

Nasal Colivelin Treatment Ameliorates Memory Impairment Related to Alzheimer's Disease

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Humanin (HN) and its derivatives, such as Colivelin (CLN), suppress neuronal death induced by insults related to Alzheimer's disease (AD) by activating STAT3 *in vitro*. They also ameliorate functional memory impairment of mice induced by anticholinergic drugs or soluble toxic amyloid- β (A β) *in vivo* when either is directly administered into the cerebral ventricle or intraperitoneally injected. However, the mechanism underlying the *in vivo* effect remains uncharacterized. In addition, from the standpoint of clinical application, drug delivery methods that are less invasive and specific to the central nervous system (CNS) should be developed. In this study, we show that intranasally (i.n.) administered CLN can be successfully transferred to CNS via the olfactory bulb. Using several behavioral tests, we have demonstrated that i.n. administered CLN ameliorates memory impairment of AD models in a dose-responsive manner. Attenuation of AD-related memory impairment by HN derivatives such as CLN appears to be correlated with an increase in STAT3 phosphorylation levels in the septohippocampal region, suggesting that anti-AD activities of HN derivatives may be mediated by activation of STAT3 *in vivo* as they are *in vitro*. We further demonstrate that CLN treatment inhibits an A β induced decrease in the number of choline acetyltransferase (ChAT)-positive neurons in the medial septum. Combined with the finding that HN derivatives upregulate mRNA expression of neuronal ChAT and vesicular acetylcholine transporter (VACHT) *in vitro*, it is assumed that CLN may ameliorate memory impairment of AD models by supporting cholinergic neurotransmission, which is at least partly mediated by STAT3-mediated transcriptional upregulation of ChAT and VACHT.

Neuropsychopharmacology (2008) 33, 2020–2032; doi:10.1038/sj.npp.1301591; published online 10 October 2007

Keywords: Alzheimer's disease; humanin; activity-dependent neurotrophic factor; Colivelin; memory impairment

INTRODUCTION

Development of effective Alzheimer's disease (AD) therapy is urgently required because AD currently affects nearly 2% of the population in industrialized countries (Mattson, 2004). AD is characterized clinically by progressive memory impairment and pathologically by senile plaques, neurofibrillary tangles, and neuronal death. Several lines of evidence have also revealed that dysfunction in the cholinergic system of the basal forebrain is involved in the pathogenesis of AD (Bartus *et al*, 1982; Coyle *et al*, 1983). Progressive cholinergic denervation in the cerebral cortex as well as decreased levels of acetylcholine (ACh) and choline acetyltransferase (ChAT) have been reported to be associated with AD-relevant cognitive impairment (Bartus

et al, 1982; Coyle *et al*, 1983). A mutually aggravating relationship between cholinergic dysfunction and pathological changes in AD has also been reported. Dysfunction of cholinergic neurotransmission may contribute to the development of amyloid plaque pathology (Caccamo *et al*, 2006) and toxic soluble amyloid- β (A β) disrupts the cholinergic neurotransmission (Bales *et al*, 2006). Moreover, acetylcholinesterase (AChE) inhibitors, such as donepezil, improve cognitive function in AD patients to some extent (Lane *et al*, 2004; Petersen *et al*, 2005; Winblad *et al*, 2006). Taken altogether, supporting cholinergic neurotransmission is deemed to be a promising strategy for AD therapy, especially for patients in the early stage of the disease, when massive and irreversible brain atrophy has not yet taken place.

Humanin (HN), originally identified from an occipital lobe of an AD patient, abolishes neuronal cell death induced by various familial AD (FAD) causative genes and A β (Hashimoto *et al*, 2001a, b; Nishimoto *et al*, 2004). Colivelin (CLN), the strongest HN derivative so far developed, is a fusion peptide composed of a potent HN derivative named AGA-(C8R)HNG17 attached to the C terminus of activity-dependent neurotrophic factor (ADNF) (Brenneman and

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Received 14 February 2007; revised 21 August 2007; accepted 26 August 2007

Gozes, 1996; Brenneman *et al*, 1998; Chiba *et al*, 2004, 2005, 2006; Matsuoka *et al*, 2006). Our earlier studies indicated that HN and CLN suppressed AD-relevant neuronal death by activating STAT3 *in vitro* (Chiba *et al*, 2005; Hashimoto *et al*, 2005; Matsuoka *et al*, 2006). It has been also demonstrated *in vivo* that HN derivatives, including CLN, suppress spatial working memory impairment induced by blockers to the cholinergic system or intracerebroventricular (i.c.v.) injection of toxic A β peptides (Mamiya and Ukai, 2001; Krejcová *et al*, 2004; Chiba *et al*, 2005). Considering that both insults cause memory impairment *in vivo* by inducing the dysfunction of the cholinergic system without significant neuronal death, HN derivatives are likely to support cognitive function by upregulating the cholinergic neurotransmission *in vivo*. However, the molecular mechanism underlying this activity remains unknown.

From the standpoint of clinical applications for HN derivatives, it is also important to establish drug delivery methods that are less invasive and central nervous system (CNS) specific to administer CLN. Drugs for AD must be safely and continuously used over a long period, and i.c.v. or i.p. injection is not appropriate. Intranasal (i.n.) administration is one of the less invasive delivery methods for peptide drugs (Illum, 2000; Thorne *et al*, 2004). It bypasses the blood–brain barrier (BBB), which otherwise restricts entrance of molecules from the bloodstream to the CNS. A number of neurotrophic peptides—including vasoactive intestinal peptide, ADNF, and insulin-like growth factor-1—have been successfully delivered to the brain or the CSF by i.n. administration (Gozes *et al*, 1996, 2000; Thorne *et al*, 2004). Given that highly lipophilic small peptides can be relatively easily delivered to the CNS by i.n. administration, HN derivatives such as CLN may be efficiently transported to the CNS by this method.

In this study, we demonstrate the therapeutic efficacy of i.n. CLN treatment compared with those of i.n. ADNF or AGA-(C8R)HNG17 using two types of AD models: anticholinergic drugs (cholinotoxin)-induced models (Chiba *et al*, 2005) and a repetitive A β injection model (Yamada *et al*, 2005). We also characterize the molecular mechanism underlying CLN- or AGA-(C8R)HNG17-mediated support of cognitive function in terms of upregulation of the cholinergic neurotransmission, putatively mediated by STAT3.

METHODS

Peptides and Materials

Colivelin (CLN, SALLRSIPAPAGASRLLLLTGEIDL), ADNF (SALLRSIPA), and AGA-(C8R)HNG17 (PAGASRLLLLTGEIDL) were synthesized as described previously (Chiba *et al*, 2005). A rabbit polyclonal anti-HN antibody (PO4) was described previously (Tajima *et al*, 2002). A rabbit polyclonal anti-ADNF antibody was raised against synthetic ADNF. Affinity-purified polyclonal antibody against ChAT was purchased from Chemicon (Temecula, CA). A rabbit polyclonal antibody against phospho-STAT3 (Tyr⁷⁰⁵) and a rabbit monoclonal antibody against total STAT3 (79D7) were from Cell Signaling Technology (Beverly, MA). A β 25–35 was purchased from Peptide Institute (Osaka, Japan).

Scopolamine hydrobromide and 3-quinuclidinyl benzilate (3-QNB) were from Sigma (St Louis, MO). Other reagents used in the study were all commercially available.

Animals and Treatments

This study was conducted in accordance with the Policies on the Use of Animals and Humans in Neuroscience Research, the Society for Neuroscience and Guideline for the Care and Use of Laboratory Animals of KEIO University School of Medicine. All experimental procedures were approved by the Institutional Animal Experiment Committee at KEIO University.

CD-1 (ICR) mice at the age of 7 weeks were purchased from Charles River Japan Inc. (Kanagawa, Japan). Animals were housed in a specific pathogen-free animal facility as described previously (Kawasumi *et al*, 2004; Yamada *et al*, 2005). Cholinotoxin models were described previously (Chiba *et al*, 2005). Briefly, CD-1 mice at the age of 8 weeks were given 10 μ l vehicle (sterile deionized distilled water (ddw) containing 5% sefsol and 20% isopropanol) i.n. (Gozes *et al*, 1996, 2000) with or without indicated amounts of synthetic CLN peptides twice, at 24 h and 30 min before the Y-maze test (YM), as depicted in Figure 2a. Scopolamine (1 mg/kg) dissolved in 0.2 ml sterile saline was subcutaneously (s.c.) injected at 30 min before YM whereas 3-QNB (0.5 mg/kg) dissolved in 0.2 ml sterile ddw containing 20% methanol was i.p. injected at 15 min before YM to induce amnesia.

The AD mouse model by repetitive i.c.v. injection of A β was described previously (Yamada *et al*, 2005). Briefly, CD-1 mice at the age of 8 weeks were stereotaxically implanted with cannulas (C315GS-4; PlasticOne Inc., Roanoke, VA) in the left lateral ventricle (anterior–posterior, +0.3 mm; lateral, 1.0 mm; and horizontal, 3.0 mm from the bregma). Ten days after implantation, animals were randomly assigned to the control group, A β -injected group, or the A β -injected plus CLN-treated group, receiving i.c.v. injection of either 3 μ l sterile ddw or 1 nmol A β 25–35 peptide in 3 μ l ddw, every other day for 3 weeks (10 times) together with i.n. administration of 10 μ l vehicle with or without 1 nmol CLN once a day for 3 weeks, as depicted in Figure 7a.

Behavioral Tests

An open field test (OF) was performed as described previously (Kawasumi *et al*, 2004; Yamada *et al*, 2005) to habituate the mice to following experimental conditions. YM was performed as described previously (Yamada *et al*, 2005). Briefly, the apparatus for YM is made of three gray plastic arms (40 cm long, 12 cm high, 3 cm wide at the bottom, and 10 cm wide at the top). Mice were allowed to explore the maze freely for 8 min. Spontaneous alternation percentage (SA%), an index of spatial working memory, was defined as the ratio of the arm choices that differed from the previous two choices (correct choices) to total choices during the run (total arm entry, 2).

A memory assessment version of the elevated-plus maze (EPM) was performed as described (Itoh *et al*, 1991). The testing apparatus, set at 100 cm above the floor, was made of four arms crossed perpendicularly at the center. Two arms were open (50 \times 10 cm gray plastic floor plates without

walls), while the other two were closed (with 10 cm high gray plastic walls). Mice were individually placed at the end of the open arm and allowed to explore the maze freely for 90 s. The test was performed twice at a 24-h interval without any specific restriction. The first session was performed after scopolamine treatment by the same procedure as that depicted in Figure 2a. Examined parameters were (1) transfer latency, defined as the time elapsed until the first entry to the closed arms; (2) duration of the first stay in a closed arm, defined as the time from first entry to a closed arm to first escape from the arm; and (3) cumulative time spent in open/closed arms.

A passive avoidance (PA) task was performed as described (Jhoo *et al*, 2004). The PA apparatus consisted of two chambers separated with a gray plastic guillotine door; one (11 cm long \times 5 cm wide, with 13 cm walls) was illuminated and the other (25 cm long \times 25 cm wide, with 20 cm walls) was darkened with a constant current shock generator. The test was performed twice at a 24-h interval without any specific restriction. In an acquisition trial (day 1), each mouse with or without s.c. scopolamine administration (0.5 mg/kg) was confined in the illuminated chamber for 15 s and then the door was opened for the mouse to move freely into the dark chamber. Soon after the mice entered the dark chamber, the door was closed and an inescapable electric shock (0.3 mA, 3 s, once) was delivered through the floor grid. Each mouse was again tested in the apparatus (day 2, a retention trial) 24 h later. Entry latency (or step-through latency) defined as elapsed time until the mice entered into the dark chamber (maximum 300 s) was measured as an index of contextual memory.

A radial arm maze (RAM) task was performed as described (Kawasumi *et al*, 2004). Eight arms were connected radially to an octagonal center platform, on which a transparent guillotine door was placed to retain animals on the platform. Each arm was made of a 40 \times 7 cm gray plastic floor without walls. The apparatus was elevated at 100 cm from the floor. Mice were deprived of chow for 12 h before the test; to motivate them, small chow pellets (10 mg) were placed at the end of each arm. Mice were first confined at the central octagon individually for 10 s, and then were allowed to explore the maze freely until they finished choosing all eight arms. The task ended when the tested mouse chose all arms (maximum 300 s). Examined parameters were (1) total time required to finish the task (Total time) and (2) working memory error percentages in the first eight arm entries (Error % in the first eight entries).

Biochemical Measurements of Serum ALT/GPT, AST/GOT, and CRE

To examine the acute side effects of CLN, male CD-1 mice at the age of 8 weeks were singly intravenously (i.v.) administered with either 0.1 ml saline or saline containing 200 nmol CLN. Serum of the treated mice was sampled for biochemical analysis of alanine aminotransferase (ALT, or GPT), aspartate aminotransferase (AST, or GOT), and creatinin (CRE) by Fuji Dri-Chem 3000 (Fuji-Film, Tokyo, Japan, $N=3$), 6 h after the injection. For subacute side effect analysis, male CD-1 mice at the age of 8 weeks were i.n. administered with 10 μ l vehicle or vehicle containing 5 nmol CLN once a day for 1 week.

Histological Analysis

Immunohistochemical (IHC) analysis was performed as described previously (Chiba *et al*, 2005; Yamada *et al*, 2005). Under general anesthesia with diethyl ether, mice were transcardially perfused with phosphate-buffered saline (PBS), and fixed by ethanol containing 5% acetic acid. Paraffin-embedded sections at 10 μ m were prepared and were soaked with the appropriate primary antibodies (anti-ChAT antibody, 1:50 dilution; anti-ADNF antibody, 1:50; PO4, 1:100) and the appropriate second antibody. Immunoreactivity was visualized with the ABC method (for ChAT, Vectastain Elite Kit, Vector, CA, USA) or the FITC-labeled anti-rabbit secondary antibody (for anti-ADNF antibody and PO4). ChAT-immunoreactive neurons in the medial septa of five coronal sections were counted, and the averages of the total numbers of ChAT-positive neurons in three mice per treatment group ($N=3$) were compared (Yamada *et al*, 2005).

Immunoblot Analysis of STAT3 Phosphorylation

After i.n. treatment of several peptides, mice were transcardially perfused with PBS. The olfactory bulbs and hippocampuses of the mice were then sampled and frozen by liquid nitrogen. For immunoblot analysis, brain samples were lysed in a lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, protease inhibitors, 1 mM EDTA, phosphatase inhibitor cocktails 1 and 2 (Sigma)). Samples were then subjected to normal SDS-PAGE and blotted onto polyvinylidene fluoride membranes. The membranes were soaked with anti-phospho-STAT3 antibody (1:1000) and then with horseradish peroxidase-labeled anti-rabbit IgG antibody (1:5000) (Bio-Rad Laboratories, Hercules, CA, USA). Immunoreactive bands were detected with ECL Western Blotting Detection Reagents (Amersham Bioscience, Uppsala, Sweden). Total STAT3 was visualized with the specific antibody (1:3000) on the identical membrane after stripping of anti-p-STAT3 antibody. Densitometric analysis of the results was carried out with NIH Image (ver. 1.62) software.

Assessment of mRNA Levels (RT-PCR)

Total RNA was extracted from PC12 cells (1.2×10^5 cells per well in a six-well plate, cultured in DMEM containing 10% fetal bovine serum) that had been treated with or without 100 pM of CLN, ADNF, or AGA-(C8R)HNG17 by the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First-strand cDNAs were synthesized from 1 μ g total RNA using Omniscript reverse transcriptase according to the manufacturer's protocol (Quiagen, Valencia, CA). PCR amplification with Taq DNA polymerase (Sigma) was performed under the following condition of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s, repeated 25 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 35 cycles for ChAT and acetylcholine transporter (VACHT). The following sequences were used for forward and reverse primer: ChAT, forward 5'-GGGTGATCTGTTCACTCAGTTGAG-3', and reverse 5'-CTCTGTAAGCCTGTAGTAAGCC-3' (669 bp); VACHT, forward

5'-AGCGGGCCTTTCATTGATCG-3', and reverse 5'-GGCGC ACGTCCACCAGGAAGG-3' (814 bp); and GAPDH, forward 5'-GAACATCATCCCTGCATCC-3', and reverse 5'-GCTTCA CCACCTTCTTGATG-3' (179 bp).

AChE Activity Assay (Ellman's Method)

The reaction mixture was prepared as follows: phosphate buffer containing 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, 1 µg/ml, Sigma), 0.05% acetylthiocholine (Sigma), and indicated concentrations of tacrine (Sigma) or CLN (total volume; 100 µl). The mixture was incubated for 15 min at 37°C. AChE (acetylcholinesterase; 1 µl, final; 0.01 U/µl, purified from electric eel, Sigma) was then added to the mixture and further incubated for 15 min. Reaction was stopped by rapid icing. The absorbance at 416 nm of the mixture represented the AChE activity.

Statistics

All values in the figures of the *in vitro* study indicate means ± standard deviation (SD), and the values of the *in vivo* study indicate means ± standard errors of means (SEM). Statistical analysis was performed with one-way analysis of variance (ANOVA), in which α value was set at 0.05, followed by Tukey/Kramer's *post hoc* multiple comparison test unless specifically indicated in the figure legends. Statistical analysis of RAM data was performed with two-way repeated-measures ANOVA followed by Tukey/Kramer's *post hoc* test. All data were analyzed using StatView (ver.5.0.1) software.

RESULTS

Intranasally Administered CLN is Transported to CNS via the Olfactory Bulb

In our earlier study, we reported that i.c.v. injection of CLN almost completely antagonized $\text{A}\beta$ neurotoxicity *in vivo* (Chiba *et al*, 2005). To develop a less invasive drug delivery system for CLN, we investigated whether i.n. administered CLN was transported to the CNS by immunofluorescence (IF)-based visualization of CLN in the olfactory bulb. Coronal sections of paraffin-embedded brains of mice with i.n. administration of vehicle or CLN were soaked with anti-ADNF antibody that recognizes ADNF domain of CLN and then visualized by FITC-labeled secondary antibody. IF intensity was significantly higher in the medial regions of the olfactory bulbs of the CLN-treated mice than those of the vehicle-treated control mice (Figure 1a). We also observed granular increase in IF intensity in the cerebral cortices and an overall increase in IF intensity around the optic chiasm. A similar IF staining pattern was obtained with anti-HN antibody (PO4) (Figure 1b). We confirmed not only that treatment with a secondary antibody alone did not result in similar IF staining but also that the immunoreactivity to anti-ADNF antibody was preabsorbed by synthetic ADNF and that to PO4 was preabsorbed by synthetic HN peptide (Figure 1c). Considering that the two antibodies used here recognize distinct epitopes corresponding to ADNF (N-terminal) and AGA-(C8R)HNG17 (C-terminal) portions of CLN, it is highly likely that i.n.

administered CLN reached the brain regions stained with both antibodies. Although detailed mechanisms of CLN's delivery to the CNS remain to be examined, it is suggested that i.n. administered CLN may be delivered highly specifically to the CNS through some cerebral nerves, including the olfactory and the optic nerve (Illum, 2000; Thorne *et al*, 2004).

Intranasally Administered CLN Ameliorates Scopolamine-Induced Spatial Working Memory Impairment in a Y-Maze Test

Dysfunction of the cholinergic system in the basal forebrain has been implicated in AD-relevant memory impairment (Bartus *et al*, 1982; Coyle *et al*, 1983). In accordance, administration of cholinotoxins such as scopolamine, 3-QNB, and ethylcholine aziridium (AF64A) has been reported to induce memory impairment in both rodents and humans (Fisher *et al*, 1989; Krejcová *et al*, 2004). We examined the effect of i.n. administration of CLN on scopolamine-induced working memory impairment in a YM (Figure 2a and b). As reported in earlier studies (Mamiya and Ukai, 2001; Chiba *et al*, 2005), s.c. administration of scopolamine significantly reduced the SA%, an index of the spatial working memory in YM, suggesting that scopolamine impaired the spatial working memory. Intranasal administration of CLN significantly attenuated scopolamine-induced decrease in SA% in a dose-dependent manner (Figure 2b). Intranasally administered CLN itself did not significantly affect SA% of mice without scopolamine treatment (Figure 2c), supporting the idea that CLN antagonized scopolamine-induced memory impairment.

Comparison of the Pharmacological Effect of CLN via Various Drug Delivery Routes

To assess the advantages of i.n. administration, we next compared the effect of i.n. CLN treatment with those of intraperitoneal (i.p.) or s.c. administration of CLN at the same dose (1 nmol per administration, twice at a 24-h interval) in the scopolamine model (Figure 2d). Scopolamine again significantly reduced SA% in YM. Neither i.p. nor s.c. administration of CLN ameliorated scopolamine-induced decrease in SA%, while i.n. treatment of CLN at the same dose did so in a statistically significant manner (Figure 2d), suggesting that i.n. administration may be a more efficient drug delivery route for CLN to the CNS.

Assessment of the Pharmacological Effect of CLN by Other Behavioral Tests Evaluating Distinct Memory Paradigms

To confirm the efficacy of CLN, we next examined the effect of i.n. administered CLN on scopolamine-induced memory impairment using other behavioral tests. We first performed 'EPM', a widely used test for anxiety, which can also be used as a fear-motivated spatial learning test, as reported in an earlier study (Itoh *et al*, 1991). This test consists of two identical sessions, separated by a 24-h interval. In the second session, mice with normal memory that learned the EPM apparatus in the first session tend to avoid open arms

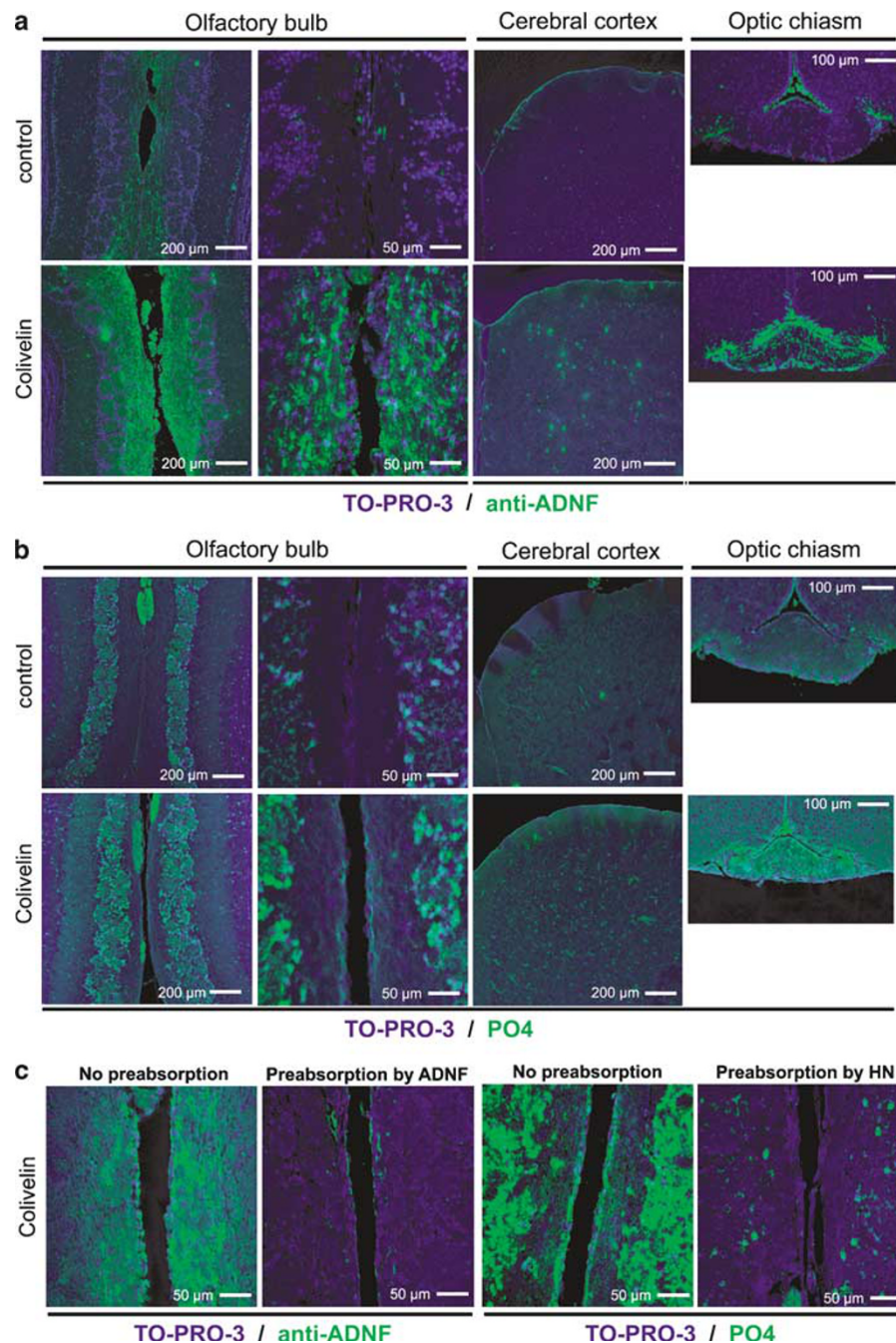


Figure 1 CNS delivery of Colivelin (CLN) by i.n. administration. Intranasally administered CLN in the brain was immunohistochemically visualized. CLN (5 nmol) was i.n. administered to mice 30 min before being killed. Paraffin-embedded coronal sections of the olfactory bulbs (left panel, bar: 200 μ m; right panel, bar: 50 μ m), the cerebral cortices (primary motor cortex, bar: 200 μ m), or the optic chiasm (bar: 100 μ m) of vehicle-treated and CLN-treated mice were immunohistochemically stained with an anti-activity-dependent neurotrophic factor (ADNF) antibody (a, green) or an antibody raised against humanin (HN) peptide (PO4) (b, green) to detect administered CLN peptide. Nuclear staining was performed with TO-PRO-3 (blue). Preincubation (or preabsorption) of the anti-ADNF antibody and PO4 with their synthetic antigenic peptides (ADNF or HN at 10 μ M) almost completely diminished IF intensity observed in the olfactory bulbs of mice with i.n. administration of CLN (c).

because mice fear high places. Accordingly, 'transfer latency' from the open arm to the closed arm and 'time spent in open arms' were significantly shortened (Figure 3a and c) while 'duration of the first stay in closed arm' and 'time spent in closed arms' were significantly prolonged (Figure 3b and c) in the control mice. In clear contrast, there were no significant changes in the four examined parameters in scopolamine-treated mice, indicating that

scopolamine treatment completely inhibited memory acquisition in the first session (Figure 3a–d). Mice treated with scopolamine together with i.n. CLN showed memory acquisition to an extent similar to the control mice (Figure 3a–d), indicating that CLN antagonized scopolamine-induced inhibition of memory acquisition in the EPM.

We also performed a PA task to evaluate contextual memory (Figure 4a). In the acquisition trial (day 1), there

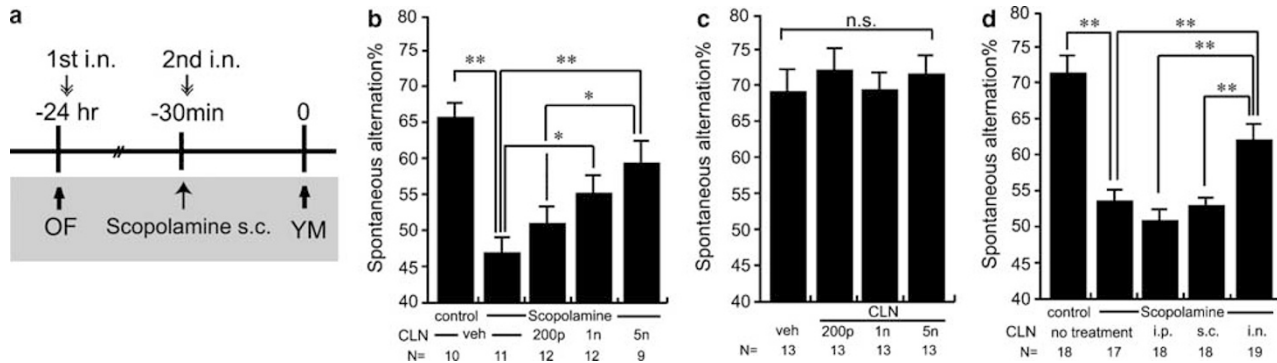


Figure 2 Effect of i.n. administered Colivelin (CLN) on a cholinotoxin model in a Y-maze test (YM). The design of the experiment is depicted in (a). Open field (OF) test was performed to habituate mice to the experimental conditions. We examined the effect of i.n. administered CLN on scopolamine (1.0 mg/kg, s.c.)-induced working memory impairment in YM (b). Indicated doses of CLN were administered per application. Spontaneous alternation percentages (SA%) of each treatment group are shown as means \pm SEM. ANOVA revealed a significant effect of treatments on SA% ($F_{(4,49)} = 8.730$; $P < 0.0001$). $*P < 0.05$, $**P < 0.01$ by Fisher's PLSD multiple comparison test. Effect of i.n. CLN itself on SA% was also examined (c). There was no significant effect of the CLN dose on SA% ($F_{(3,48)} = 0.287$; $P = 0.8344$). 'n.s.' means 'not significant'. We also compared the effects of CLN via different delivery routes (i.p., s.c., or i.n.) at the same dose (1 nmol/application, two times as depicted in (a)) on scopolamine-induced memory impairment (d). ANOVA revealed a significant effect of treatments on SA% ($F_{(4,85)} = 23.242$; $P < 0.0001$). $**P < 0.01$ by Tukey/Kramer's *post hoc* test.

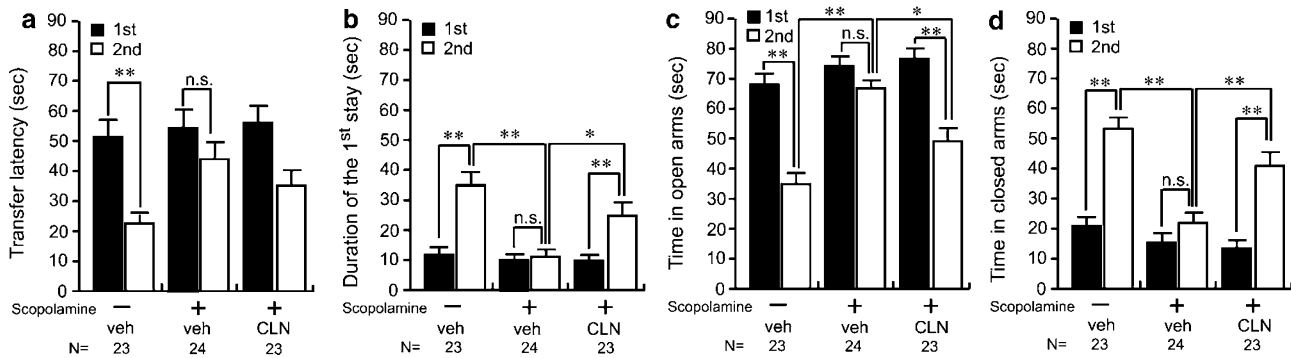


Figure 3 Effect of i.n. administered Colivelin (CLN) on a cholinotoxin model in an elevated-plus maze (EPM). Effect of i.n. administered CLN was examined in EPM, which assesses fear-motivated spatial learning. Scopolamine (1.0 mg/kg s.c.) was administered 30 min before the first session. Intranasal administration of vehicle (veh) or CLN (1 nmol per administration) was performed twice as depicted in Figure 2a. The closed column shows results in the first session and the open column shows those in the second session. Data are shown as means \pm SEM. ANOVA revealed a significant effect of treatments on the examined parameters: transfer latency into a closed arm ($F_{(5,134)} = 5.761$; $P < 0.0001$) (a, 'transfer latency'), duration of the first stay in the closed arm ($F_{(5,134)} = 12.168$; $P < 0.0001$) (b, 'first stay'), cumulative time spent in the open arms ($F_{(5,134)} = 23.058$; $P < 0.0001$) (c, 'time in open arms'), and cumulative time spent in the closed arms ($F_{(5,134)} = 23.027$; $P < 0.0001$) (d, 'time in closed arms'). $*P < 0.05$, $**P < 0.01$ by Tukey/Kramer's *post hoc* test. 'n.s.' means not significant.

were no differences in the entry latencies among the four treatment groups (Figure 4b). In the retention trial, the entry latency of the mice receiving s.c. saline and i.n. vehicle administration (control mice) was robustly prolonged compared to that in the acquisition trial, suggesting that the control mice acquired contextual memory in PA task on day 1 and that the memory was retained at least for 24 h. The entry latency in the mice receiving s.c. scopolamine and i.n. vehicle administration in the retention trial was slightly prolonged compared to that in the acquisition trial but was significantly shorter than that in the retention trial of the control mice, indicating that scopolamine impaired memory function. CLN treatment significantly attenuated scopolamine-induced decrease in the entry latency in the retention trial (Figure 4b), suggesting that CLN suppresses scopolamine-induced memory deficits in PA. There was no significant difference in the entry latency between mice receiving s.c. saline/i.n. CLN treatment and mice receiving s.c. saline/i.n. vehicle treatment.

We further performed an RAM task to assess memory performances; acquisition of reference memory and spatial working memory. Low-dose scopolamine (0.2 mg/kg/day) or saline were daily injected to mice and their memory performances were evaluated by the task once a day for 6 consecutive days. Their memory performances were further tested by the task for additional 2 days without the injection of scopolamine or saline to monitor the disappearance of the scopolamine's effect. CLN or vehicle was daily i.n. administered for 8 days. Total times required for the mice receiving s.c. saline and i.n. vehicle treatment (saline/vehicle group) to finish the task were significantly shortened and reached a plateau on the fourth day of the trial (day 4) (Figure 5b). Such learning pattern in the total time was similarly observed in another parameter of the test, error percentage in the first eight entries (Figure 5c). Total times required for the mice receiving s.c. saline and i.n. CLN (saline/CLN group) to finish the task were mostly similar to those for the saline/vehicle group, while those required for

the mice receiving s.c. scopolamine and i.n. vehicle (scopolamine/vehicle group) were not shortened until day 8, indicating that acquisition of reference memory was completely blocked by scopolamine treatment. Error percentages in the scopolamine/vehicle group were larger than those of the saline/vehicle group day 1 through day 7 (statistically significant on days 1, 4, 5, 6, and 7), indicating that scopolamine treatment also impaired spatial working

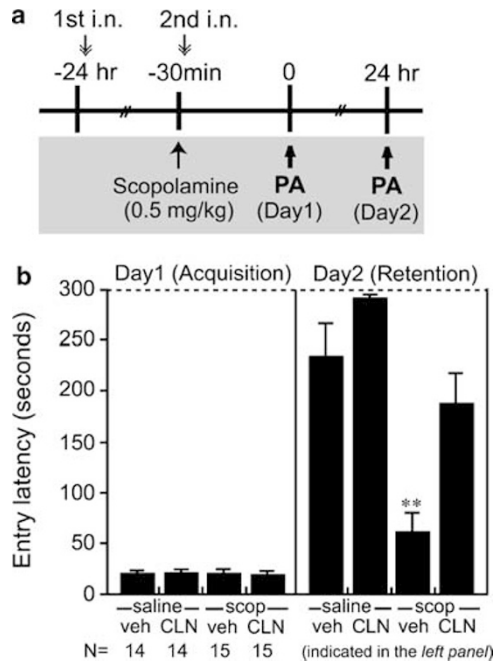


Figure 4 Effect of i.n. administered Colivelin (CLN) on a cholinotoxin model in a passive avoidance (PA) task. The design of the experiment is depicted in (a). We examined the effect of i.n. administered CLN on scopolamine (0.5 mg/kg, s.c.)-induced memory impairment in PA. Entry latencies in the acquisition trial and the retention trial were measured (b). Data are shown as means \pm SEM. ANOVA revealed a significant group difference ($F_{(3,54)} = 17.897$; $P < 0.0001$). $**P < 0.01$ (vs saline plus vehicle, saline plus CLN, or scopolamine plus CLN group) by Tukey/Kramer's *post hoc* test.

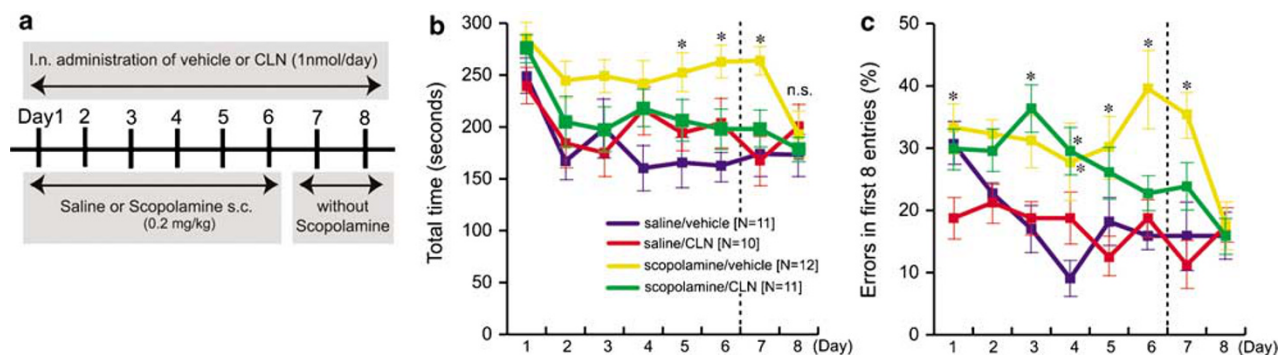


Figure 5 Effect of i.n. administered Colivelin (CLN) on a cholinotoxin model in a radial-arm maze (RAM). The design of the experiment is depicted in (a). We examined the effect of i.n. administered CLN on scopolamine (0.2 mg/kg, s.c.)-induced memory impairment in RAM. Examined parameters are total time spent in choosing all arms (b, 'total time') and working memory error percentage in the first eight entries (c, 'error%' in the first eight entries', defined as the percentage of overlapped entries in the first eight entries). Saline s.c. plus vehicle i.n. ('control mice', $N = 11$), blue line; saline s.c. plus CLN i.n. ($N = 10$), red line; scopolamine s.c. plus vehicle i.n. ($N = 12$), yellow line; scopolamine s.c. plus CLN i.n. ($N = 11$), green line. Data are shown as means \pm SEM. Two-way repeated-measures ANOVA was performed on 'total time' ($F_{(3,40)} = 9.621$; $P < 0.0001$ for groups, $F_{(7,40)} = 5.896$; $P < 0.0001$ for days, and $F_{(21,40)} = 0.922$; $P = 0.5627$ for interactions between groups and days) and 'error%' ($F_{(3,40)} = 16.602$; $P < 0.0001$ for groups, $F_{(7,40)} = 3.579$; $P = 0.001$ for days, and $F_{(21,40)} = 1.736$; $P = 0.0254$ for interactions between groups and days). Tukey/Kramer's *post hoc* test was performed for multiple group comparison ($*P < 0.05$, vs the control mice).

memory. Note that both parameters in the scopolamine/vehicle group returned to the levels equivalent to those of the saline/vehicle group on day 8, 2 days after the last injection of scopolamine. Intranasal CLN treatment showed substantial rescue effects on scopolamine-induced memory impairment: shortened total times day 2 through day 7 and decreased error percentages on days 6 and 7 (Figure 5b and c), indicating that i.n. CLN treatment inhibited both working and reference memory impairment caused by scopolamine.

CLN's Activity is Stronger Than ADFN or AGA-(C8R)HNG17 *In Vivo*

Colivelin has been reported to exhibit its neuroprotective activity by activating the downstream prosurvival signals of putative ADFN receptor and HN receptor *in vitro* (Chiba *et al*, 2005; Matsuoka *et al*, 2006). To delineate the relative *in vivo* potency of CLN and its component peptides, we compared the effect of i.n. administered CLN with those of its component peptides, ADFN and AGA-(C8R)HNG17 on working memory impairment induced by another cholinotoxin, 3-QNB (Figure 6a). As expected from the results in Figure 2b, short-term i.n. administration of CLN again significantly attenuated memory impairment caused by 3-QNB in a dose-responsive fashion (Figure 6c). Note that i.n. administration of 1 nmol CLN exhibited more potent effect than donepezil (10 mg/kg). In contrast, short-term i.n. administration of neither ADFN nor AGA-(C8R)HNG17 inhibited 3-QNB-induced memory impairment at all (Figure 6d and e), indicating that only CLN showed some pharmacological effect, unlike its component peptides *in vivo*.

Activity-dependent neurotrophic factor has been reported to exhibit a neuroprotective effect on memory impairment induced by another cholinotoxin, AF64A, when i.n. administered daily for longer than 1 week (Gozes *et al*, 2000). Combined with the negative finding shown in Figure 6d, that short-term administration of ADFN did not show any pharmacological effect on cholinotoxin-

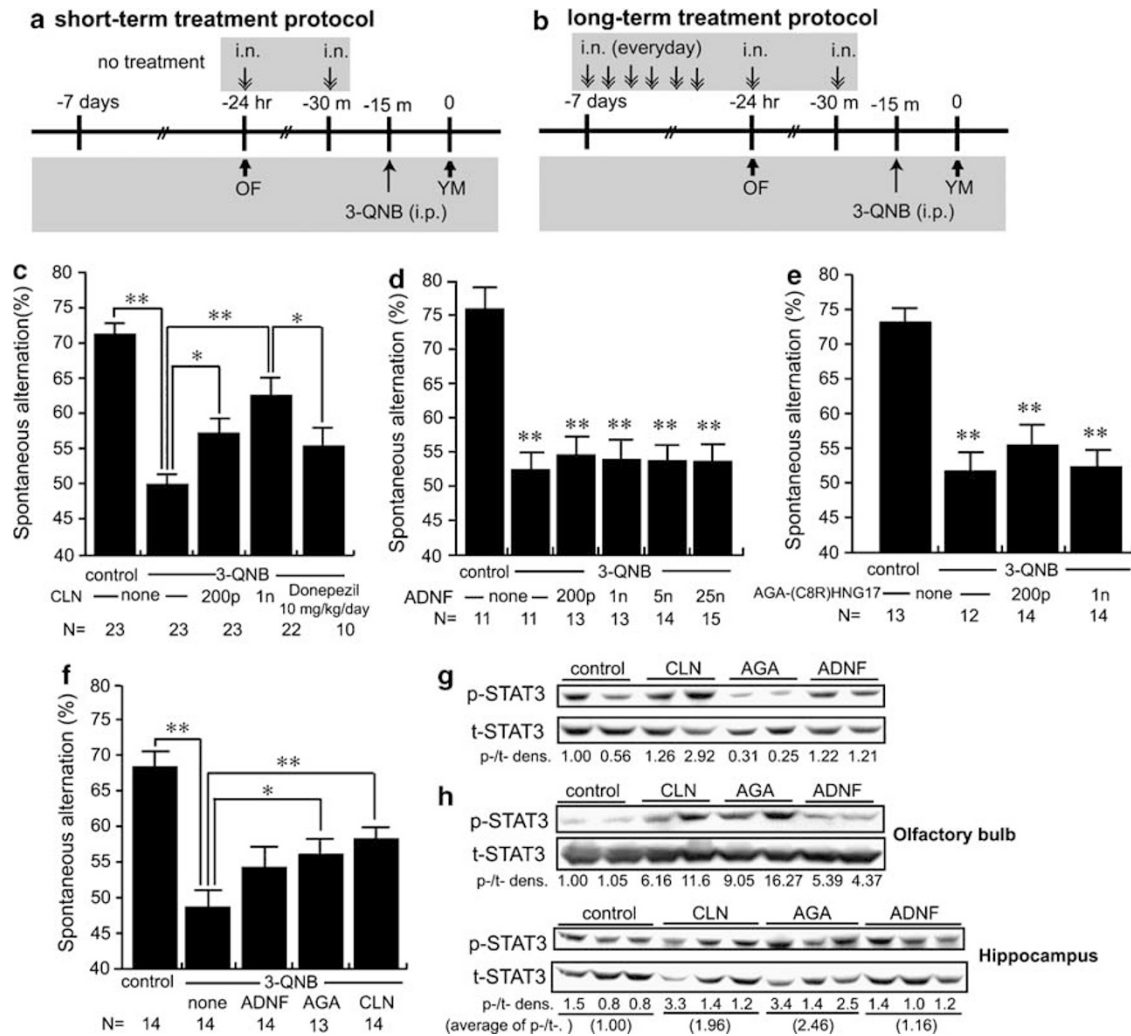


Figure 6 Characterization of Colivelin (CLN)-mediated memory improvement. Experimental designs for short-term and long-term peptide treatments on 3-quinclidinyl benzilate (3-QNB, 0.5 mg/kg, i.p.)-induced memory impairment are depicted (a and b). Effect of short-term i.n. administration of CLN (c), activity-dependent neurotrophic factor (ADNF) (d), or AGA-(C8R)HNG17 (e) at indicated doses per application in a cholinotoxin (3-QNB) model was examined. Donepezil (10 mg/kg) was i.p. administered as a positive control. High hydrophobicity of AGA-(C8R)HNG17 prevented us from examining its effect at higher doses. Spontaneous alternation percentage (SA%) of each treatment group is shown as means \pm SEM. One-way ANOVA revealed significant effects of treatments on SA%: $F_{(4,63)} = 11.817$; $P < 0.0001$ for (c), $F_{(5,71)} = 6.113$; $P < 0.0001$ for (d), and $F_{(4,48)} = 9.488$; $P < 0.0001$ for (e). $*P < 0.05$, $**P < 0.01$ by Tukey/Kramer's *post hoc* test (for (d) and (e)), $**P < 0.01$ vs control). Effect of long-term i.n. administration of the peptides was also examined (f) (ANOVA: $F_{(4,64)} = 8.559$, $P < 0.0001$). $*P < 0.05$, $**P < 0.01$ by Fisher's PLSD multiple comparison test. Immunoblot analysis of the lysates from olfactory bulbs of the mice treated with short-term i.n. administration of vehicle (control), CLN, ADNF, or AGA-(C8R)HNG17 (AGA) (two lanes each), was carried out with antibodies against both phosphorylated (p-) and total (t-) STAT3 (g). Immunoblot analysis of the brain lysates derived from olfactory bulbs and hippocampus of the mice treated with long-term i.n. administration was also carried out with antibodies against both p-STAT3 and t-STAT3 (h). Lysates were sampled 30 min after the second i.n. treatment of the peptides. Values indicated under panels for immunoblot analysis are the densitometrically calculated ratios of p-STAT3 to t-STAT3 expression levels.

mediated memory impairment, it is speculated that repetitive administration of ADNF may increase its efficacy, presumably because it increases the concentrations of ADNF *in vivo*. Based on this speculation, we administered ADNF, AGA-(C8R)HNG17, or CLN to the mice once a day for 1 week at a dose of 1 nmol per treatment (long-term treatment) to potentiate the effects of the peptides, as depicted in Figure 6b. We then found that long-term i.n. administration of CLN or AGA-(C8R)HNG17 showed significant inhibition of memory impairment caused by 3-QNB while that of ADNF appeared to show a marginal but statistically insignificant inhibition of it (Figure 6f). Thus, it is likely that the efficacy of i.n. treatment of the peptides can be potentiated by increasing administration frequency.

STAT3 is Involved in CLN-Mediated Inhibition of Cholinotoxin-Induced Memory Impairment

We further examined levels of STAT3 (Tyr⁷⁰⁵) phosphorylation, which represents activated STAT3 levels, in the brains of mice i.n. administered with CLN, ADNF, or AGA-(C8R)HNG17 because it has been shown that STAT3 is an essential effector for intracellular signals activated by HN and its derivatives *in vitro* (Chiba *et al*, 2005; Hashimoto *et al*, 2005; Matsuoka *et al*, 2006). Short-term i.n. administration of CLN tended to increase the STAT3 phosphorylation levels in the olfactory bulb while neither that of ADNF nor AGA-(C8R)HNG17 did so (Figure 6g). We also found that long-term i.n. administration of AGA-

(C8R)HNG17 as well as that of CLN significantly induced STAT3 phosphorylation in both the olfactory bulb and the hippocampus, indicating that STAT3 phosphorylation levels correlated positively with SA% obtained in YM. Therefore, we speculate that the pharmacological effect exhibited by the peptides *in vivo* was at least partially due to STAT3 activation (Figure 6h).

Intranasal Administration of CLN Protects the Cholinergic Systems from Toxicity by A β 25–35

Intracerebroventricular injection of toxic A β peptides has been reported to impair spatial working memory in rodents (Maurice *et al*, 1996; Yamada *et al*, 1999, 2005). We have recently established a toxic A β -induced mouse model of AD by using repetitive i.c.v. injections of a small amount of toxic A β via a cannula implanted into the lateral ventricle (Chiba *et al*, 2005; Yamada *et al*, 2005). Using this model, we examined the therapeutic effect of i.n. administered CLN on A β toxicity *in vivo*. We i.c.v. injected 1 nmol of the A β 25–35 peptide every other day for 3 weeks (totally 10 times) together with or without i.n. administration of 1 nmol CLN once a day for 3 weeks, as schematically shown in Figure 7a. Repetitive A β 25–35 injection efficiently decreased SA% compared to the vehicle-injected controls (Figure 7b). SA% of A β -injected mice with i.n. CLN treatment was almost equal to that of the water-injected control mice, suggesting that i.n. administration of CLN completely suppressed memory impairment induced by repetitive i.c.v. injection of toxic A β (Figure 7b). To confirm the effect of i.n. CLN treatment on A β -induced memory impairment, we also performed PA (Figure 7c). In the retention trial, A β injection significantly reduced entry latency into the darkened chamber and i.n. CLN treatment inhibited A β -induced reduction of entry latency, indicating that i.n. CLN treatment attenuated contextual memory impairment caused by A β detected in PA as well as A β -induced spatial working memory impairment in YM.

As reported in earlier studies (Yamada *et al*, 1999, 2005), i.c.v. injection of A β downregulates the expression level of ChAT in neurons of the medial septum. There is a positive correlation between the extent of spatial working memory impairment and the decrease in numbers of ChAT-positive neurons in the medial septum. By IHC analysis of the medial septa of A β 25–35-injected mice with or without i.n. administration of CLN (Figure 7d and e), we observed a significant decrease in the numbers of ChAT-immunoreactive neurons in the medial septa of A β 25–35-injected mice (232.3 ± 16.0 ChAT-positive neurons), compared to the controls (555.6 ± 57.4 , $P < 0.01$) (Figure 7e). In accordance with the SA% in YM, there were nearly as many ChAT-positive neurons in A β 25–35-injected mice with i.n. administration of CLN (514.0 ± 21.1) as those in the controls, suggesting that i.n. CLN treatment blocked the decrease in the number of ChAT-positive neurons, caused by repetitive i.c.v. injection of A β 25–35 (Figure 7e).

To elucidate the mechanism underlying CLN-mediated inhibition of A β 25–35-induced decrease in the number of ChAT-positive neurons, we next examined mRNA expression of ChAT and GAPDH in PC12 cells treated with 100 pM CLN, ADNF, or AGA-(C8R)HNG17 by semiquantitative RT-PCR (Figure 7f). We then found a significant increase in the

mRNA expression levels of ChAT in PC12 cells treated with 100 pM CLN compared to those of the control cells. mRNA expression levels of ChAT were also upregulated by 100 pM AGA-(C8R)HNG17, but not by 100 pM ADNF. CLN and AGA-(C8R)HNG17 treatment, but not ADNF treatment, also increased mRNA expression levels of vesicular VAcHT as well (Figure 7f). It is reasonable that CLN simultaneously upregulates mRNA expression levels of VAcHT as well as those of ChAT because mRNA expression of these molecules has been reported to be regulated in the same fashion (Oda, 1999). Note that mRNA expression levels of GAPDH were not affected by any treatment (Figure 7f). Taken together, these results suggest that CLN-induced upregulation of both ChAT and VAcHT expressions, which is likely to be due to STAT3 activation, may contribute to CLN-mediated suppression of AD-relevant cholinergic dysfunction.

CLN does not Inhibit AChE Activity

It is also possible that CLN supports cholinergic neurotransmission by mechanisms other than upregulation of ChAT expression. To investigate whether CLN directly inhibits activity of the acetylcholinesterase (AChE), a key enzyme in neuronal choline degradation and a target of currently used anti-AD drugs, we examined the effect of CLN treatment on the AChE activity by Ellman's method (Figure 8). In the assay, we detected a significant increase in absorbance at 416 nm when acetylthiocholine, DTNB or Ellman's reagent, and AChE were mixed *in vitro*. Absorbance at 416 nm was dose dependently reduced by a AChE inhibitor tacrine, suggesting that tacrine inhibited AChE activity. In clear contrast, CLN did not inhibit AChE activity.

CLN Treatment did not Cause Obvious Side Effects

From the standpoint of clinical application of CLN, it is very important to delineate its potential toxic side effects. For that purpose, we preliminarily examined side effects of CLN to liver and kidney by measuring serum ALT (GOT), AST (GPT), and CRE (Figure 9a–c). To assess acute side effects, we injected 200 nmol of CLN i.v. to CD-1 mice and sampled their sera 6 h after the injection. No significant differences in the three parameters were observed between sera of vehicle-treated control mice and those of CLN-treated mice. Similarly, subacute toxicities of CLN to liver or kidney were not recognized in the sera after i.n. administration of 5 nmol CLN once a day for 1 week. In addition, no behavioral abnormality or apparent health disturbance was documented in daily observation.

DISCUSSION

We have here established the efficacy of i.n. treatment of CLN against AD-relevant memory impairment. Delivery of peptide drugs into the CNS is always troublesome because BBB blocks their transportation. CLN has the highest potency among the HN derivatives thus far developed (EC₅₀ is <100 fM) and is therefore effective against AD-relevant memory impairment with i.p. or i.v. administration, which enables a very small fraction of CLN to enter the CNS (Chiba *et al*, 2005). It is apparent, however, that a

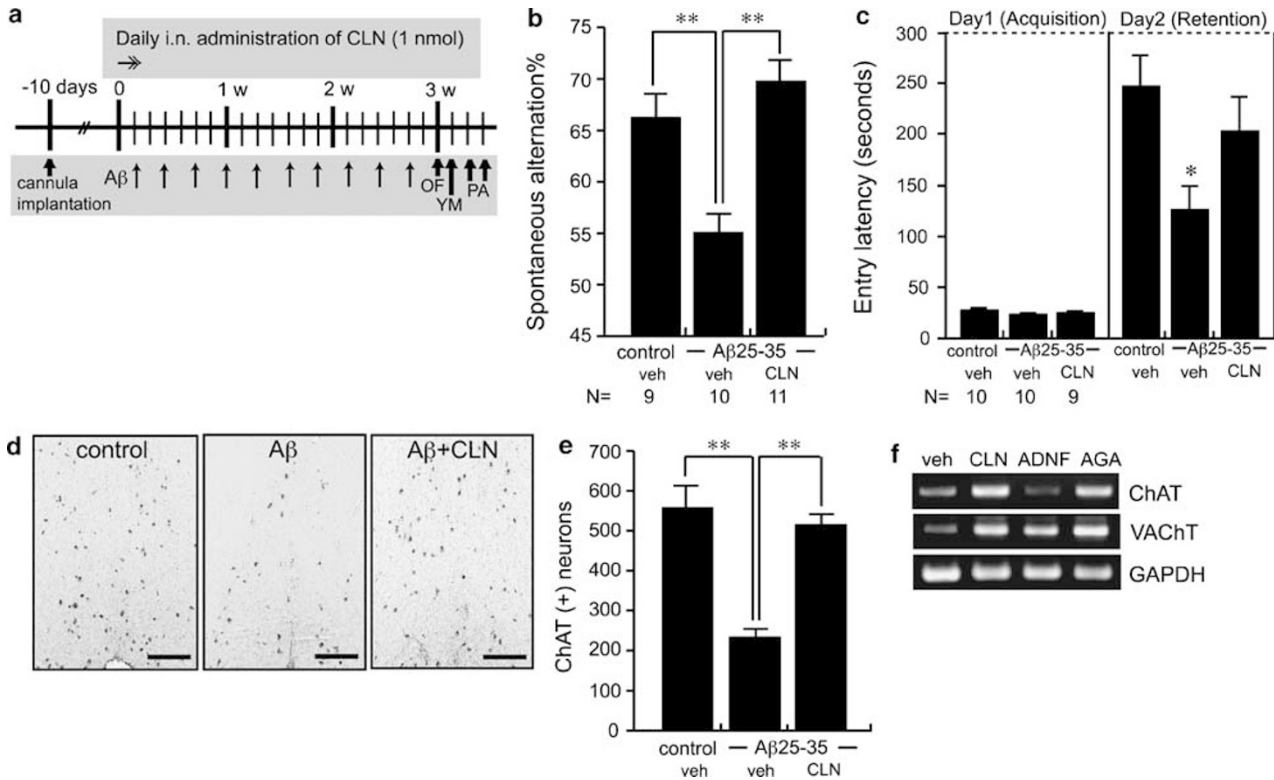


Figure 7 Effect of i.n. Colivelin (CLN) treatment on the repetitive $A\beta$ injection model. We examined the effect of i.n. administered CLN on $A\beta$ 25-35-induced working memory impairment. The experimental design is depicted in (a). Spontaneous alternation percentage of mice with repetitive i.c.v. injection of sterile water or $A\beta$ 25-35 together with i.n. administration of vehicle (veh) or CLN (1 nmol per application) is shown as means \pm SEM (b). One-way ANOVA revealed a significant effect of treatments ($F_{(2,44)} = 14.917$; $P < 0.0001$). $**P < 0.01$ by Tukey/Kramer's *post hoc* test. Passive avoidance test (PA) was also performed to examine the effect of i.n. CLN on memory impairment caused by repetitive $A\beta$ i.c.v. administration (c). Entry latencies, an index of contextual memory in PA, are shown as means \pm SEM (ANOVA: $F_{(2,26)} = 4.775$; $P = 0.0171$). $*P < 0.05$ by Dunnett's *post hoc* test (vs control). Brain sections of the medial septum were immunohistochemically stained with anti-choline acetyltransferase (ChAT) antibody (d; bar: 100 μ m). ChAT-immunoreactive neurons in the medial septum were counted and compared among the groups (e). Data are shown as means \pm SEM ($**P < 0.01$). (f) RT-PCR amplification of ChAT, vesicular acetylcholine transporter (VACHT), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs was performed using PC12 cells stimulated for 24 h with peptides (lanes: vehicle, veh; 100 pM Colivelin, CLN; 100 pM activity-dependent neurotrophic factor (ADNF), ADNF; 100 pM AGA-(C8R)HNG17, AGA).

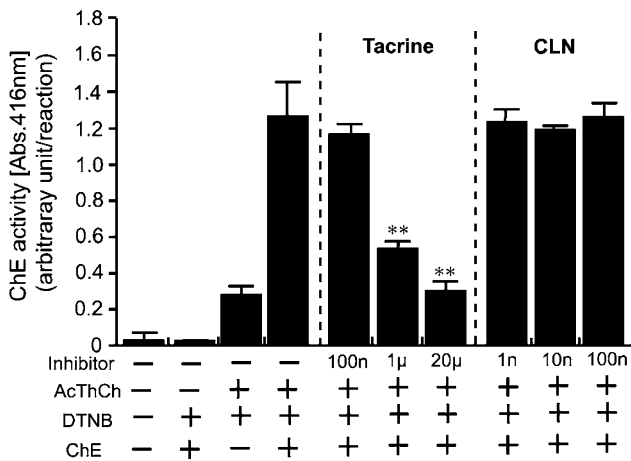


Figure 8 Effect of Colivelin (CLN) on acetylcholinesterase (AChE) activity. We measured AChE activity with or without a AChE inhibitor tacrine or CLN at indicated concentrations by Ellman's method ($N = 3$). Data are shown as means \pm SD ($**P < 0.01$). AcThCh, acetylthiocholine; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid).

course of AD patients, anti-AD drugs should be noninvasively administered. Accordingly, we searched for drug delivery systems more selective and less invasive than i.c.v., i.v., or i.p. administrations. We have here found that i.n. administration may match these conditions.

We tried to prove the entrance of CLN into the CNS. It is true that we do not have direct evidence that intact CLN reached CNS by i.n. administration. IHC analysis, however, indicated that CNS areas specifically immunoreactive to two CLN antibodies recognizing distinct epitopes (ADNF and AGA-(C8R)HNG17 portions) of CLN were largely overlapped (Figure 1). Combined with another finding by behavioral tests that i.n. ADNF or i.n. AGA-(C8R)HNG17 has by far weaker activity than i.n. CLN against memory impairment (Figure 6c-e), it is reasonable to think that intact CLN, but not a mixture of degraded peptides containing ADNF or HN epitope, reached CNS and improved memory impairment. As reported in earlier studies (Illum, 2000; Thorne *et al*, 2004), CLN immunoreactivity was predominantly detected in the medial and ventral regions of the olfactory bulbs of the mice with i.n. CLN treatment (Figure 1a and b), suggesting that i.n. administered CLN was likely to be delivered to CNS by mechanisms called the dendritic transporting processes of the olfactory sensory neurons (Illum, 2000; Thorne *et al*,

large amount of potent HN derivatives in the systemic blood stream by i.p. or i.v. administration causes side effects in extra-CNS organs. Furthermore, given the long clinical

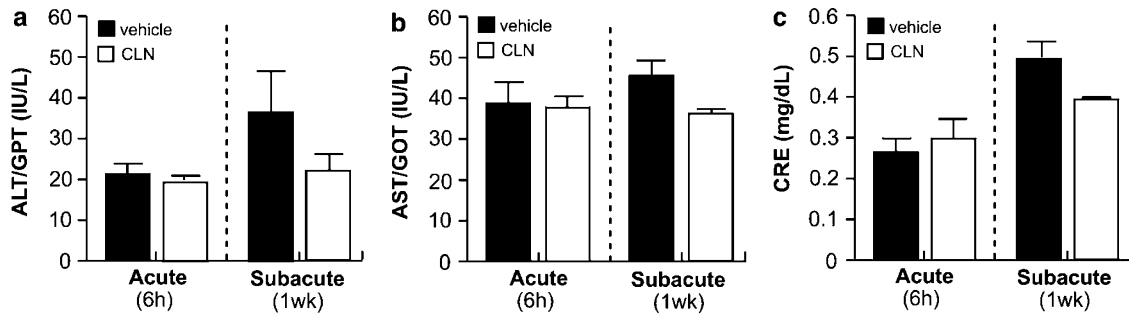


Figure 9 Estimation of acute and subacute toxicities of Colivelin (CLN) monitored by serum markers. To examine acute and subacute toxicities of CLN to liver and kidney, we measured serum alanine aminotransferase (ALT/GPT) (a), aspartate aminotransferase (AST/GOT) (b), and creatinin (CRE) (c, $N=3$ each). To monitor acute toxicities, we i.v. injected 200 nmol of CLN to CD-1 mice at 8 weeks of age and collected sera 6 h after the treatment. To monitor subacute toxicities, we i.n. administered CLN (5 nmol per application) once a day for 1 week, followed by serum collection. Data are shown as means \pm SD. Standard values measured in normal CD-1 mice at 31 weeks of age are 33 ± 7 for ALT/GPT, 49 ± 5 for AST/GOT, and 0.31 ± 0.03 for CRE, respectively (CLEA Japan Inc.).

2004). Although the detailed mechanisms underlying nose-to-brain transport remain elusive, they are thought to consist of both an extracellular transportation system, by which it may take several minutes for peptides to reach the CNS, and an intracellular transportation system, by which it may take more than 6 h for peptides to reach the CNS, based on previous observations by electron microscopy (Broadwell and Balin, 1985; Balin *et al.*, 1986). We also observed substantial immunoreactivity around the optic chiasm. Combined with the previous finding that the trigeminal nerve is another route for peptides to the CNS (Illum, 2000; Thorne *et al.*, 2004), we speculate that the optic nerve may also serve as a route.

We also compared the efficacy of i.n. administration of CLN with two other drug delivery routes, i.p. and s.c. (Figure 2d). We observed some pharmacological effect of CLN by i.n. administration on scopolamine-induced memory impairment, confirming that i.n. administration is a highly specific method of delivering peptide to the CNS. It is, however, important to confirm that the efficacy of i.n. administration is not limited to rodents. The routes from the nasal cavity to CNS are thought to remain in humans although human olfactory nervous systems are relatively degenerated compared with those of rodents. In support of the issue, i.n. administration of insulin in humans has been reported to improve memory without obvious systemic side effects such as hypoglycemia (Benedict *et al.*, 2004). Future investigation on the pharmacodynamics of CLN is warranted.

Colivelin, consisting of ADFN and AGA-(C8R)HNG17, exhibits its neuroprotective activity by activating both ADFN receptor- and HN receptor-mediated prosurvival pathways *in vitro* (Chiba *et al.*, 2005; Matsuoka *et al.*, 2006). Both HN derivatives and ADFN-related peptides have also been reported to attenuate cholinotoxin-induced memory impairment *in vivo* (Gozes *et al.*, 2000; Chiba *et al.*, 2005). We found that short-term i.n. CLN treatment ameliorated the AD-relevant memory disturbance while short-term i.n. treatment with the individual components of CLN at the same dose did not reproduce the pharmacological effect of CLN (Figure 6c–e). The results indicate that i.n. administration of CLN exhibits a more potent pharmacological effect than those of ADFN or AGA-(C8R)HNG17 *in vivo*, presumably because the concentration of CLN is more

easily elevated by i.n. administration to its effective level in CNS than those of its component peptides. In accordance with this idea, we also found that long-term i.n. administration of AGA-(C8R)HNG17 significantly and that of ADFN marginally attenuated memory impairment caused by 3-QNB (Figure 6f), suggesting that repetitive i.n. administration increases the efficacy of the neuroprotective peptides by increasing intracranial concentrations. In addition, the fusion of AGA-(C8R)HNG17 to ADFN (CLN) may enhance the *in vivo* ADFN's rescue effect on ALS model mice (Chiba *et al.*, 2004, 2006). Taken together, it is possible that the ADFN portion of CLN may contribute to the pharmacological effect of CLN on AD model mice.

The targets of CLN remain enigmatic because neither the receptor of ADFN nor that of HN has been identified. Recently, it has been reported that ADFN binds to and stabilizes tubulin to protect cells from various types of cellular stress (Holtser-Cochav *et al.*, 2006). ADFN-related peptide, NAP (NAPVSIPQ) also specifically binds to tubulin at an NAP-binding site as ADFN does, which may explain, in part, the broad neuroprotective activity offered by these peptides. In this paradigm, ADFN should pass through the plasma membrane. However, we have no evidence so far that CLN, a 26-amino-acid peptide, also physically goes through the plasma membrane and binds to tubulin. Our *in vitro* observation suggested that activation of the Ca^{2+} /calmodulin-dependent protein kinase IV (CaMKIV) is essential in the neuroprotective effect of ADFN although the precise mechanism remains uncharacterized (Chiba *et al.*, 2004, 2005). The involvement of tubulin and CaMKIV in the pharmacological effect of CLN *in vivo* remains to be addressed.

The HN receptor is thought to be on the cell membrane (Hashimoto *et al.*, 2001a, b; Nishimoto *et al.*, 2004; Matsuoka *et al.*, 2006) although it remains unidentified. Our recent studies have demonstrated that HN signals against AD-relevant neuronal death are intracellularly mediated by STAT3 *in vitro* (Chiba *et al.*, 2005; Hashimoto *et al.*, 2005). Earlier studies established the therapeutic effect of HN derivatives on functional memory deficits caused by AD-relevant insults, such as cholinotoxins and soluble A β (Mamiya and Ukai, 2001; Krejcova *et al.*, 2004; Chiba *et al.*, 2005). It has long been unclear, however, how HN derivatives exert their effects *in vivo*. In this study, we

observed upregulation of STAT3 phosphorylation levels only in the olfactory bulbs of mice with i.n. CLN treatment when they were treated with the short-term i.n. protocol (Figure 6g), suggesting that elevation of STAT3 phosphorylation levels may be correlated with the pharmacological effect of CLN. This hypothesis was further supported by another observation, that STAT3 phosphorylation levels in the olfactory bulbs and the hippocampuses were upregulated in mice with i.n. administration of CLN or AGA-(C8R)HNG17 under the long-term protocol (Figure 6h). Additional detailed mechanisms underlying the pharmacological effect of CLN remain to be addressed in future investigation.

Given that HN derivatives significantly antagonize cholinotoxin-induced memory impairment, it is natural to think that they directly affect the cholinergic systems. In support of this idea, i.n. administered CLN inhibited the downregulation of ChAT expression in the medial septa by A β treatment (Figure 7d and e), consistent with our previous finding that i.c.v. injection of CLN antagonizes A β -induced downregulation of ChAT expression (Chiba *et al*, 2005). We further found that mRNA expression of ChAT and VAcHT in PC12 cells was upregulated by treatment with CLN or AGA-(C8R)HNG17 (Figure 6e). Together with an earlier finding that leukemia inhibitory factor, which activates STAT3, maintains ChAT expression (Cheema *et al*, 1998); it is suggested that activation of STAT3 transcriptionally upregulates ChAT and VAcHT expression. Considering that ChAT catalyzes ACh synthesis and VAcHT uptakes ACh to synaptic vesicles (Oda, 1999), we speculate that CLN-mediated STAT3 activation increases presynaptic ACh storage and supports cholinergic neurotransmission.

In addition, it is reported that STAT3 may bind to the 5' upstream region of the *presenilin 1* (*PS1*) gene, a causative gene of FAD, and regulate its transcription (Rogaev *et al*, 1997). Given that PS1 is a major component of the γ -secretase that cleaves amyloid precursor protein and contributes to the generation of A β (Takasugi *et al*, 2003), activation of STAT3 might affect memory impairment related to AD via transcriptional regulation of PS1.

In summary, we provide the first evidence that HN derivatives can be i.n. administered for treatment of AD. Especially, i.n. CLN treatment shows great promise in development of a novel AD therapy. We also demonstrate that HN derivatives may trigger STAT3-mediated signals *in vivo* as well as *in vitro*, which may consequently increase presynaptic ACh storage by transcriptionally upregulating ChAT and VAcHT expression and promote cholinergic neurotransmission.

ACKNOWLEDGEMENTS

We thank Dr Ikuo Nishimoto, who initiated the work and unfortunately passed away on 17 October 2003. We are indebted to Drs Yasuo Ikeda and Masaki Kitajima for essential help. We also thank Dr Etsuro Ogata, and Mr Yoshiomi and Mrs Yumi Tamai for indispensable support; Dr Dovie Wylie for expert assistance; Ms T Hiraki, H Matsumoto, T Yoshida-Nishimoto, and S Uchida for expert assistance; and all members of the Department of

Anatomy for essential cooperation. This work was supported in part by grants from the Japan Society for the Promotion of Science, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), the KEIO Gijuku Academic Development Funds (TC), and in part by Noevir Co., Ltd.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare that except for income received from our primary employer no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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