitor of protein kinase A (PKA), was purchased from Alexis Corporation (Basel, Switzerland). Rat CGRP was purchased from Peptide Institute Inc. (Osaka, Japan). Human recombinant IGF-I was kindly supplied by Astellas Pharma Inc. (Tokyo, Japan). All other reagents were of analytical grade.

**Generation of CGRP-Deficient Mice.** The generation of CGRP-deficient \( \text{CGRP}(-/-) \) mice was described previously (Oh-hashi et al., 2001). The mouse CT/\text{CGRP} genomic DNA was cloned from a BALB/c mouse genomic library in EMBL3 using synthetic oligonucleotide probes derived from the mouse CT/\text{CGRP} cDNA sequence. A 7.0-kilobase fragment containing exons 3 to 5 of the mouse CT/\text{CGRP} gene was subcloned into pBluescript phagemid (Stratagen, La Jolla, CA). A targeting vector was constructed by replacing the 1.6-kilobase XbaI-XbaI fragment encompassing exons 5, which is specific for CGRP, with the neomycin resistance gene and flanking the thymidine kinase gene. This plasmid was linearized with NotI and introduced into 129/Sv-derived SM-1 embryonic stem cells by electroporation, after which the cells were selected in medium containing 418 (300 µg/ml) and ganciclovir (2 µM). Homologous recombinants were identified by PCR and Southern blot analysis. Targeted embryonic stem cell clones were injected into C57BL/6 mouse blastocysts to generate chimeric mice. Male chimeras were genotyped by Southern analysis with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. F1-derived mice were then mated to C57BL/6 females, and germline transmission was achieved. Littermates obtained by breeding heterozygotes with the genetic background of the 129/Sv background were used for phenotypic analysis. Only males were used in this study.

**Genotype Determination of CGRP\((-/-)\) Pups.** Genomic DNA was extracted from tails of mice as described previously (Oh-hashi et al., 2001) and was used for PCR analysis. PCR was performed using the external primers of the replaced gene fragment. The wild-type allele and the mutant allele gave different band sizes. Primer sequences and PCR conditions have been described previously (Oh-hashi et al., 2001).

**Isolation and Culture of DRG Neurons.** DRG neurons from the lumbar, cervical, and thoracic region were dissected from wild-type mice as described previously (Harada et al., 2006). In brief, DRG was placed in ice-cold sterile calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA). Ganglia were chopped and incubated at 37°C for 15 minutes in Dulbecco's PBS containing 20 U/ml papain (Worthington Biochemical Corporation, Lakewood, NJ). The tissue was then incubated at 37°C for 15 min in Dulbecco's PBS containing 4 mg/ml collagenase type II (Worthington Biochemical Corporation). The tissue was incubated for an additional 30 min in Dulbecco's PBS containing 2000 U/ml dispase I.
CGRP (Godo Shusei, Tokyo, Japan) at 37°C. Individual cells were then dissociated by trituration through a fire-polished Pasteur pipette. After centrifugation at 250 g for 5 min, the resultant pellet was washed twice in serum-free Ham’s F-12 medium (HyClone Laboratories, Logan, UT). Cells were plated on 60-mm polystyrene dish precoated with Vitrogen collagen (Cohesion Technologies, Inc., Palo Alto, CA) in Ham’s F-12 medium containing 10% supplemented calf serum, 2 mM glutamine, and 50 ng/ml mouse 2.5S nerve growth factor (Millipore, Billerica, MA). After 24 h, the culture medium was removed and replaced every 2 days.

**Resting DRG Neurons in Culture and cAMP Measurement.** After 5 days in culture, the medium was aspirated gently and washed with serum free Ham’s F-12 medium. Cells were incubated with donepezil (1–100 μM), tacrine (1–100 μM), or vehicle for 30 min in Ham’s F-12 medium containing 1% supplemented calf serum without nerve growth factor. After incubation, supernatants were sampled and stored at −20°C for CGRP measurement. To determine whether donepezil increased CGRP release from DRG neurons via TRPV1 activation, we examined the effect of CPZ, an inhibitor of TRPV1 activation (Okajima and Harada, 2006), on donepezil-induced CGRP release from DRG neurons. CGRP levels were determined using a specific enzyme immunoassay kit (SPI-BIO, Massy Cedex, France). Recent studies demonstrated that cAMP plays a critical role in CGRP release from sensory neurons by phosphorylating TRPV1 through activation of PKA (Okajima and Harada, 2006), and cAMP-dependent PKA activation is critically involved in CGRP production in DRG neurons (Okajima and Harada, 2006). Therefore, we measured the intracellular cAMP levels in DRG neurons. We examined the effect of KT5720 on CGRP release from DRG neurons at a concentration of 10 μM, as described previously (Harada et al., 2006). After collection of supernatants, plates were placed on ice, media were removed, and cells were washed by ice-cold PBS. Thereafter, ice-cold 65% ethanol was added to each well and placed on ice. Ethanol was collected and dried under nitrogen gas.

Intracellular levels of cAMP were determined using a specific enzyme immunoassay kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer’s instructions.

**Animal Model.** Age-matched (10–12 weeks old, 21–24 g) male C57BL/6 wild-type (Nihon SLC, Hamamatsu, Japan) and CGRP−/− mice were used in each experiment. They were maintained under standard conditions of temperature (23–25°C) and on a 12-h light/dark cycle. Food and water were provided ad libitum. The care and handling of the animals were in accordance with the National Institute of Health guidelines. All of the experimental procedures described below were approved by the Nagoya City University Animal Care and Use Committee. The animals were sacrificed at the end of each treatment period. Mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Separate sets of animals were used for each measurement in the present study.

**Administration and Preparation of Various Agents.** Capsaicin was dissolved in 10% Tween 80/10% ethanol (10%) and diluted with normal saline. KT5720 and CPZ were dissolved in 10% Tween 20/10% methanol (10%) with normal saline. Donepezil or tacrine was added to the food powder up to final dosage of 1.5 mg/kg per day. Mice were allowed free access to normal food or drug-added food for 4 weeks. CGRP was dissolved in sterile distilled water and injected intraperitoneally 30 min before the behavioral test at a dosage of 10 μg/kg. IGFI-1 and β-actin cDNA generated by PCR were used as standards for quantitation of sample cDNA. Copy numbers of cDNA for IGFI-1 were standardized by those for β-actin from same sample.

**Sensory Denervation by Neonatal Capsaicin Treatment in the WT Mice.** Sensory denervation by neonatal capsaicin administration was performed according to a previously described method (Buck and Burks, 1986). Neonatal male WT mouse pups (C57BL/6; Nihon SLC) were weighted and injected subcutaneously into the back with 50 mg/kg capsaicin on postnatal day 2. When these mice reached 10 to 12 weeks of age, they were tested for sensory denervation by applying 0.1 mg/ml capsaicin in saline to the eyes; absence of blinking or scratching confirmed sensory denervation. Capsaicin-treated mice that showed any blinking or scratching were excluded from the study.

**Determination of Hippocampus Tissue Levels of CGRP.** Tissue levels of CGRP were determined in animals by modification of the methods described previously (Harada et al., 2006). The tissues were weighed and then homogenized in a Polytron-type homogenizer (2 times of 15 s) using 1 ml of 2 N acetic acid. The homogenates were bathed in 90°C water for 20 min and then centrifuged at 4500g for 10 min. CGRP was extracted from the supernatant by using reversed-phase C18 columns (GE Healthcare). Columns were prepared by washing with 5 ml of methanol, followed by 10 ml of water before use. The samples were applied onto the column, followed by washing with 20 ml of 0.1% trifluoroacetic acid. CGRP was eluted with 3 ml of 60% acetonitrile in 0.1% trifluoroacetic acid, and the solvent was evaporated under a stream of nitrogen gas. The concentration of CGRP was assayed by using a specific enzyme immunoassay kit (SPI-BIO). The antisera cross-reacts 100% with rodent α- and β-CGRP according to the manufacturer’s data sheet. Results are expressed as micrograms of CGRP per gram of tissue.

**Determination of Hippocampus IGF-I Level.** Tissue levels of IGF-I were determined in animals by modification of the methods as described previously (Harada et al., 2007). The hippocampus was minced and homogenized in a Polytron-type homogenizer (2 times of 15 s) using 1 ml of 1 N acetic acid according to the manufacturer’s instruction. The homogenate was then centrifuged at 4500g for 10 min. The supernatants were kept in a deep freezer at −80°C. The concentration of IGF-I was assayed by using a specific enzyme immunoassay kit (Diagnostic Systems Laboratories Inc., Webster, TX).

**Quantitative mRNA Analysis.** Quantitative mRNA analysis was performed as described previously (Harada et al., 2007). The tissue was weighed and immersed in liquid nitrogen. Total RNA was extracted from the hippocampus with TRIzol reagent (Invitrogen) according to the manufacturer’s instruction. RNA extracted was used as a template for cDNA reverse transcription. Sample cDNAs were amplified in the model 7700 Sequence detector (Applied Biosystems, Foster City, CA) with primers, dual-labeled fluorogenic probes, and a Taqman PCR Reagent Kit (Applied Biosystems). Thermal cycler conditions were 10 min at 95°C for deactivation preceding 40 cycles for 15 s at 95°C for denaturation and 1 min at 60°C for both annealing and extension. Known concentrations of serially diluted IGF-I and β-actin cDNA generated by PCR were used as standards for quantitation of sample cDNA. Copy numbers of cDNA for IGF-I were standardized by those for β-actin from same sample.

**Immunohistochemical Staining of IGF-I and Glial Fibrillar Acidic Protein in Hippocampi.** The double labeling of immunofluorescent technique was used for immunohistochemical staining of various tissues with anti-IGF-1 antibody (Harada et al., 2007). Mice were perfused with 4% paraformaldehyde in phosphate buffer. Tissue blocks were immersed in the same perfusate at 4°C overnight and stored thereafter in a 20% sucrose solution. The tissue blocks of mouse brain were frozen in dry ice-cooled optimal cutting temperature compound (Tissue Tec; Bayer Corp., Emeryville, CA). Frozen coronal sections (18 μm thick) obtained by use of the freezing microtome were stored at −80°C before immunofluorescence. Sections were rinsed in PBS and then incubated for 1 h with PBS, 0.2% Triton X-100, and 0.5% blocking reagent (Roche Diagnostics, Basel, Switzerland) at room temperature. They were incubated overnight at 4°C with mouse anti-IGF-I monoclonal antibody (1:200; Millipore) and rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:1000; Dako Denmark A/S, Glostrup, Denmark). The sections were treated with secondary antibodies Alexa Fluor 568 anti-rabbit IgG for GFAP and Alexa Fluor 488 anti-mouse IgG for IGF-I (1:500; Invitrogen) for 1 h at room temperature. The number of both IGF-I- and GFAP-positive cells was counted in the granule cell layer and in the hilus. The number of cells was counted using one 18 μm-thick section per animals.
c-fos Immunohistochemistry. Immunohistochemical staining of c-fos in mice brain was performed according to the method as described previously (Linden et al., 2004). Mice were anesthetized (5% isoflurane) and decapitated. Brains were rapidly removed and frozen in dry ice-cooled optimal cutting temperature compound (Tissue Tec; Bayer Corp.). Frozen coronal sections (18 μm thick) obtained by use of the freezing microtome were stored at −80°C before immunofluorescence. Slides were removed from the freezer, allowed to air-dry for 15 min, and fixed for 10 min in ice-cold 4% paraformaldehyde in PBS (pH 7.4). Sections were rinsed in PBS and then incubated for 1 h with PBS, 0.2% Triton X-100, and 0.5% blocking reagent (Roche Diagnostics) at room temperature. They were incubated overnight at 4°C with rabbit anti-c-fos polyclonal antibody (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The sections were treated with secondary antibody Alexa Fluor 568 anti-rabbit IgG (1:500; Invitrogen) for 1 h at room temperature. After staining, samples were examined under a fluorescence microscope (Axio Imager A1; Carl Zeiss GmbH, Jena, Germany).

Double Stain Immunohistochemistry for CD31, GFAP, or Calbindin-D28k with BrdU. We used BrdU labeling to monitor proliferation and double immunofluorescent labeling for BrdU and cell-specific markers to determine the phenotype of the progenitor progeny. During the last 5 days of each treatment period, the animals for determination of BrdU-labeled nuclei received a daily intraperitoneal injection of BrdU at a dosage of 50 mg/kg. Angiogenesis and neurogenesis in the mouse brains was monitored by BrdU incorporation into the nuclei of dividing cells as described previously (Aberg et al., 2000). Samples were treated for DNA denaturation in the following manner: tissues were incubated in 50% formamide in 2× SSC buffer (1× SSC, 0.3 M NaCl, and 0.03 M sodium citrate) at 65°C for 2 h, rinsed in PBS, and then incubated for 30 min with 2 N HCl at 37°C. They were rinsed for 10 min at room temperature in 0.1 M boric acid (pH 8.4). The tissue was rinsed with PBS three times followed by incubation in PBS containing 0.2% Triton X-100, and 0.5% blocking reagent (Roche Diagnostics) for 1 h and then with primary rat anti-CD31 monoclonal antibody (1:200; BD Biosciences Pharmingen, San Diego, CA), with mouse anti-BrdU monoclonal antibody (1:100; Invitrogen) or rabbit anti-GFAP polyclonal antibody (1:1000; Dako Denmark A/S) or mouse anti-calbindin-D28k monoclonal antibody (1:200; Abcam, Inc., Cambridge, UK), or with rat anti-BrdU monoclonal antibody (1:400; Abcam, Inc.) overnight at 4°C. The sections were treated with secondary antibodies Alexa Fluor 594 anti-rat IgG for CD31, Alexa Fluor 488 anti-mouse IgG for BrdU (anti-mouse), Alexa Fluor 568 anti-rabbit IgG for GFAP, Alexa Fluor 488 anti-mouse IgG for calbindin-D28k, and Alexa Fluor 594 anti-rat IgG for BrdU (anti-rat) (1:500; Invitrogen) for 1 h at room temperature. After staining samples were examined under a fluorescence microscope (Axio Imager A1; Carl Zeiss GmbH), BrdU-positive cells were counted in the granule cell layer and in the hilus. The number of cells was counted using one 18 μm-thick section per animal.

Morris Water Maze Task. Behavioral testing was conducted as described previously (Ryan et al., 2008). We used a circular pool (150 cm diameter). The pool was filled with water at 30°C and contained a round-shaped transparent acrylic platform (10 cm diameter). In the task, the platform was submerged 1 cm below the surface of the water and located in the southeast quadrant of the pool throughout the trials. After a mouse was put into the pool, each had a maximum of 90 s to locate and climb onto the platform (one trial). When a mouse located the platform, it was allowed to stay on it for 20 s. The mouse that did not find the platform in the allowed time was placed on it by the experimenter and left there for 20 s. Latency to reach the platform was monitored. Each mouse was subjected to one trial per day. The task consisted of 5 days of trials. Two hours after the last trial, the probe test was carried out. For this test, the platform was removed from the pool, and the trial was performed with the cutoff time of 90 s. The time spent in the target area (zone radius: 30 cm, three times the target diameter) was recorded as a percentage of the trial time in the pool.

Statistical Analysis. Data are expressed as the mean ± S.D. The results were compared using an analysis of variance followed by Scheffé’s post hoc test. A level of p < 0.05 was considered statistically significant.

Results

Effects of Donepezil and Tacrine on CGRP Release and Cellular cAMP Levels in DRG Neurons Isolated from WT Mice. Donepezil at concentrations of 1, 10, and 100 μM increased the release of CGRP from DRG neurons isolated from WT mice (Fig. 2A). Tacrine did not increase the CGRP release from DRG neurons (Fig. 2A). Cyclic AMP has been shown to play an important role in the release of CGRP from sensory neurons upon activation (Okajima and Harada, 2006). Donepezil may increase CGRP release from DRG neurons by activating PKA because donepezil was found to increase cellular cAMP levels in DRG neurons. To examine this possibility, we analyzed the effect of pretreatment with the PKA inhibitor KT5720 on donepezil-induced CGRP release from DRG neurons isolated from WT mice. Donepezil-induced increase in CGRP release...
from DRG neurons was completely reversed by pretreatment with KT5720 (Fig. 3).

Effects of Donepezil and Tacrine on Tissue Levels of CGRP, IGF-I, and IGF-I mRNA in the Hippocampus of WT Mice, CGRP(+/−) Mice, and WT Mice Subjected to Functional Denervation. To examine whether donepezil increases tissue levels of CGRP, IGF-I, and IGF-I mRNA in the hippocampus through sensory neuron stimulation in mice, we determined tissue levels of these substances in the hippocampus after oral administration of donepezil for 4 weeks in WT mice, CGRP(+/−) mice, and WT mice subjected to functional denervation. At baseline, tissue levels of CGRP, IGF-I, and IGF-I mRNA in the hippocampus of WT mice were significantly higher than those in the hippocampus of CGRP(+/−) mice and WT mice subjected to functional denervation (p < 0.01) (Fig. 4). Administration of donepezil significantly increased hippocampal tissue levels of CGRP, IGF-I, and IGF-I mRNA in WT mice (p < 0.01), whereas such increases were not observed in the hippocampus of CGRP(+/−) mice and WT mice subjected to functional denervation (Fig. 4). Increase in the levels of CGRP, IGF-I, and IGF-I mRNA was not observed in WT mice after tacrine administration for 4 weeks (Fig. 4).

Effects of Donepezil and Tacrine on Immunohistochemical Expression of IGF-I in the Hippocampus of WT Mice, CGRP(+/−) Mice, and WT Mice Subjected to Functional Denervation. Increase in the immunohistochemical expression of IGF-I in the dentate gyrus (DG) was observed after 4 weeks of donepezil administration in WT mice. IGF-I immunoreactivity was colocalized with immunoreactivity for the astrocyte marker GFAP in WT mice (Figs. 5, A—C, and 6), and increase in the IGF-I immunoreactivity colocalized with GFAP immunoreactivity was observed after donepezil administration in WT mice (Figs. 5, D—F, and 6). Such an increase in IGF-I expression was not observed in WT mice after tacrine administration for 4 weeks (Figs. 5, G—I, and 6). An increase of IGF-I expression was not observed in DG after donepezil administration in CGRP(+/−) mice and the WT mice subjected to functional denervation (data not shown).

Effect of Donepezil on c-fos Expression in the Spinal and Supraspinal Nervous Tissues of WT Mice, CGRP(+/−) Mice, and WT Mice Subjected to Functional Denervation. To analyze the mechanism and pathway of the relay system that leads to the increase in hippocampal IGF-I production in WT mice administered donepezil, we determined c-fos expression in the spinal and supraspinal nervous tissues of WT mice, CGRP(+/−) mice, and WT mice subjected to functional denervation after 4 weeks of oral administration of donepezil. In WT mice, increase in c-fos expression was observed in the dorsal horns (laminae I–II) of the spinal cord and, supraspinally, in the solitary tract nucleus (NTS), parabrachial nuclei (PBN), and the hippocampus (Figs. 7 and 8); no such increase in c-fos expression was noted in WT mice after administration of tacrine for 4 weeks (Fig. 8). An increase in c-fos expression was not observed in the same tissues after donepezil admin-
administration in CGRP(−/−) mice and WT mice subjected to functional denervation (data not shown).

Effects of Donepezil and Tacrine on Hippocampal Angiogenesis and Neurogenesis in WT Mice, CGRP(−/−) Mice, and WT Mice Subjected to Functional Denervation. The number of BrdU-immunoreactive cells in the DG was significantly higher in WT mice than in CGRP(−/−) mice and WT mice subjected to functional denervation (p < 0.01) (Fig. 9A). Further significant increase in the number of BrdU-immunoreactive cells was observed in the DG of WT mice after 4 weeks of administration of donepezil (p < 0.01), whereas no such increase was observed in CGRP(−/−) mice and WT mice subjected to functional denervation (Fig. 9A).

Colocalization of BrdU immunoreactivity with immuno-reactivity for the vascular endothelial cell marker CD31, the granule cell marker calbindin-D28k, and the astrocyte marker GFAP was examined to determine the phenotype of progenitor cell progeny in the DG after donepezil administration in WT mice, CGRP(−/−) mice, and WT mice subjected to functional denervation (Fig. 9, B–D). Significantly higher numbers of BrdU+/CD31+, BrdU+/calbindin-D28k+, and BrdU+/GFAP+ cells were observed in the DG of WT mice than in those of CGRP(−/−) mice and WT mice subjected to functional denervation (p < 0.01) (Fig. 9, B–D). A significant increase in the number of BrdU+/CD31+ and BrdU+/calbindin-D28k+ cells, but not BrdU+/GFAP+ cells, in the DG was observed after 4 weeks of administration of donepezil in WT mice, whereas no such increase in the number of BrdU+/CD31+ and BrdU+/calbindin-D28k+ cells was noted in WT mice administered tacrine for 4 weeks (Fig. 9, B–D). No increase in the number of any of these cells in the DG was observed after donepezil administration in CGRP(−/−) mice and WT mice subjected to functional denervation (Fig. 9, B–D).

Effects of Donepezil and Tacrine on the Spatial Learning Function in WT Mice, CGRP(−/−) Mice, and WT Mice Subjected to Functional Denervation. To determine whether donepezil improves the cognitive function in mice by stimulating sensory neurons, we examined the effect of donepezil administration on spatial learning in WT mice, CGRP(−/−) mice, and WT mice subjected to functional denervation using the Morris water maze test for 5 consecutive days. In animals not administered donepezil, a significant improvement in spatial learning on days 3, 4, and 5 compared with that on day 1 was observed in WT mice (p < 0.01) (Fig. 10A); no such improvement was noted through the 5 days in CGRP(−/−) mice and WT mice subjected to functional denervation (Fig. 10, B and C). In WT mice, the improvement in spatial learning on days 2, 3, 4, and 5 was...
In the present study, donepezil increased CGRP release from DRG neurons isolated from WT mice in vitro. Pretreatment with CPZ, an inhibitor of TRPV1 activation, reversed the donepezil-induced increase in the CGRP release from DRG neurons, suggesting that TRPV1 activation may be critically involved in the donepezil-induced increase of CGRP release from sensory neurons. The mechanism(s) by which donepezil activates TRPV1 is still not fully understood. PKA activation has been shown to induce phosphorylation of TRPV1, thereby sensitizing the sensory neurons to activation by endogenous agonists (Okajima and Harada, 2006). Consistent with this hypothesis are observations in the present study demonstrating that donepezil increased cellular cAMP levels in DRG neurons and that the donepezil-induced increase of CGRP release from DRG neurons was completely inhibited by the PKA inhibitor KT5720. These observations suggest that donepezil may increase the release of CGRP from sensory neurons by activating PKA through an increase in cellular cAMP levels in sensory neurons.

The hippocampal tissue levels of IGF-I and IGF-I mRNA were significantly lower in CGRP(−/−) mice and WT mice subjected to functional denervation than in WT mice. Oral administration of donepezil increased the hippocampal tissue levels of CGRP, IGF-I, and IGF-I mRNA and the immunohistochemical expression of IGF-I in WT mice, but not in the CGRP(−/−) mice and WT mice subjected to functional denervation. These observations strongly suggest that donepezil administration may induce the transcription and production of IGF-I in the hippocampus by increasing hippocampal CGRP levels. Consistent with this notion is our previous report demonstrating that stimulation of sensory neurons by capsaicin administration induced transcription and production of IGF-I in various tissues by increasing CGRP levels (Harada et al., 2007).

IGF-I immunoreactivity was colocalized with immunoreactivity for the astrocyte marker GFAP in the DG of WT mice administered donepezil. This observation is consistent with reports showing that astrocytes can produce IGF-I in the hippocampus (Ye et al., 2004). CGRP has been shown to increase cAMP levels via CGRP receptor activation in astrocytes (Lazar et al., 1991). Because cAMP plays an important role in IGF-I production (Vignery and McCarthy, 1996), stimulation of sensory neurons by donepezil may increase CGRP levels in the hippocampus, thereby increasing IGF-I production via increasing cAMP levels in astrocytes. Precisely which cells produce CGRP in the hippocampus of WT mice administered donepezil is presently unknown.

To examine the mechanism(s) by which afferent sensory information arising from stimulation by donepezil is transmitted to the hippocampus, we analyzed c-fos expression in spinal and supraspinal nervous tissues in WT mice, CGRP(−/−) mice, and WT mice subjected to functional denervation after 4 weeks of administration of donepezil. In WT mice, increase in c-fos expression was observed in the dorsal horns (laminae I–II) of the spinal cord and, supraspinally, in the NTS, PBN, and hippocampus after donepezil administration. These observations strongly suggest that nociceptive information arising from stimulation with donepezil in the gastrointestinal tract may be transmitted via the spinopara-
brachial circuits, including NTS as a relay point (Castle et al., 2005).

Nonprincipal neurons have been shown to be immunoreactive for CGRP in the mouse hippocampus (Sakurai and Kosaka, 2007). Hippocampal nonprincipal neurons are innervated by GABAergic neurons projecting from the medial septum (Gulyas et al., 1991). Because the medial septum receives sensory input from PBN where the spinothalamic tracts terminate (Castle et al., 2005), the increase in hippocampal tissue CGRP levels in WT mice administered donepezil may be a consequence of activation of the hippocampal nonprincipal neurons by GABAergic neurons projecting into them from the medial septum. These observations suggest that CGRP released from the hippocampal nonprincipal neurons acts on astrocytes via CGRP receptors, thereby increasing IGF-I production in the hippocampus of WT mice administered donepezil.

In contrast with observations in WT mice administered donepezil, induction of c-fos expression was not observed in the spinal or supraspinal nervous tissues of CGRP(-/-) mice and WT mice subjected to functional denervation after donepezil administration. These observations suggest that donepezil may stimulate sensory neurons in the gastrointestinal tract, thereby increasing hippocampal IGF-I production, and that CGRP may function as a transmitter in the pathway involved in this sensory nervous relay system. Consistent with this hypothesis are reports demonstrating CGRP expression at synaptic contacts between the primary afferent sensory neurons and spinothalamic tract neurons in the dorsal horn of the spinal cord (Carlton et al., 1990), in spinothalamic tract cells, and in nerve fibers originating from the PBN (Tie-Jun et al., 2001).

The number of CD31+ cells, calbindin-D28k+, and GFAP+ cells in the BrdU-immunoreactive cells of the DG in CGRP(-/-) mice and WT mice subjected to functional denervation was significantly lower than the corresponding number in WT mice, suggesting that CGRP and/or IGF-I may be deeply related to neural stem cell proliferation in the mouse hippocampus. Consistent with this hypothesis is a report demonstrating that IGF-I is necessary for angiogenesis in the adult mouse brain (Lopez-Lopez et al., 2004) and for neural stem cell proliferation acting via the mediation of epidermal growth factor and fibroblast growth factor-2 (Arsenijevic et al., 2001).

Donepezil increased the number of BrdU+ cells as well as BrdU+ and calbindin-D28k+ double-positive cells, but not that of BrdU+ and GFAP+ cells, in the DG of WT mice. Tacrine had no such effect on the number of either BrdU+ cells or BrdU+ and calbindin-D28k+ double-positive cells. Donepezil had no effect on the number of these cells in the DG of CGRP(-/-) mice and WT mice subjected to functional denervation. Peripheral infusion of IGF-I was shown to selectively induce angiogenesis via a VEGF-dependent mechanism in the adult mouse brain (Lopez-Lopez et al., 2004) and neurogenesis in the adult rat hippocampus (Aberg et al., 2000). These observations strongly suggest that stimulation
of sensory neurons by donepezil may induce angiogenesis and neurogenesis by inducing IGF-I production via an increase in CGRP levels in the mouse hippocampus. Because angiogenesis has been shown to provide a favorable environment for neuronal stem cell proliferation via activation of a VEGF-dependent mechanism (Palmer et al., 2000), the hippocampal neurogenesis induced by donepezil administration in WT mice may be mediated by angiogenesis.

IGF-I exerts beneficial effects against the decline of cognitive function by inducing neurogenesis in the hippocampus (Aberg et al., 2000), suggesting that donepezil may improve the cognitive function by inducing IGF-I production through promoting CGRP release in the mouse hippocampus. Consistent with this hypothesis are observations in the present study demonstrating that donepezil significantly improved spatial learning function in WT mice but not in CGRP(−/−) mice.
mice and WT mice subjected to functional denervation. These observations suggest that stimulation of sensory neurons by donepezil may increase the release of CGRP, inducing IGF-I production in the mouse hippocampus, thereby improving cognitive function.

Acetylcholine is one of the most important neurotransmitters involved in learning and memory (Kotani et al., 2006). Activation of the central cholinergic function has been considered to enhance hippocampal neurogenesis, thereby contributing to the improvement in cognitive function. In this context, donepezil, a selective acetylcholinesterase inhibitor, ameliorates cognitive impairment in Alzheimer’s disease (Winblad et al., 2006). Tacrine, another selective acetylcholinesterase inhibitor, did not stimulate sensory neurons as shown in the present study. Tacrine also was shown to have no effect on hippocampal tissue levels of CGRP, IGF-I, and IGF-I mRNA and spatial learning in WT mice. These observations strongly suggest that stimulation of sensory neurons by donepezil may not be dependent on its acetylcholinesterase-inhibiting activity, but rather on some other unknown pharmacological actions.

Taken together, the observations of the present study strongly suggest that peripheral sensory nerve stimulation with donepezil may increase tissue levels of CGRP in the hippocampus, thereby inducing IGF-I production as well as promoting angiogenesis and neurogenesis to produce improvement in cognitive function in mice.

Of the various regulators of IGF-I production, GH is probably the most important, capable of regulating it via endocrine and paracrine mechanisms (Okajima and Harada, 2008). IGF-I production in the brain is not reduced in GH receptor-knockout mice (Lupu et al., 2001). These observations suggest that hippocampal IGF-I production may be GH-independent and that sensory neurons may be involved at least in part in the GH-independent IGF-I production mechanism(s) in the hippocampus.

Because the serum levels of IGF-I in patients with Alzheimer’s disease have been shown to be significantly lower than those in controls without dementia (Watanabe et al., 2005), the observations of the present study indicate that pharmacological stimulation of sensory neurons may be useful for improving cognitive function via promotion of angiogenesis and neurogenesis in the hippocampus through induction of IGF-I production in patients with Alzheimer’s disease.

References


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