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Nasal Cavity Administration of Melanin-Concentrating Hormone Improves Memory Impairment in Memory-Impaired and Alzheimer's Disease Mouse Models

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Abstract

Melanin-concentrating hormone (MCH) is a highly conserved neuropeptide known to exhibit important functions in the brain. Some studies have reported that MCH improves memory by promoting memory retention. However, the precise molecular mechanisms by which MCH enhances memory impairment have yet to be fully elucidated. In this study, MCH was administered to the scopolamine-induced memory-impaired mice via the nasal cavity to examine the acute effects of MCH and Alzheimer's disease (AD) mouse models to evaluate the chronic effects of MCH. MCH improved memory impairment in both models and reduced soluble amyloid beta in the cerebral cortex of APP/PS1 transgenic mice. In vitro assays also showed that MCH inhibits amyloid beta-induced cytotoxicity. Furthermore, MCH increased long-term potentiation (LTP) in the hippocampus of wild-type and 5XFAD AD mouse model. To further elucidate the mechanisms of the chronic effect of MCH, the levels of phosphorylated CREB and GSK3β, and the expression of BDNF, TrkB and PSD95 were examined in the cerebral cortex and hippocampus. Our findings indicate that MCH might have neuroprotective effects via downstream pathways associated with the enhancement of neuronal synapses and LTP. This suggests a therapeutic potential of MCH for the treatment of neurodegenerative diseases such as AD.

Keywords Alzheimer's disease (AD) · Cognitive function · Melanin-concentrating hormone (MCH) · Scopolamine · Long-term potentiation (LTP)

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Introduction

The melanin-concentrating hormone (MCH) is a highly conserved neuropeptide in animals and has been implicated in multiple brain functions [1, 2]. Located in the lateral hypothalamus and zona incerta, MCH-producing neurons are connected to various areas related to memory and learning processes including the cerebral cortex, hippocampal formation, medial, lateral and basolateral nuclei of the amygdala, and nucleus accumbens [3]. The hippocampus is known to play a critical role in spatial learning and memory and is also involved in episodic learning and memory, while the amygdala and prefrontal cortex are involved in emotional memories [4, 5].

Some studies have reported that the activation of MCH-producing neurons enhances learning and memory [6–10]. Moreover, direct administration of MCH peptides to the CA1 region of the hippocampus increased the response latency in a step-down inhibitory avoidance test, which evaluates both short-term and long-term learning and memory processes [7, 8]. In this test, MCH was associated with increased potentiation and nitric oxide and cGMP levels in the hippocampus of rats with memory impairment [8].

Two MCH receptors, MCH receptor 1 (MCH-R1) and MCH receptor 2 (MCH-R2), have been reported in mammals; however, only MCH-R1 is capable of binding to MCH peptide in non-human species such as rodents and rabbits [11]. In MCH-R1 knockout mice where MCH neurotransmission is selectively blocked, impaired memory retention was apparent when tested using a passive avoidance paradigm [6]. Furthermore, high levels of MCH-R1 mRNA were observed in the CA1 region, subiculum, cerebral cortex, basolateral amygdala, and nucleus accumbens of the wild-type rat, all of which are important in learning and memory processes [12–14]. Moreover, an in vitro study has shown that MCH holds an important role in the induction of long-term potentiation (LTP) by modulating hippocampal synaptic transmission via *N*-methyl-D-aspartate (NMDA) receptor-dependent pathway [10]. These findings may suggest that increased MCH and MCH receptors may correlate with increased neurotransmission.

Previous studies have suggested that MCH affects cognitive performance in Parkinson's disease (PD) [15] and Alzheimer's disease (AD) [16], but not in Huntington's disease [17]. Further investigation on MCH could lead to a better understanding of its role in other neurodegenerative diseases. Thus, we evaluated the acute and chronic neuroprotective effects of MCH in in vitro and in vivo AD models in this study. To examine acute effects of MCH in vitro, MCH was co-treated with amyloid beta in SH-SY5Y cells and primary cortical neurons, and then the cell viability was examined. Moreover, we tested whether MCH induces LTP in the hippocampal slice of wild-type or 5xFAD AD animal model and whether intranasal administration of MCH reverses scopolamine-induced memory impairment in mice. For chronic effects of MCH against memory impairments, two AD animal models were used; amyloid beta-injected AD mice and APPswe/PSEN1dE9 (APP/PS1) transgenic mice, overexpressing a chimeric mouse/human amyloid precursor protein (APP) and mutant human presenilin 1 (PS1). Designed to explore the potential effects of MCH on memory and learning processes, this study will provide a clue on whether MCH has a therapeutic potential for the treatment of memory-related disorders such as AD.

Materials and Methods

Viability Test on SH-SY5Y Cells and Primary Cortical Neurons

SH-SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin antibiotics (Hyclone Laboratories Inc., Logan, UT, USA). SH-SY5Y cells were seeded at 2×10^5 cells per well in a 48-well plate and grown at 37 °C in an incubator with 5% CO_2 for 24 h. Cell viability was calculated by measuring the mitochondrial dehydrogenase activity retained in the cultured cells, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Biosesang, Gyenggido, Korea). SH-SY5Y cells were incubated in 2 ml MTT solution (0.5 mg/ml) for 3 h while maintaining 5% CO_2 at 37 °C. Then, the MTT medium was carefully removed, and 1 ml of dimethylsulfoxide (DMSO) was added to the cells to solubilize the formazan. The absorbance was read at 540 nm. All experiments were repeated at least three times. Before the treatment of MCH (dissolved in saline; Tocris Biosciences, Bristol, UK), the SH-SY5Y cells were differentiated with 10 μM of retinoic acid (Sigma-Aldrich) for 24 h. Then the differentiated cells were treated with 25 μM of amyloid beta $(A\beta)_{25–35}$ (Sigma-Aldrich) plus 0, 5, 10, 20, and 50 nM of MCH for 24 h, and the cell viability was measured by MTT assay.

Primary cortical neurons were prepared using a method described by Kim et al. (2011) with a slight modification [18]. Briefly, on embryonic day 15, embryos were dissected from pregnant C57BL/6J female mice. The cortical regions of embryonic brains were isolated in calcium/magnesium-free Hank's balanced salt solution (HBSS). The cells were plated at 2.5×10^4 cells/cm^2 in 24-well plates coated with 15 μg/ml poly-l-Lysine (Invitrogen, Carlsbad, CA, USA). The cells were placed in N2 media supplemented with B27 (Invitrogen) at 37 °C under 5% CO_2. The medium was changed every 2 days. On day 5, the cells were treated with 20 μM of amyloid beta $(A\beta)_{25–35}$ (Sigma-Aldrich) plus 0, 1, and 2 μM of MCH for 24 h, and the cell viability was measured by MTT assay.

APPswe/PSEN1dE9 Mice

All animal works followed the protocols approved by the Institutional Animal Care and Use Committee at Dongguk University (No. 2016-1193) and the National Institute of Health guidelines. Male double transgenic mice expressing APP/PS1 with a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and were bred with wild-type female mice from the Jackson Laboratory at Dongguk University. The animals were housed under the constant temperature at 22 ± 1 °C, relative humidity at 55 ± 1%, and 12-h light/12-h dark cycle (lights on at 7:00 a.m.). They were allowed free access to food and water. Previously, we examined the neuroprotective effects of intranasally administrated MCH in PD animal mice [15]. In that study, we screened for the effective dose of MCH (1, 5, 10 μg) when mice were treated daily. Among them, 1 μg of MCH treatment over 1 month showed strong therapeutic effect against PD. Thus, we selected 1 μg of MCH, 3 times/week for long-term treatment over a period of 3 months in this study to examine the sustained effects of MCH and to minimize side effects. MCH (1 μg/30 μl, dissolved in saline) was administered intranasally into mice. Saline was used as a negative control (control, $n = 6$). Four-month-old APP/PS1 female transgenic mice were used and treated with intranasal administration of MCH at a dose of 1 μg 3 times a week for 3 months ($n = 6$). To examine the nutritional status, body weight and food intakes were recorded each week at the same time.

Scopolamine-Treated Mice

Scopolamine hydrobromide (Sigma-Aldrich), a muscarinic cholinergic receptor antagonist, impairs learning and memory in animals [19]. Thus, the Institute of Cancer Research (ICR) mice (Koatech, Gyenggido, Korea) were injected with saline-dissolved scopolamine intraperitoneally (i.p.) at a dose of 0.5 mg/kg to examine acute effects of MCH against memory impairment. The mice were divided into four groups ($n = 6$/group): control group (control, 30 μl saline), MCH-treated group (MCH, intranasal, 1 μg/30 μl saline), scopolamine-treated group (Scop, i.p., 0.5 mg/kg), and MCH plus scopolamine-treated group (M + S). MCH was administered through the nasal cavity 30 min before scopolamine treatment. All animals were injected with scopolamine except the control group. Within 30 min after the scopolamine injection, a Morris water maze test and passive avoidance test were conducted. Then, the mice were sacrificed by decapitation.

Aβ-Injected Mice

To generate an AD animal model, $A\beta_{1-40}$ (American Peptide Co., Sunnyvale, CA, USA) was administered by intracerebroventricular (*i.c.v.*) injection at 400 pmol of concentration. As an inactive control against $A\beta_{1-40}$, $A\beta_{40-1}$ (American Peptide Co.), the N − C inverted sequence of $A\beta_{1-40}$, was used. Each peptide was dissolved in 0.1 M PBS (pH 7.4), and aliquots were stored at − 20 °C. Each aliquot was allowed to aggregate in distilled water at 37 °C for 4 days. Two-month-old C57BL/6J mice (Koatech) were administered 400 pmol of $A\beta_{40-1}$ or $A\beta_{1-40}$ by *i.c.v.* injection as described by Chauhan et al. [20]. A total volume of 5 μl was injected in conscious mice at the bregma using a 10-μl microsyringe (Hamilton, Reno, NV, USA) fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. The injection site and needle track were visible and verified at the time of dissection. After the injection of Aβ, different concentrations of MCH (1 or 5 μg/30 μl, $n = 4$) were administered intranasally to mice daily for 7 days. Saline was used as a negative control ($n = 4$), and donepezil hydrochloride (Eisai Korea, Seoul, Inc., Korea) ($n = 4$), an acetylcholinesterase inhibitor [21] known to enhance learning and memory function in rodents, was used as a positive group.

Glucose and Cholesterol Measurements

The blood samples were collected from test animals. Glucose, total cholesterol, and triglyceride (TG),) levels were measured using kits from Asanpharm (Seoul, Korea) according to the manufacturer's instructions.

Passive Avoidance Test

On the training day, the mice were placed in a light chamber for 30 s. Then, the middle door was lifted, and the mice were allowed to enter the dark chamber. When the mice entered the dark chamber with all four paws, the dividing door was lowered, and a 50-V foot shock was delivered for 3 s. After the foot shock, the mice were removed and placed back to the home cage. Next day, the mice were returned to the light chamber. After 5 s, the middle door was opened, and the latency to enter the dark chamber was recorded.

Morris Water Maze Test

A round pool (97 cm in diameter and 60 cm in height) was used for Morris water maze test. Briefly, the pool was filled to a depth height of 30 cm with clouded water, composed of powdered milk, at 20 ± 1 °C. The platform (6 cm in diameter and 29 cm in height) was placed 1 cm below the water surface in the center of one quadrant of the maze. On the first day, mice received a swimming training for 60 s in the absence of the platform. The mice were subjected to four successive test trials. The trial sessions began on day 2 for 4 days. During each trial, the escape latency was measured for each mouse. The measurements were averaged for each trial session per each mouse. The time interval between each trial was 30 s. Once the mouse found the platform, the mouse was allowed to

stay on for 10 s. If the mouse failed to locate the platform within 60 s, the mouse was brought onto the platform for 10 s. On the last day of training, the mice were subjected to a probe trial session, in which the platform was submerged under the water and mice were allowed to swim in search of the platform for 60 s. The swimming time was recorded in the pool quadrant, where the platform had previously been exposed.

$A\beta_{1-42}$ Measurement

The cerebral cortices were immediately dissected and frozen. Tissues were stored at − 80 °C until use. For insoluble fraction, the tissue was homogenized in 8 vol (wt/vol) of cold 5 M guanidine HCl/50 mM Tris–HCl and mixed at room temperature for 4 h. For soluble fraction, the tissue was homogenized in lysis buffer containing 50 mM Tris-base (pH 7.5), 10 mM sodium pyrophosphate, 1% NP-40, and protease inhibitor cocktail (Invitrogen). Following centrifugation at 12,000×*g* for 20 min at 4 °C, each fraction was diluted with sample buffer (Invitrogen) and used for the measurement of $A\beta_{1-42}$ levels by enzyme-linked immunosorbent assay (ELISA) (Invitrogen).

Immunohistochemistry

One hemisphere of each mouse brain was post-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight. Then, the fixed brain tissue was kept in 30% sucrose solution until tissues sunk down. The tissues were cut coronally into 40-μm-thick sections. Then, the sections were blocked with 2% normal goat serum and 2% bovine serum albumin in PBS-T for 30 min to avoid a nonspecific reaction. The sections were incubated with monoclonal anti-Aβ protein antibody (1:200 in PBS-T; Santa Cruz, CA, USA) at 4 °C for 16 h. Then, the sections were stained with ABC methods (Vectastain Elite ABC kit; Vector Laboratories, Inc., CA, USA) and developed with diaminobenzidine (Sigma, MO, USA). The stained sections were finally visualized using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Immunoblot Analysis

Tissue extracts were obtained from the cerebral cortex and hippocampus of APP/PS1 transgenic mice. The tissues were homogenized in lysis buffer containing 50 mM Tris-base (pH 7.5), 10 mM sodium pyrophosphate, 1% NP-40, and protease inhibitors. Tissue lysates (30 μg) were electrophoresed using SDS gels and transferred to nitrocellulose membranes according to the standard protocol. Then, the membranes were incubated with anti-phospho-CREB, anti-CREB, anti-phospho-MAPK, anti-MAPK, anti-phospho-GSK3β, anti-GSK3β (Cell Signaling Technology, Beverly, MA, USA), anti-BDNF, anti-TrkB, anti-PSD95, and anti-β-actin (Abcam, Cambridge, UK) antibodies for 16 h at 4 °C. After washing with 1× TBS-T buffer, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibody (Thermo Fisher Scientific, Waltham, MA, USA). Then, the signal was visualized by a chemiluminescence kit (Super Signal West Pico; Pierce), and images were obtained using Molecular Imager ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA). The signal intensity was analyzed using the Image Lab TM software version 2.0.1 (Bio-Rad).

Hippocampal Slice Preparation

Wild-type (C57BL/6J, 2–3 months old) and 5XFAD (APP KM670/671NL (Swedish), 4~5 months old) mice were obtained from The Jackson Laboratory and used in this experiment. The animals were housed in individually ventilated cages with free access to food and water. The animals were maintained under 12-h/12-h light/dark cycle (light phase commencing at 8:00 p.m.) and at 22~30 °C. The animals were sacrificed between 9 and 10 a.m. by cervical dislocation. All animal experiments followed the protocols approved by the Institutional Animal Care and Use Committee of Chonnam National University Medical School (CNU IACUC-H-2018-1).

After cervical dislocation, the brain was quickly removed and transferred to ice-cold artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgSO_4$, 2 mM $CaCl_2$, and 10 mM glucose. A mid-sagittal cut was made in the brain, and one hemisphere was stored in the ice-cold aCSF until further analysis. Hippocampal slices were prepared by cutting the other hemisphere transversely (400-μm thick) using a Mcllwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Guildford, Surrey, UK). Then, the slices were stabilized in aCSF for 1 h while constantly perfused in 95% O_2/5% CO_2 mixture at room temperature.

Electrophysiology

Hippocampal slices were stored in aCSF at 20–25 °C for 1–2 h before transferring to the recording chamber. For recording, the tissues were submerged in aCSF flowing at 2 ml/min at 30 °C, and the field excitatory postsynaptic potentials (fEPSP) were recorded in the CA1 region using glass electrodes containing 3 M NaCl. A stimulating electrode in CA2 was used to evoke field EPSPs (constant voltage, 10 μs duration, repeated at 15-s intervals). LTP was evoked by two trains of tetanus stimuli (each 100 Hz, 1 s; repeated after a 30-s interval). After establishing the stable baseline for 30 min, fEPSP was further recorded for at least 60 min in the presence of 100 nM or 200 nM of MCH. Then, the slope of the evoked

field potential responses was measured and expressed relative to the normalized preconditioning baseline. Data were collected by NI USB-6251 (National Instruments, Austin, TX, USA) and amplified by Axopatch 200B (Axon Instruments, Union City, CA, USA). Data were captured and analyzed using WinLTP (www.winltp.com).

Statistical Analysis

One-way ANOVA and *t* test were used to analyze the data. All data are expressed as means ± SEMs. *P* values < 0.05 were considered as statistically significant values.

Results

Effects of MCH on the Viability of SH-SY5Y Cells and Primary Cortical Neurons

First, we generated Aβ-induced toxicity in neuronal cells to analyze the neuroprotective effect of MCH in vitro. SH-SY5Y neuroblastoma cells were differentiated into neuronal cells in the presence of 10 uM of retinoic acid and treated with varying concentrations of MCH (5, 10, 20, and 50 nM). There was no cytotoxic effect of MCH only treatment in the cells (Fig. 1a, left panel). The treatment with $A\beta_{25–35}$ resulted in a significant decrease in cell viability but the reduced cell viability was significantly improved with 5 and 10 nM of MCH co-treatments (Fig. 1a, right panel).

To further demonstrate the neuroprotective effect of MCH, primary cortical neurons were cultured from mouse embryos and treated with 25 μM of $A\beta_{25–35}$ and 1 or 2 μM of MCH. Exposure of primary cortical neurons to the $A\beta_{25–35}$ protein substantially decreased cell viability by 40%. However, this decrease was restored after treatment with MCH a higher concentration of MCH (~ 20% recovery for 2 μM, Fig. 1b). These results suggest that MCH has a neuroprotective effect on acute toxicity in neurons.

Effects of MCH in Scopolamine-Induced Cognition Impairment Mouse Models

A passive avoidance test was performed to examine the effects of MCH on scopolamine-induced memory impairment. Scopolamine (0.5 mg/kg) was injected i.p. into mice to induce memory deficit by blocking cholinergic muscarinic receptors in the central nervous system [19]. As shown in Fig. 2a, no significant difference was found between the groups under an untrained condition. The step-through latency time following training in the scopolamine group was not significantly different from the initial latency time. However, the latency time to enter the dark chamber was significantly longer in the mice receiving 1 μg of MCH (M + S group), compared with the initial latency time. This result suggests that intranasal administration of MCH can help retain memory impaired by scopolamine treatment.

Another memory analysis paradigm, a test for retrieval memory, was performed on the Morris water maze to further examine the effect of MCH on memory retention. On day 3, the control and MCH groups spent less time on an escape platform compared with the scopolamine-treated groups. On day 4, the scopolamine-treated mice continued to show increased swimming time on the platform (Fig. 2b). However, the MCH co-treatment group significantly lowered the latency time, suggesting improvement of retrieval memory by MCH (Fig. 2b).

Effects of MCH in an Aβ-Injected AD Mouse Model

Each mouse was injected at the bregma with Aβ peptide to generate an AD model. The effects of MCH were examined on this model using the passive avoidance test, with donepezil as a positive control. The mice injected with $A\beta_{1–40}$ exhibited a notable memory deficit in the test but not $A\beta_{40–1}$. However, the latency time of both the donepezil (D)- and MCH-treated (M) groups was significantly longer than that of the $A\beta_{1–40}$-treated group (Fig. 2c). Interestingly, mice receiving 1 μg of

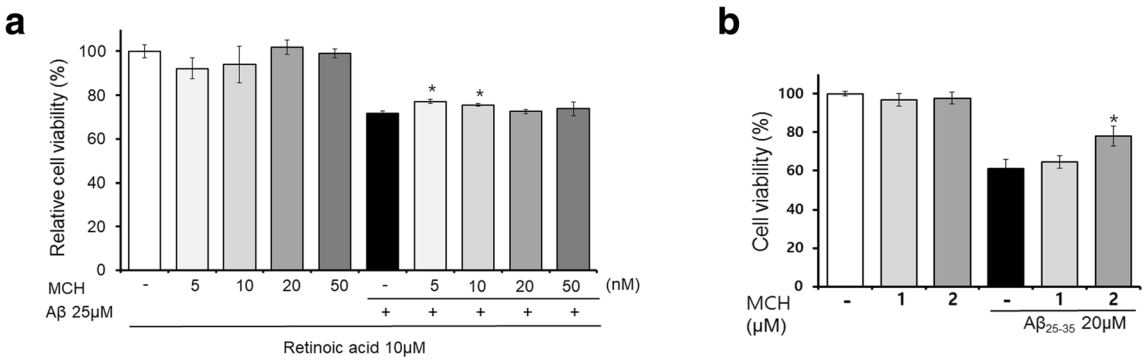

Fig. 1 MCH reduced $A\beta_{25–35}$-induced cell death in neuronal cells. **a** SH-SY5Y cells were differentiated with 10 μM of retinoic acid for 24 h and then cells were stimulated with 25 μM of $A\beta_{25–35}$ plus 0, 5, 10, 20, and 50 nM of MCH. After 24 h, cell viability was measured by MTT assay. **b** Five days after seeding the primary cortical neurons, cells were stimulated with 20 μM of $A\beta_{25–35}$ plus 0, 1, or 2 μM of MCH for 24 h and cell viability was assayed by MTT. Data represents the mean ± SEM of three independent experiments. *$P < 0.05$ vs. $A\beta_{25–35}$

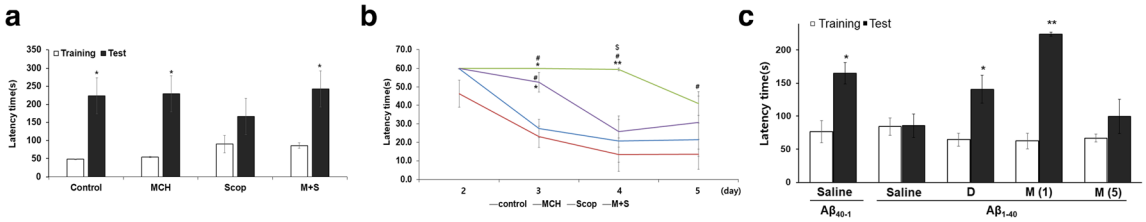

Fig. 2 Effects of MCH in mouse models with reduced cognitive function. To observe the effect of MCH on the transient cognitive decline by scopolamine, latency time for each group was measured in passive avoidance test (each group, $n = 6$) (**a**) and water maze behavior test (**b**). Saline (control), MCH 1 μg, (MCH), scopolamine (Scop), and MCH 1 μg + scopolamine (M + S). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. control, $^{\#}P < 0.05$ vs. MCH, $^{\$}P < 0.05$ vs. M + S. **c** Each mouse was injected at the bregma with $A\beta_{1–40}$ peptide to generate an AD model. The effects of MCH were examined on this model using the passive avoidance test, with donepezil as a positive control. The mice injected with $A\beta_{1–40}$ exhibited a notable memory deficit in the test but not $A\beta_{40–1}$. However, the latency time of both the donepezil (D)- and MCH-treated (M) groups was significantly longer than that of the $A\beta_{1–40}$-treated group. MCH administration (1 μg) increased the latency time in the Aβ-injected mice. The effects of MCH were compared with donepezil, a positive control. Data represents the mean ± SEM. Saline ($n = 4$), donepezil, D ($n = 4$), MCH 1 μg, M (1) ($n = 4$), and MCH 5 μg, M (5) ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. $A\beta_{1–40}$

MCH had a longer latency time than those treated with donepezil. However, 5 μg of MCH treatment did not show any significant effect.

Neuroprotective Effects of MCH in APP/PS1 Transgenic Mice

To investigate the long-term effects of MCH treatment, 4-month-old APP/PS1 female transgenic mice were injected intranasally with 1 μg of MCH 3 times a week for 3 months. To understand the role of MCH in glucose/lipid metabolism, we analyzed the blood samples of APP/PS1 female transgenic mice for glucose, total cholesterol, and triglyceride (TG) levels. Blood glucose and total cholesterol levels did not change after MCH treatment (Fig. 3b–d). However, mice treated with MCH exhibited a significantly lower level of TG (Fig. 3c).

Then, the passive avoidance test was performed to examine the effects of MCH on memory when the APP/PS1 transgenic mice reach the age of 7 months old. Before MCH treatment, both the control and MCH-treated groups exhibited poor performance in the test at 4 months, suggesting memory loss in APP/PS1 mice. However, a significant improvement in latency time was observed after the MCH treatment, but not in the control group (Fig. 4a). These findings suggest that the long-term treatment of MCH may reverse the memory loss of APP/PS1 transgenic mice.

The whole brain tissues of APP/PS1 transgenic mice were perfused and subjected to immunohistochemistry. The cortical and hippocampal tissues were immunostained with the anti-

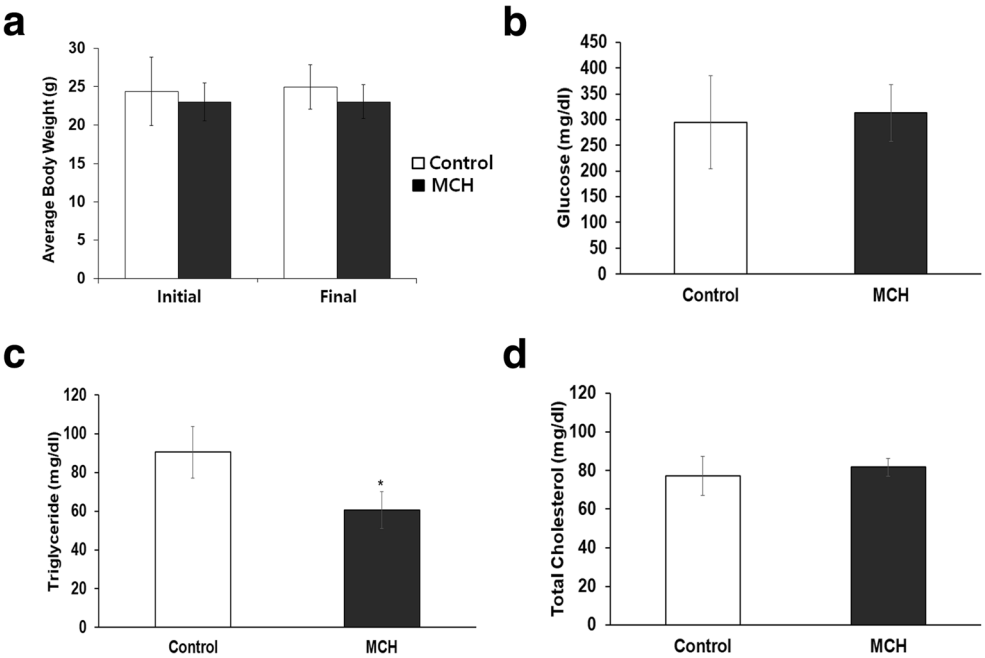

Fig. 3 The effects of MCH on energy homeostasis in APP/PS1 transgenic mice. After administration of MCH (1 μg) over 3 months, body weight (**a**), glucose (**b**), TG (**c**), and total cholesterol (**d**) levels were examined in the blood samples of 7-month-old APP/PS1 transgenic mice. Data represents the mean ± SEM ($n = 6$). $^{*}P < 0.05$ vs. control

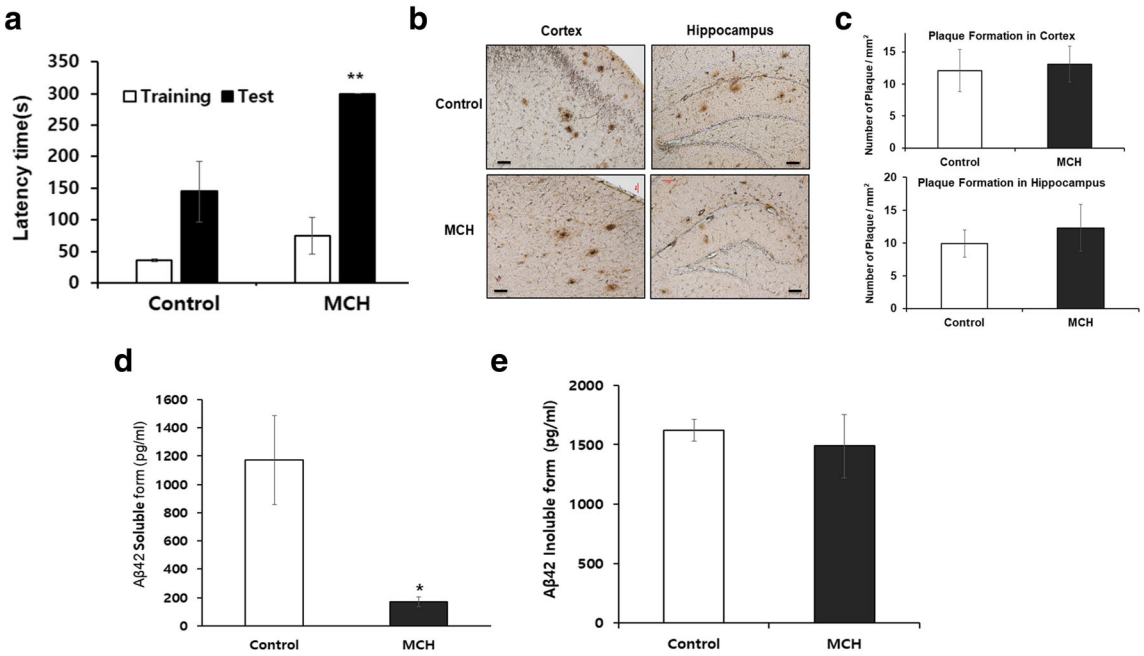

Fig. 4 MCH administration enhanced memory impairment and reduced soluble Aβ in APP/PS1 transgenic mice. **a** After administration of MCH (1 μg) over 3 months, passive avoidance test was carried out in 7-month-old transgenic mice. The latency time was increased by MCH treatment compared with the control. **b** Immunohistochemistry revealed $A\beta_{40}$ plaques in the cerebral cortex or hippocampus of MCH-treated and control mice. Scale bar, 100 μm. **c** The number of plaques were counted and visualized in a bar graph. The soluble form (**d**) and insoluble form (**e**) of Aβ were quantified using a 96-well microplate and represented as the mean ± SEM ($n = 6$). *$P < 0.05$, **$P < 0.001$ vs. control

Aβ antibody. As shown in Fig. 4b, a similar immunoreactivity was observed for Aβ in two groups. The quantification result of the average number of plaques also showed that there was no difference in plaque formation between the control and MCH-treated groups in the cerebral cortex and hippocampus (Fig. 4c).

Next, both soluble and insoluble $A\beta_{1-42}$ levels were measured to examine whether MCH affects the Aβ toxicity in the brain. MCH administration significantly decreased the level of $A\beta_{1-42}$ in the brain compared with the vehicle-treated mice (Fig. 4d), while the level of insoluble $A\beta_{1-42}$ did not change (Fig. 4e). These results indicate that MCH treatment might facilitate the reduction of soluble $A\beta_{1-42}$ in the brain.

To investigate whether MCH mediates the neuroprotective effects through CREB, MAPK, and GSK3β, we determined the phosphorylation levels of CREB, MAPK, and GSK3β in the cerebral cortex and hippocampus of APP/PS1 transgenic mice exposed to MCH or vehicle. The immunoblot analysis showed that MCH increased the phosphorylation of CREB, MAPK, and GSK3β in the cortical and hippocampal tissues (Fig. 5).

To identify the signaling pathway that mediates the MCH-induced phosphorylation of CREB, MAPK, and GSK3β, the protein levels of BDNF, TrkB, and postsynaptic density protein 95 (PSD95) were measured in the cerebral cortex and hippocampus of the mice treated with MCH. As shown in Fig. 6, MCH administration significantly elevated the levels of BDNF, TrkB, and PSD95 compared with the control group in both tissues.

Effects of Acute MCH Administration on LTP

To evaluate the effect of MCH on synaptic function, we treated hippocampal slices prepared from the wild-type mice acutely with either 100 nM or 200 nM of MCH for 1 h and analyzed LTP. Treatment with 200 nM of MCH resulted in an increase in hippocampal LTP compared with untreated control slices. However, no significant changes were observed in slices treated with 100 nM of MCH (control 147 ± 5%, $n = 5$, closed circle; MCH (100 nM) 151.7 ± 3, $n = 6$, open circle; MCH (200 nM) 169.3 ± 3% $n = 6$, open triangle, $P < 0.005$) (Fig. 7a).

Next, we examined this LTP-enhancing effect of MCH using the 5XFAD transgenic mice, an AD mouse model that displays synaptic impairment. Acute hippocampal slices obtained from 4 to 5-month-old 5XFAD mice were treated with MCH, and LTP analyses were performed. Results showed that 200 nM of MCH treatment significantly improved the impaired LTP in 5XFAD mouse hippocampal slices. However, no changes in LTP were observed at a concentration of 100 nM compared with the control (control 120.5 ± 4%, $n = 5$, closed circle; MCH (100 nM) 122.8 ± 2, $n = 6$, open circle; MCH (200 nM) 150.9 ± 4% $n = 6$, open triangle, $P < 0.01$) (Fig. 7b).

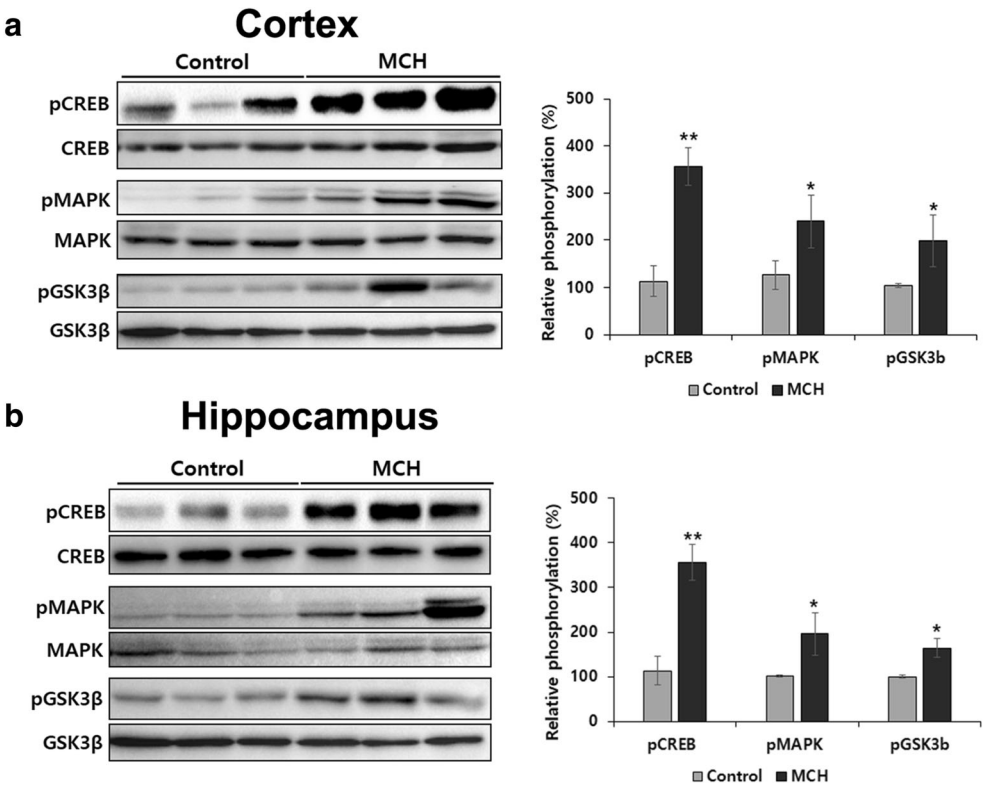

Fig. 5 MCH administration increased the phosphorylation of CREB, MAPK, and GSK3β in the cerebral cortex and hippocampus of APP/PS1 transgenic mice. **a** The cortical and **b** hippocampal lysates were electrophoresed and immunoblotted with each antibody. The amount of phosphorylated proteins was normalized against that of the total proteins (right panel). Data represents the mean ± SEM ($n = 6$). $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control

Discussion

We found that intranasal administration of MCH helped with memory retention in memory-impaired mouse models and AD models. It increased phosphorylation of CREB and GSK3β, and induced BDNF, TrkB, and PSD95 expression, and finally reduced amyloid-beta-induced toxicity in the cerebral cortex and hippocampus in AD mice. Moreover, MCH induced LTP in the AD hippocampus, indicating that chronic administration of MCH enhances brain functions involved in learning and memory processes in AD.

The MCH system, including MCH peptide, MCH receptors, and MCH-related peptides, is responsible for the tuning of arousal and modulates cognitive-promoting neuronal

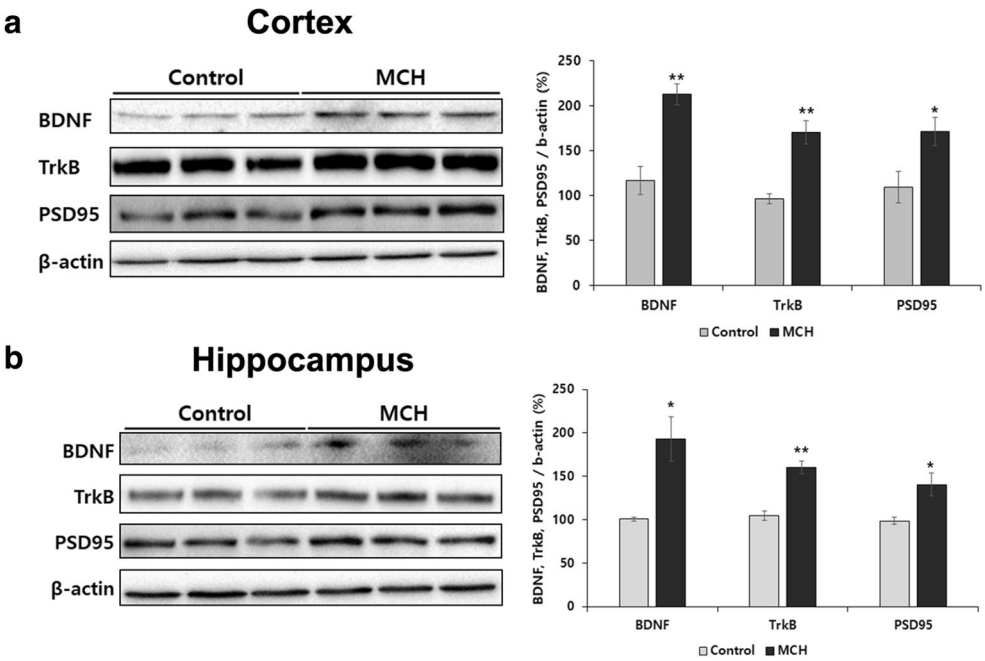

Fig. 6 MCH administration increased the expression of BDNF, TrkB, and PSD95 in the brain of APP/PS1 transgenic mice. **a** The cortical and **b** hippocampal lysates were electrophoresed and immunoblotted with each antibody. The amount of each protein was normalized against that of β-actin (lower panel). Data represents the mean ± SEM ($n = 6$). $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control

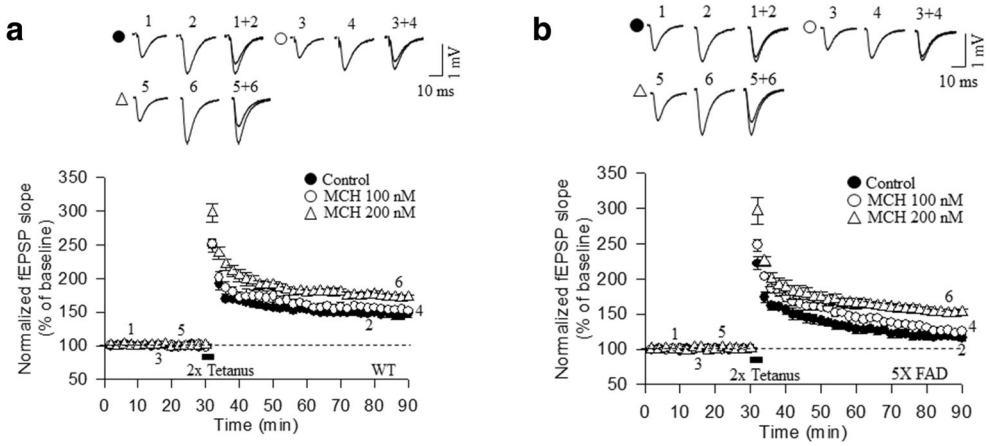

Fig. 7 Acute MCH treatment improves hippocampal LTP in both wild-type and AD mice. **a** LTP induction was observed in the CA1 region of the hippocampus following the delivery of high-frequency stimulation. MCH (200 nM) exposure over 1 h significantly enhanced the level of LTP induction (open triangle), compared with the control (closed circle) or lower level of MCH (100 nM) treatment (open circle). **b** A higher level of MCH (200 nM) treatment also enhanced LTP induction in the hippocampal slices prepared from 5XFAD transgenic mice (open triangle), while 100 nM of MCH (open circle) did not compared with the untreated counterpart (closed circle)

populations from the brainstem and forebrain structures [21]. Moreover, activation of MCH-producing neurons appears to increase learning and memory processes [22]. Our study demonstrated that MCH co-treatment reversed scopolamine-induced memory retention. Administration of scopolamine induces dementia model showing AD-related cognitive impairment profiles and oxidative stress [23]. Previously, we found that MCH has neuroprotective effects in SH-SY5Y cells and primary dopamine neurons against neurotoxin-induced toxicity [15]. In this study, MCH showed acute neuroprotective effects in SH-SY5Y cells and primary cortical neurons against Aβ-induced toxicity (Fig. 1), suggesting that MCH treatment may acutely modulate oxidative stress via MCHR and its downstream signaling cascade in the scopolamine-induced mice and reverse memory impairment.

Furthermore, some studies have reported that MCH decreases the LTP threshold by increasing hippocampal synaptic transmission through an NMDA receptor-dependent pathway and that MCH administration elicits an increase in NMDA receptor subunit 1 (NR1) expression, which is essential for NMDA receptor function and memory formation [10, 24]. In this study, we demonstrated that MCH (200 nM) significantly increases LTP in hippocampal slices from 5XFAD mice, suggesting that MCH acutely increases hippocampal LTP and thereby contributes to the induction of PSD.

The essential roles of BDNF and its receptor TrkB in complex learning and synaptic plasticity mediated by the hippocampus have been demonstrated by studies using BDNF knockout [25] and TrkB knockout mice [26]. The regulation of synaptic plasticity by neurotrophins and neutrophin receptor genes is widely believed to regulate the neural activity in the hippocampus [25, 26]. In the present study, long-term treatment of MCH upregulates BDNF and TrkB in the cortex and hippocampus of APP/PS1 Tg mice (Fig. 6). In addition, MCH induced PSD95 in the brain of AD mice. PSD95, as a major component of PSD, anchors AMPA receptors at the synapse on which glutamate acts to mediate excitatory synaptic transmission [27]. Thus, the overexpression of PSD95 enhances the amplitude of the excitatory postsynaptic current, similar to the strengthening of LTP [27]. An enduring increase in synaptic strength, that is LTP, is correlated with an increase in the expression of PSD [28]. Thus, these results are strengthening the notion that MCH may increase neurotransmission by enhancing the neuronal synapses.

To find out the signaling pathway by which MCH improves memory retention, we analyzed the activities of signaling proteins such as CREB, MAPK, and GSK3β. CREB is known to play an important role in numerous signal transduction pathways, including neurotransmission. In addition, CREB is a downstream substrate and effector of multiple signaling pathways. A large number of neurotransmitters converge on CREB via various kinases, such as the MAPK, PKC, PKA, ERKs, and PI3K/AKT/GSK3β pathways in neurons [29, 30]. Thus, the phosphorylation of CREB may be indicative of neural activity and is involved in the activation of many genes, including BDNF [31]. Our results suggest that the MCH-induced phosphorylation of CREB is mediated by MAPK and PI3K/Akt/GSK3β pathways and might contribute to the production of BDNF.

MCH is also known to modulate homeostasis and metabolism; increased food intake or sleep was reported in MCH-treated rats [32, 33]. However, we found that MCH did not have any significant effects on body weight (Fig. 3a) and sleeping behavior (data not shown), suggesting that the long-term MCH treatment does not affect the overall energy expenditure in transgenic mice. This contradictory observation could be explained by the dose difference; a higher dose (30 μg) of nasal administration of MCH induced body weight

gain (data not shown). Thus, the present study implies that low doses (1 or 5 μg) of MCH may not cause adverse effects such as an alteration in energy homeostasis.

In the present work, we developed a novel therapeutic strategy using the intranasal route to treat AD. Previously, we reported that MCH is well delivered and distributed around cortex and hippocampus after intranasal application of FITC-labeled MCH at 30 min [34], suggesting that intranasal administrated MCH distributes a whole brain distribution.

Recent studies have shown that high levels of blood TG are associated with rapid cognitive decline [35]. In this study, we found that the long-term treatment of MCH reduces blood TG levels (Fig. 3c), suggesting that MCH may improve cognitive functions by reducing circulating TG. However, it remains to be elucidated how MCH reduces TG level.

In summary, we found that MCH has acute neuroprotective effects in SH-SY5Y and primary cortical neurons against Aβ-induced toxicity. In addition to these in vitro results, intranasal administration of MCH acutely enhanced memory retention and impairment in the scopolamine-induced and Aβ-induced AD mouse models. Moreover, chronic administration of MCH reduced soluble Aβ in the cerebral cortex of APP/PS1 transgenic mice. The enhancement of cognitive function and reduction of amyloid pathology are likely mediated by the phosphorylation of CREB and GSK3β and induction of BDNF, TrkB, and PSD95. Furthermore, MCH acutely increased LTP in the hippocampus of wild-type and AD model mice. These novel findings show that MCH may exert neuroprotective effects via downstream pathways related to the enhancement of neuronal synapses and LTP, and present the nasal administration of MCH as a therapeutic candidate for treating AD.

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Compliance with Ethical Standards All animal experiments in this study followed the "Guide for Animal Experiments" provided by the Korean Academy of Medical Sciences. All animal protocols were approved by the Institutional Animal Care and Use Committee of Dongguk University, South Korea (IACUC-2016-1193), and Chonnam National University Medical School (CNU IACUC-H-2018-1).

Conflict of Interest The authors declare that they have no conflict of interest.

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