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Memantine reduces the production of amyloid-β peptides through modulation of amyloid precursor protein trafficking

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ABSTRACT

Memantine, an uncompetitive glutamatergic N-methyl-D-aspartate (NMDA) receptor antagonist, is widely used as medication for the treatment of Alzheimer's disease (AD). It has been reported that memantine reduces amyloid-β peptide (Aβ) levels in both neuronal cultures and in brains of animal models of AD. However, the underlying mechanism of these effects is unclear. Here we examined the effect of memantine on Aβ production. Memantine was administered to 9-month-old Tg2576 mice, a transgenic mouse model of AD, at 10 or 20 mg/kg/day in drinking water for 1 month. Memantine significantly reduced the amounts of both CHAPS-soluble and CHAPS-insoluble Aβ in the brains of Tg2576 mice. Memantine at 10 mg/kg/day for 1 month also reduced the levels of insoluble Aβ42 in the brains of aged F344 rats. Moreover, memantine reduced Aβ and sAPPβ levels in conditioned media from rat primary cortical cultures without affecting the enzymatic activities of α-secretase, β-secretase, or γ-secretase. Notably, in a cell-surface biotinylation assay, memantine increased the amount of amyloid precursor protein (APP) at the cell surface without changing the total amount of APP. Collectively, our results indicate that chronic treatment with memantine reduces the levels of Aβ both in AD models and in aged animals, and that memantine affects the endocytosis pathway of APP, which is required for β-secretase-mediated cleavage. This leads to a reduction in Aβ production. These results suggest that memantine reduces Aβ production and plaque deposition through the regulation of intracellular trafficking of APP.

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia and is characterized by a progressive loss of memory and neuronal loss in the presence of senile plaques and neurofibrillary tangles. Senile plaques consist of amyloid-β peptide (Aβ), which is produced upon the proteolysis of amyloid precursor protein (APP). APP is proteolyzed by one of two mutually exclusive pathways. Non-amyloidogenic APP processing due to α-secretase cleavage is considered the major pathway. The amyloidogenic pathway involves sequential cleavages of APP initially by β-secretase (β-site APP cleaving enzyme 1 [BACE1]) and further by γ-secretase (Chow et al., 2010; Tomita, 2014), which leads to production of the Aβ peptide. Aβ is a neurotoxic peptide (Chen et al., 2000; Deshpande et al., 2006), and there is growing evidence suggesting that Aβ oligomers induce acute synaptic dysfunction through the modulation of N-methyl-D-aspartate (NMDA) receptor activity, which results in the interruption of neural networks (Lacor et al., 2007; Li et al., 2009; Palop and Mucke, 2010).

Memantine, an uncompetitive NMDA receptor antagonist is thought to selectively block the excitotoxic effects of glutamate, including the pathological influx of Ca^{2+} and oxidative stress in postsynaptic neurons, while preserving physiological transmission, which is essential for normal cellular function (Parsons et al., 1999). Numerous studies in neural cultures have indicated that memantine attenuates the deleterious effects of Aβ, which include reductions in neurite outgrowth (Hu et al., 2007), loss of the spine cytoskeletal protein drebrin (Lambert et al., 2007), and the disruption of the axonal trafficking of dense-core vesicles and mitochondria (Decker et al., 2010). Neuroprotective effects of memantine against Aβ toxicity have been shown in several animal studies. Memantine inhibited neuronal damage and learning deficits in rats hippocampally injected with aggregated Aβ and the NMDA-receptor agonist ibotenic acid

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(Nakamura et al., 2006). Further, memantine rescued neocortical cholinergic fibers, attenuated microglial activation, and improved the impaired learning in rats injected intracerebroventricularly (i.c.v.) with Aβ oligomers (Nyakas et al., 2011).

Several studies indicate that memantine leads to a reduction in Aβ levels in the brains of animal models of AD, such as APP/PS1 Tg mice (Scholtzova et al., 2008; Alley et al., 2010), Tg2576 mice (Dong et al., 2008), and triple-transgenic mice, which express 3 dementia-related transgenes (APP_{SWE}, presenilin-1_{M146V}, and tau_{P301L}) (Martinez-Coria et al., 2010). However, there are neither studies using aged wild-type animals nor a unified view of mechanisms by which memantine reduces Aβ levels in the brains of these animals. In this study, concurrent with the clinical use of memantine for patients with AD, we studied the effects of memantine on brain-Aβ levels in Tg2576 mice and aged F344 rats, which are shown to have insoluble Aβ42 in their brains. Additionally, we examined the effects of memantine on the process of Aβ production using primary neuronal cultures.

2. Materials and methods

2.1. Compounds

Memantine hydrochloride was obtained from Daiichi Sankyo Propharma Co., Ltd. (+)-MK-801 hydrogen maleate and D-AP5 were purchased from Sigma-Aldrich and Tocris, respectively.

2.2. Animals

Tg2576 mice (Taconic Biosciences, Inc.), F344/N Slc rats (Japan SLC, Inc.), and Slc: Wistar rats (Japan SLC, Inc.) were used in our experiments. The animals were housed in standard environmental quarters maintained at a constant temperature, humidity, and light/dark cycle. They were maintained with free access to water and food. All experimental procedures were performed in accordance with the guidelines for animal experiments of The University of Tokyo or Daiichi-Sankyo Co., Ltd., and were approved by the Institutional Animal Care and Use Committee/ethics committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo or Daiichi-Sankyo Co., Ltd., Tokyo, Japan.

2.3. Effects of memantine in Tg2576 mice

The Tg2576 mouse is one of the most well-characterized and widely used mouse models of AD (Hsiao et al., 1996), shows increases in Aβ starting before 6 months of age, and has amyloid plaques beginning at 9–12 months of age (Kawarabayashi et al., 2001). In the clinic, therapeutic intervention using memantine begins in patients who have Aβ plaques in their brains. Thus, to assess the therapeutic effects of memantine on Aβ accumulation in Tg2576 mice that have already developed Aβ plaques in the brain, memantine was administered to 9-month-old mice. It has reported that female Tg2576 mice show a more rapid plaque deposition in the brain than do male mice (Callahan et al., 2001), so we selected female mice for short term experiment. Nine-month-old female mice were grouped into four cohorts. These cohorts were pre-treatment (n =5), vehicle-treated (n =24), memantine 10 mg/kg/day (n =24), and memantine 20 mg/kg/day (n =23). Memantine was dissolved in distilled water (DW, Otsuka) and was administered in drinking water. Water bottles were changed and weighed once per week and water consumption was calculated. Each mouse was decapitated and the cortices from each brain were dissected out before the study (pre-treatment group) or after a 1-month treatment (vehicle-treated and memantine-treated groups). Each sample was immediately frozen on dry ice and stored at −80 °C until use. Samples were homogenized in a 5-fold volume of 1% CHAPS (Sigma-Aldrich) solution containing protease inhibitors. They were then centrifuged at 100,000*g* for 1 h at 4 °C. The supernatant was collected as the CHAPS-soluble sample. The pellet was then homogenized using a 10-fold volume of 90% formic acid. After subsequent centrifugation at 100,000*g* for 1 h at 4 °C, the supernatant was neutralized with a 40-fold volume of 1 M Tris solution. The sample was further centrifuged at 9000*g* for 10 min at 4 °C, and the supernatant was collected as the CHAPS-insoluble sample. Both Aβ40 and Aβ42 levels in each CHAPS-soluble and CHAPS-insoluble samples were determined using enzyme-linked immunosorbent assay (ELISA) kits (Wako). The levels of Aβ oligomers, sAPPα, and sAPPβ were measured using ELISA kits (IBL). The data were standardized to brain tissue weight and expressed as pmol or ng/g of brain tissue.

2.4. Effects of memantine in aged F344 rats

F344 rats are reported to have age-related memory deficits (Kikusui et al., 1999) and significantly increased insoluble Aβ42 levels in their brains at age ≥22 months. This is concurrent with observations in sporadic AD (Takahashi et al., 2010). To determine whether memantine further inhibits the age-associated accumulation of Aβ in the brains of normal animals, we used F344 rats aged 22 months. In total, 22-month-old male F344 rats were divided into the 2 groups of vehicle (DW)-treated (n =4) and 10 mg/kg/day memantine-treated (n =7). Memantine was administered in drinking water. Water bottles were changed once a week. After 31 days of treatment, each rat was anesthetized and CSF was collected by a cerebellomedullary cisternal puncture using an indwelling needle. The collected CSF was centrifuged at 9000*g* for 5 min at 4 °C and the supernatant was frozen and stored at −80 °C until use. Blood was collected by cardiac puncture under anesthesia. Plasma was obtained by centrifuging the blood at 9000*g* for 5 min at 4 °C. Each rat was decapitated and the cortices of each brain were dissected out. Each sample was immediately frozen on dry ice and stored at −80 °C until use. Samples were homogenized in a 5-fold volume of 1% CHAPS solution containing protease inhibitors and centrifuged at 100,000*g* for 1 h at 4 °C. The supernatant was collected as the CHAPS-soluble sample. The pellet was then homogenized in 70% formic acid. After centrifugation at 100,000*g* for 1 h at 4 °C, the supernatant was neutralized with a 20-fold volume of 1 M Tris solution. The sample was centrifuged at 9000*g* for 10 min at 4 °C and the supernatant was collected as the CHAPS-insoluble sample. The levels of Aβ40 and Aβ42 were determined using ELISA kits (Wako). Brain Aβ levels were standardized to brain tissue weight and expressed as pmol/g of brain tissue.

2.5. Rat primary cell culture and conditions of drug treatment

Cortical neurons were harvested from embryonic day 18 Wister rat pups, as described previously, with certain modifications (Fukumoto et al., 1999; Suzuki et al., 2012). Neuronal cultures were prepared using the Nerve-Cell Culture System (Sumitomo Bakelite). Dissociated neurons were plated at 1.2×10^5 or 5×10^5 cells/cm^2 on 12-well plates or 100-mm dishes coated with poly-L-ornithine (Sigma-Aldrich) and cultured in high-glucose Dulbecco's minimal essential medium (Wako) supplemented with 50 unit/ml Penicillin, 50 mg/ml Streptomycin (Invitrogen), and 10% fetal bovine serum (FBS; Thermo Fisher Scientific). On the following day, the cultured medium was replaced with Nerve-Cell Culture Medium (Sumitomo Bakelite). Cultures were maintained at 37 °C in a 95% air/5% CO_2 humidified incubator. At 8 days *in vitro* (DIV 8), the cultured medium was replaced with medium containing test compounds or vehicle, and the medium and the cells were collected at DIV 14. The medium was centrifuged at 9000*g* for 5 min at 4 °C, and the supernatant was collected. The cells were washed with cold Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific) and removed from the plates by scraping. The cell suspension was used in immunoblot analysis.

2.6. Immunoblot analysis

The cell suspensions were dissolved in Laemmli sample buffer (Bio-Rad), and proteins were separated by SDS-PAGE. Proteins were transferred to a polyvynilidene fluoride (PVDF) membrane (Millipore). The membranes were incubated in 5% skim milk (Becton, Dickinson, and Company) or PVDF blocking reagent (for Can Get Signal) (Toyobo), treated with primary antibodies and probed with horseradish peroxidase (HRP)-conjugated secondary antibodies. To detect the expression of other proteins, membranes were incubated with stripping buffer (Thermo Fisher Scientific). Different targets were then detected using different primary antibodies. The following primary antibodies were used in this study: anti-βIII-tubulin TuJ-1 (1:5000; R & D systems, MAB1195), anti-BACE1 BACE1c (2 μg/ml; IBL, #18711), anti-APP APPc (1 μg/ml; IBL, #18961), anti-nicastrin (1:1000; Sigma-Aldrich, N1660), anti-ADAM10 (1 μg/ml; Abcam, ab1997).

2.7. In vitro α-secretase, β-secretase, and γ-secretase assays

The effects of memantine on the catalytic activities of α-secretase, β-secretase, and γ-secretase were examined using the cell membranes of human neuroglioma (H4) cells as the enzymatic source. H4 cells purchased from DS Pharma Biomedical were cultured in DMEM containing 10% FBS (Thermo Fisher Scientific). Cell membranes of H4 cells were collected as in previous reports (Hashimoto et al., 2002; Takasugi et al., 2003).

The *in vitro* α-secretase assay was performed using the SensoLyte 520 TACE (α-secretase) Activity Assay Kit (Anaspec) following the manufacturer's instructions. Cell membranes were suspended in the assay buffer included within the kit and used as the enzymatic source. Memantine (final concentration, 1–100 μM) was dissolved in dimethyl sulfoxide (DMSO, Wako). TAPI-0 (final concentration, 10 μM) was used as a positive control. Cell membranes were incubated with each drug for 30 min. Fluorescence values of the fractions were measured using 490 and 520 nm as the excitation and emission wavelengths, respectively.

To perform the *in vitro* β-secretase assay (Takasugi et al., 2011), after cell homogenization in 10 mM Tris buffer (pH 7.0), the membrane fractions were acidified using 25 mM CH3COONa (pH 4.5) and incubated with memantine (final concentration, 1–100 μM in DMSO), BACE inhibitor IV (Bi4; Calbiochem; final concentration, 10 μM in DMSO) as a positive control, or vehicle (DMSO), and the β-secretase-specific substrate JMV2236 (Bachem) at 37 °C for 3 h. Fluorescence values of the fractions were measured using 320 and 430 nm as excitation and emission wavelengths, respectively.

The *in vitro* γ-secretase assay was performed as described previously (Takahashi et al., 2003). Briefly, recombinant substrate (C100-FmH), which consists of an N-terminal methylated human βAPP (597–695), was purified from *E. Coli*. C100-FmH was then incubated with the 1% CHAPSO-solubilized membrane fraction in gamma buffer (HEPES buffer containing 0.25% CHAPSO, 5 mM EDTA, 5 mM 1,10-phenanthroline, 10 μg/ml phosphoramidon, 0.1% phosphatidylcholine, and Complete protease inhibitor) in the presence of test compounds or DMSO for the control group (final DMSO concentration, 1%) at 37 °C for 24 h. The same reaction was performed with DMSO at 4 °C for 24 h to measure the non-specific generation of Aβ (t0). The reaction was stopped by boiling for 5 min. The samples were centrifuged at 20,000*g* for 10 min at 4 °C and the levels of Aβ40 in each supernatant were measured using ELISA. The concentration of *de novo* generated Aβ40 was calculated by subtracting the mean concentration of t0 (n =2).

To determine the effects of long exposure to memantine on the catalytic activities of α-secretase, β-secretase, or γ-secretase, membranes prepared from brains of Tg2576 mice treated with memantine at 20 mg/kg/day or vehicle were used as enzymatic sources in the secretase assays.

2.8. Cell-surface biotinylation-based endocytosis assay

The cell-surface biotinylation-based endocytosis assay was performed according to the method of Bretscher and Lutter, with certain modifications (Kanatsu et al., 2014). Briefly, rat cortical primary cells were cultured as described above. At DIV 8 or DIV 14, the cultured medium was replaced with medium containing memantine (10 or 30 μM) or vehicle for 2 h and cells were washed with ice-cold PBS. The cells were then incubated with the membrane-impermeant cleavable biotin derivative Sulfo-NHS-SS-Biotin (1 mg/ml in PBS; Thermo Scientific) on ice for 30 min to label surface proteins. Excess biotin reagent was quenched by washing the cells thrice with 0.1 M glycine in ice-cold PBS. Cells were collected into tubes. After centrifugation, each pellet was lysed and sonicated in PBS containing 1% sodium dodecyl sulfate (SDS). Protein concentrations of the samples were measured using a bicinchoninic acid protein assay (Pierce) and the concentration of each sample was adjusted to the same concentration. A portion of each sample was then added to sample buffer and boiled for 1 min in 1% 2-mercaptoethanol. The resulting solution was stored as an input sample at −20 °C until use. Biotinylated proteins in the rest of the samples were captured using streptavidin sepharose (GE Healthcare) for 12 h at room temperature on a rotary mixer. The samples were eluted in SDS sample buffer by boiling for 1 min and were used as pull-down samples. Both input and pull-down samples were analyzed by immunoblotting.

2.9. Statistical analysis

Data are expressed as mean ± standard error (S.E.M.). Data were analyzed using a one-way layout and the multiple comparisons method of Dunnett. SAS System Release 8.2 (SAS Institute, Inc.) was used to perform all of the analyses and $P < 0.05$ was considered significant.

3. Results

3.1. Memantine inhibited the increases in soluble and insoluble Aβ in Tg2576 mice

First, we examined the effects of a 1-month treatment with memantine on Aβ levels in 9-month-old Tg2576 mice. Mean body weights and water consumption did not change among the groups throughout the 1-month treatment (supplementary Fig. 1). The actual doses of memantine extrapolated from the body weight and water consumption data were 8.8 and 16.1 mg/kg/day. Comparing these data to those of the pre-treatment group, we observed that the levels of insoluble Aβ40 and Aβ42 in the brains of vehicle-treated control mice had obviously increased over the 1-month treatment period. However, both doses of memantine markedly inhibited the increases in the levels of both insoluble Aβ40 and insoluble Aβ42 in the brain when compared to the vehicle-treated control group (Fig. 1A and B). Memantine also significantly and dose-dependently decreased the levels of soluble Aβ40, Aβ42, Aβ oligomers, sAPPα, and sAPPβ in the brain (Fig. 1C–G). These data suggest that chronic treatment with memantine affects APP metabolism in the brains of AD model mice.

3.2. Memantine inhibited the increase in insoluble Aβ42 in the brains of aged F344 rats

We tested the effects of memantine treatment on the endogenous murine Aβ in the brains of aged F344 rats, where we would normally observe an accumulation of insoluble endogenous murine Aβ. Memantine at 10 mg/kg/day did not affect mean body weights or water consumption throughout the 1-month treatment (supplementary Fig. 2). The estimated dose of memantine was 7.8 mg/kg/day. We

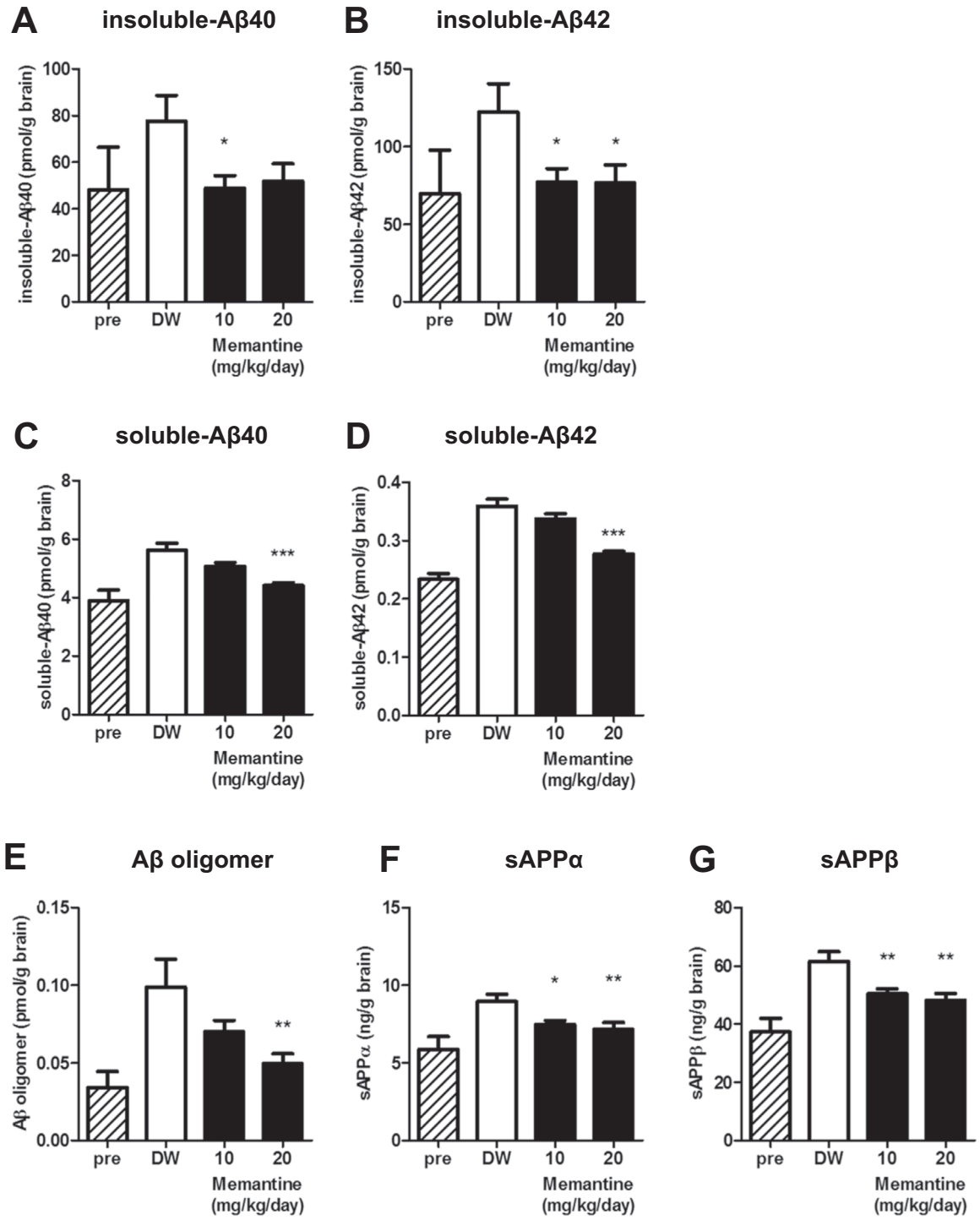

Fig. 1. Effects of 1-month administration of memantine on the levels of insoluble Aβ (A and B), soluble Aβ (C and D), Aβ oligomers (E), sAPPα (F), and sAPPβ (G) in the brains of Tg2576 mice. Data represent the mean ± S.E.M. of 5, 24, 24, and 23 mice in the pre-treatment (pre), vehicle-treated control (DW), 10 mg/kg memantine, and 20 mg/kg memantine groups, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to DW group by Dunnett test.

found that memantine significantly reduced the levels of insoluble Aβ42 in the brain (Fig. 2B). Memantine tended to reduce the levels of Aβ40 in the brain, CSF, and plasma. However, these trends were not statistically significant (Fig. 2A and C–F).

3.3. Effects of memantine on the levels of Aβ and sAPP in rat cortical primary cultures

To clarify the mechanism of Aβ reduction by memantine, we determined whether memantine reduces the levels of Aβ in rat primary cortical cultures. Starting at DIV 8, primary cortical cells were treated with memantine (10 and 30 μM) for 7 days without medium changes. The levels of Aβ40, Aβ42, and sAPPβ in the media were significantly and dose-dependently reduced by memantine (Fig. 3A). We observed no effects on cell viability (data not shown). Significant decreases in sAPPα were observed with 30 μM memantine. However, no changes were observed in the expression levels of APP, α-secretase (ADAM10), β-secretase (BACE1), or γ-secretase (Nicastrin, Nct) (Fig. 3B). These data indicate that subchronic treatment of primary neuronal cultures with memantine affects the proteolytic processing of APP.

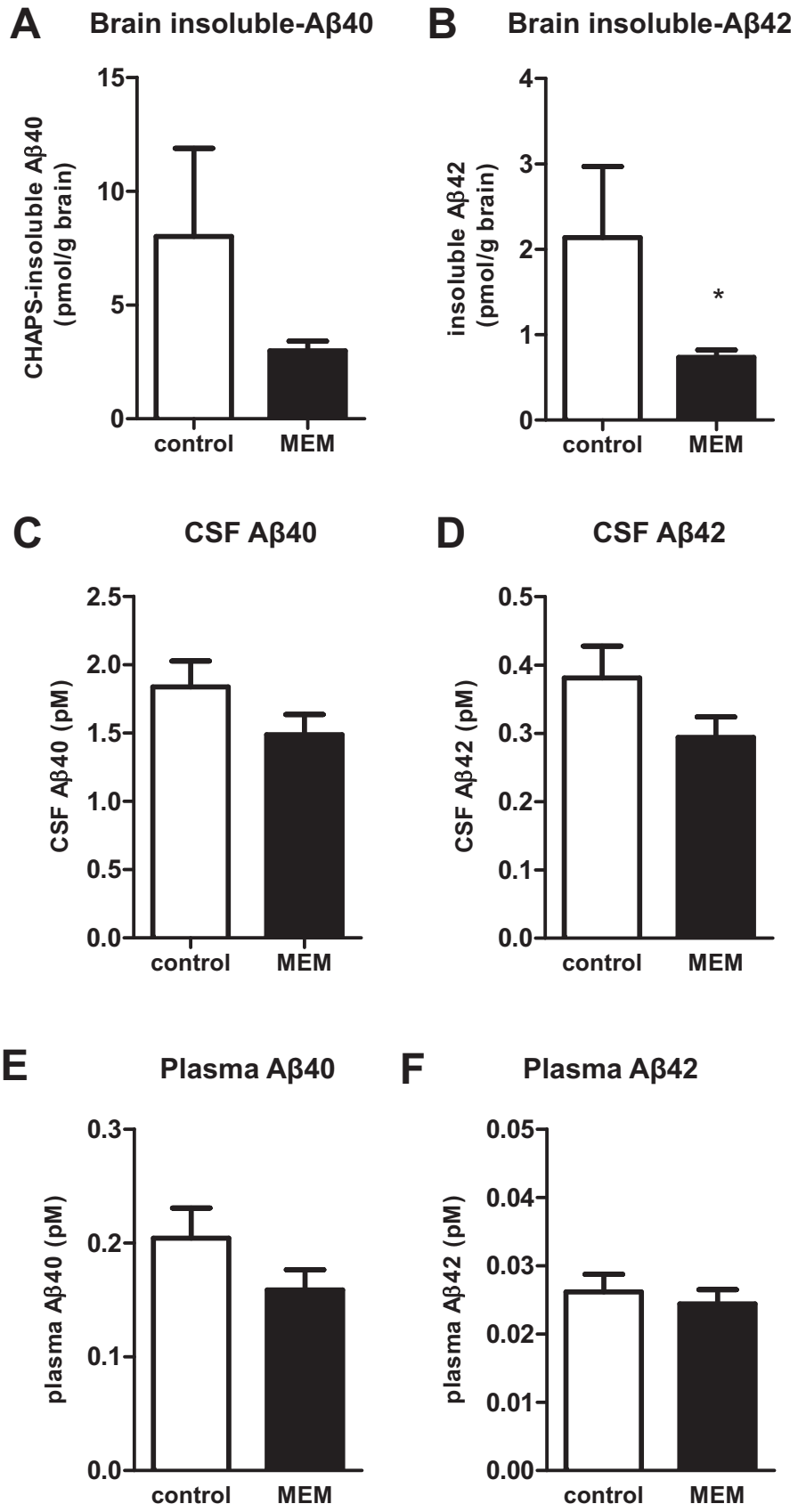

Fig. 2. Effect of 1-month administration of memantine on the levels of insoluble Aβ in brain (A and B), CSF (C and D), and plasma (E and F) of aged F344 rats. Data represent means ± S.E.M. of 4 and 7 rats in the control and memantine (MEM) groups, respectively. * $P < 0.05$, compared to the control group by *t*-test.

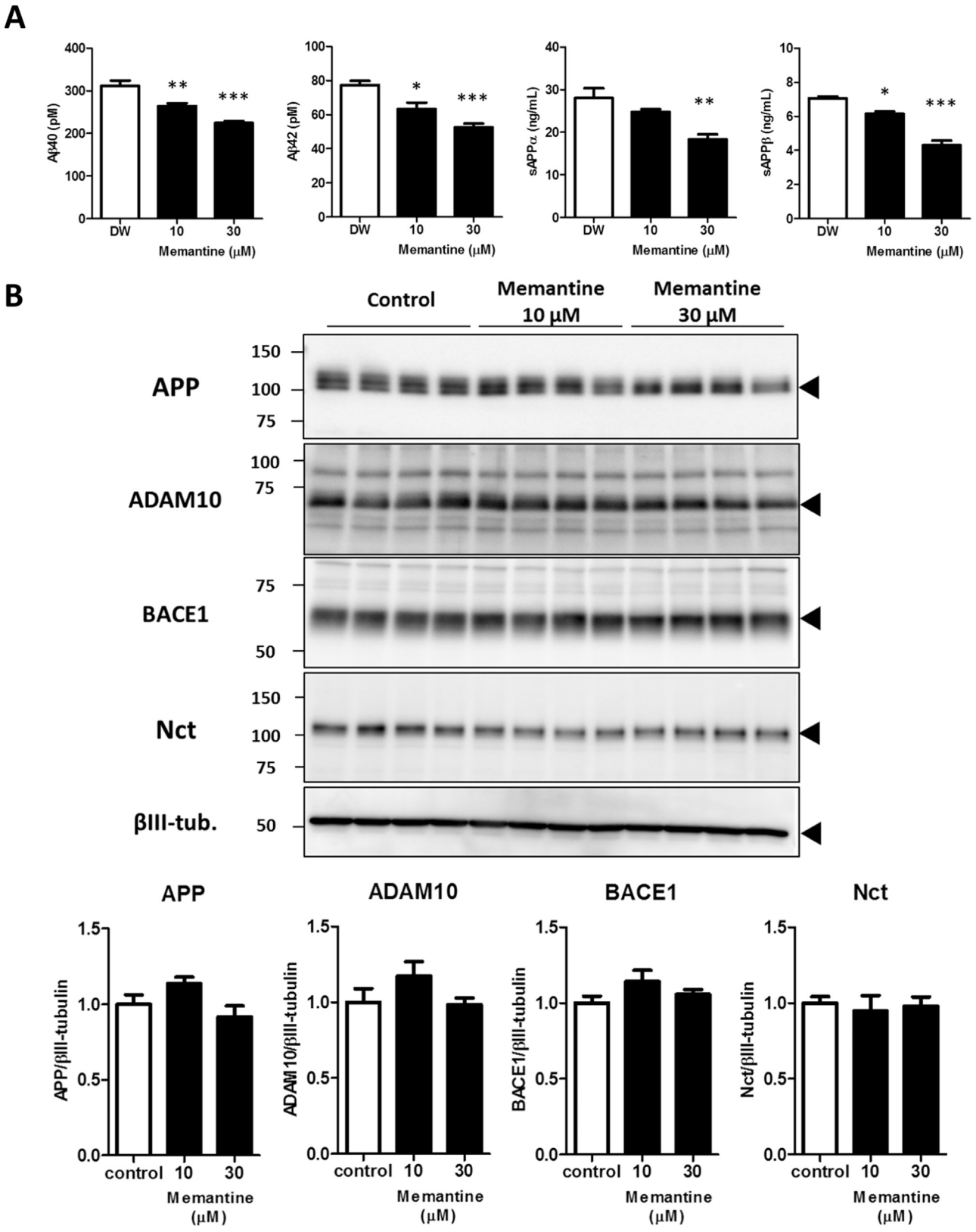

Fig. 3. Effects of memantine on Aβ and sAPP in rat primary cortical cultures. (A) Aβ40, Aβ42, sAPPα, and sAPPβ in media samples from 7-day DW- or memantine-treated rat primary cortical cultures at DIV 8. Data represent the means ± S.E.M. of 4 samples. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to the DW group by Dunnett test. (B) Expression levels of APP, ADAM10, BACE1, Nicastrin (Nct), and βIII-tubulin (βIII-tub.) in cell lysates from 7-day vehicle- and memantine-treated cultures at DIV 8. Rat primary cortical cultures were evaluated by western blot. Graphs show the quantified intensities of these proteins compared to that of βIII-tubulin. Data represent means ± S.E.M. of 4 samples.

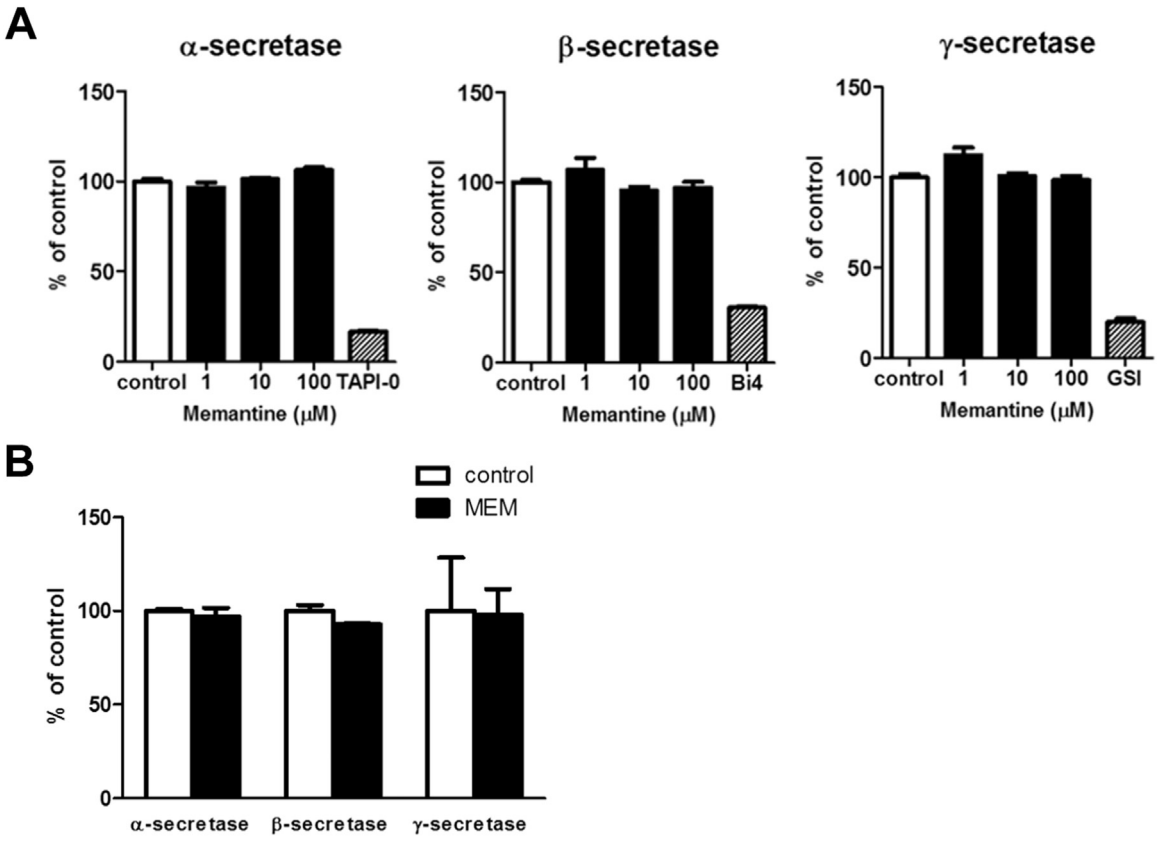

Fig. 4. Effects of memantine on the enzyme activities of α-secretase, β-secretase, and γ-secretase. (A) Membranes of human neuroglioma H4 cells were used as the enzymatic source in α-secretase, β-secretase, and γ-secretase assays. The cell membranes and each substrate were incubated with memantine (1–100 μM) or each positive control (TAPI-0, BACE inhibitor IV [Bi4], or L-685,458 [GSI]). Data represent means ± S.E.M. of 3 samples, respectively. (B) Tissue membranes from brains of mice treated with DW (control) or memantine (MEM, 20 mg/kg/day) for 1-month were used as the enzymatic sources in these α-secretase, β-secretase, and γ-secretase assays. Data represent means ± S.E.M. of 3 samples.

3.4. Effects of memantine on α-secretase, β-secretase, and γ-secretase enzyme activities

We determined whether memantine has the potential to modify the enzymatic activities of α-secretase, β-secretase, and γ-secretase. We confirmed the effects of memantine on Aβ production in the human neuroglioma cell line H4 (supplementary Fig. 3). However, coincubation with memantine at 1–100 μM did not alter the enzymatic activities of these secretases in the membrane fractions obtained from H4 cells (Fig. 4A). To further determine the effects of long-term exposure to memantine on these enzymes, we checked the catalytic activities of these enzymes in brain samples from Tg2576 mice treated with memantine at 20 mg/kg/day for 1 month. There was no difference between vehicle-treated and memantine-treated mice (Fig. 4B). These data suggest that memantine treatment reduces Aβ production without affecting the activities of APP processing enzymes.

3.5. The involvement of NMDA receptors in the effects of memantine on Aβ production

Several lines of evidence suggest that Aβ generation is regulated by neuronal activity *in vitro* (Kamenetz et al., 2003) and memantine is an uncompetitive NMDA receptor antagonist. To determine whether the effects of memantine on APP metabolism observed in this study were mediated through NMDA receptors, we examined the effects of the NMDA receptor antagonists D-AP5 and MK-801 on rat cortical primary cultures. Memantine (3–30 μM) reduced the levels of both Aβ40 and Aβ42 in a dose-dependent manner. D-AP5 (10–100 μM) and MK-801 (1–10 μM) also reduced the level of Aβ, but the both effects were limited (Fig. 5), suggesting that the effects of chronic treatment with memantine on Aβ production are mainly independent of NMDA receptor-mediated pathways.

3.6. The Effects of memantine on APP and BACE endocytosis in rat primary cortical cultures

Data obtained in this study indicate that memantine reduces the levels of Aβ by neither changing the expression levels of APP and APP-processing enzymes nor altering the activities of APP-processing enzymes. Additionally, we investigated the subcellular trafficking of APP. Cell biological analyses revealed that vesicular trafficking of APP is involved in the regulation of Aβ production (Jiang et al., 2014). Specifically, endocytosis of APP from the cell surface is a critical process in Aβ generation (Koo and Squazzo, 1994; Perez et al., 1999). To determine whether memantine affects the endocytosis of APP or BACE1, we performed a cell-surface biotinylation assay (Kanatsu et al., 2014). We found that incubation with memantine significantly increased the amount of biotinylated APP at the cell surface (pull-down) without changing the total amount of APP (input) at both DIV 8 and DIV 14. In contrast, the levels of both total and cell-surface BACE1 were almost comparable to those in control cells (Fig. 6B and C). This indicates that BACE1 trafficking was not altered. These results suggest that memantine affects the endocytosis of APP, which is required for the BACE1-mediated β-cleavage.

4. Discussion

Here we report that memantine reduces the brain levels of Aβ in Tg2576 mice that have plaques. Moreover, we show that the levels of insoluble endogenous murine Aβ were significantly reduced in aged F344 rat brains following memantine treatment. We also show that memantine affects the endocytosis of APP, but not that of BACE1. This

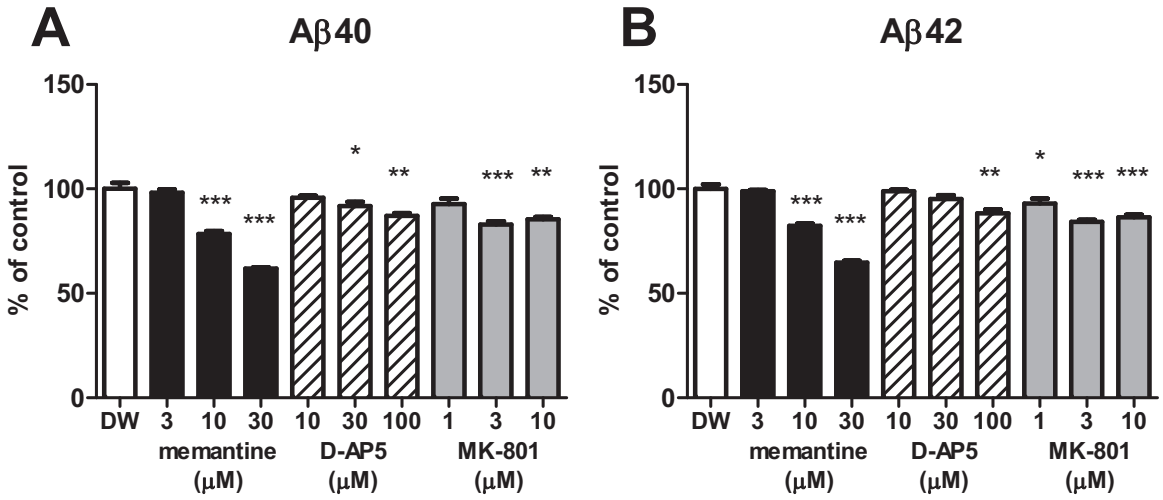

Fig. 5. Effects of NMDA receptor antagonists on Aβ in rat primary cortical cultures. Aβ40 (A) and Aβ42 (B) in media samples of rat cortical cultures at DIV 8 treated with vehicle (DW), memantine (3–30 μM), D-AP5 (10–100 μM), or MK-801 (1–10 μM) for 7 days. Data represent means ± S.E.M. of 4 samples. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to DW group by Dunnett test.

suggests that memantine may stabilize APP on the cell surface, which would lead to a reduction in Aβ production.

Our experiments with Tg2576 mice indicate that treatment with memantine inhibits the increase in insoluble Aβ in the brains of mice that have already developed Aβ plaques. This result was in agreement with a previous report indicating that treatment of Tg2576 mice with memantine at the age of 3 months, when Aβ plaques are not observed, has preventive effects (Dong et al., 2008). Memantine also reduces the levels of Aβ oligomers, which are the toxic Aβ species in the development of AD (Shankar et al., 2008). Furthermore, memantine significantly reduces insoluble Aβ42 levels in the brains of aged F344 rats, where there is accumulation of endogenous murine Aβ (Takahashi et al., 2010). Our data show that memantine reduces the levels of both insoluble Aβ and Aβ oligomers in an AD model, implying disease-modifying potential for memantine in patients with AD.

There are some possible mechanisms underlying the actions of memantine in reducing Aβ plaques in the brain. These include the inhibition of Aβ production, the inhibition of Aβ aggregation, and the acceleration of Aβ degradation. In this study, we focused on the possibility that memantine inhibits Aβ production. To clarify this, we examined the effects of memantine on Aβ production *in vitro* using rat primary cortical cultures. Memantine at 10 and 30 μM significantly reduces the levels of Aβ in the media of rat primary cortical cultures in a dose-dependent fashion. Regarding the dose used *in vivo* experiment, we measured the plasma concentration of memantine in Tg2576 mice in other experiment. After 1-month treatment with memantine at 20 mg/kg/day, the plasma concentration was 0.80 ± 0.16 μM (n=7). A previous report revealed that the concentration of memantine in brain tissue is found to be 20–30 times higher than in serum and CSF (Hesselink et al., 1999). Based on this report, the estimated concentration of memantine in the brain was 16–24 μM. This concentration in the brain may overwrap with the concentration used in the cellular experiments.

The NMDA receptor antagonists D-AP5 and MK-801 also reduced the level of Aβ, but the effect was limited, suggesting that the effects of memantine on Aβ production mainly involve an NMDA receptor-independent mechanism. Consistent with this idea, it was reported that memantine decreases the levels of Aβ in SK-N-SH cells, which have no detectable NMDA receptor activity (Pizzi et al., 2002; Alley et al., 2010).

Memantine did not affect the levels of APP. In addition, memantine had no effects on APP processing enzymes or their proteolytic activities *in vitro* or *in vivo*. We hypothesized that the Aβ-lowering effect of memantine is mediated by the modulation of APP trafficking. APP may be cleaved *via* two pathways: the non-amyloidogenic pathway and the amyloidogenic pathway (Zhang et al., 2013). The majority of cell surface APP is processed through the non-amyloidogenic pathway, whereas the internalization and recycling of cell-surface APP is required for Aβ generation and release (Koo et al., 1994). Our data indicate that memantine significantly increases the amount of APP at the cell surface without affecting BACE in the same manner. This indicates that memantine specifically affects APP endocytosis and reduces β-cleavage at the endosome. It is known that both APP and BACE1 are sorted into Rab GTPase 5-positive early endosomes (Rajendran et al., 2006). However, these trafficking pathways are differentially regulated. The internalization of APP occurs through recruitment by the adaptor-protein complex AP-2 and Dab2 for clathrin-mediated endocytosis. However, BACE1 is internalized and sorted into early endosomes *via* a route controlled by ADP-ribosylation factor-6 (Nordstedt et al., 1993; Lee et al., 2008; Sannerud et al., 2011). It has also been reported that APP and BACE1 converge upon the induction of neuronal activity (Kamenetz et al., 2003; Das et al., 2013). Stimulation of neurons with glycine results in a significant increase in APP/BACE1 convergence, and preincubation of neurons with memantine prevents this colocalization *via* an NMDA receptor-mediated pathway. However, this convergence occurs within 30 min of memantine or D-AP5 treatment; the latter had a slight effect on Aβ production in subchronic treatment experiments in our study. Considering previous findings (Pizzi et al., 2002; Alley et al., 2010), long-term treatment with memantine is likely to affect clathrin-mediated endocytosis and the subcellular localization of APP independent of NMDA receptor signaling. Notably, we observed a reduction in sAPPα production following memantine treatment *in vitro* as well as *in vivo*. It is known that the inhibition of BACE1 activity by genetic ablation or small compounds causes increases in the levels of sAPPα in neurons (Colombo et al., 2013). Additionally, this supported our notion that memantine affects cellular mechanisms instead of acting *via* simple enzymatic inhibition of APP metabolism and Aβ production. Together, our data indicate that memantine may affect the convergence of APP and BACE1 through both NMDA-dependent and NMDA-independent pathways. Nevertheless, further studies are needed to elucidate the precise molecular mechanisms underlying the actions of memantine on APP trafficking.

In summary, we show that chronic treatment with memantine not only reduces brain-Aβ levels in genetically modified mice models of AD but also inhibits age-dependent Aβ accumulation in normal animals. We found the novel mechanisms of memantine affect APP endocytosis, which impacts Aβ production in neurons. Thus, memantine has a

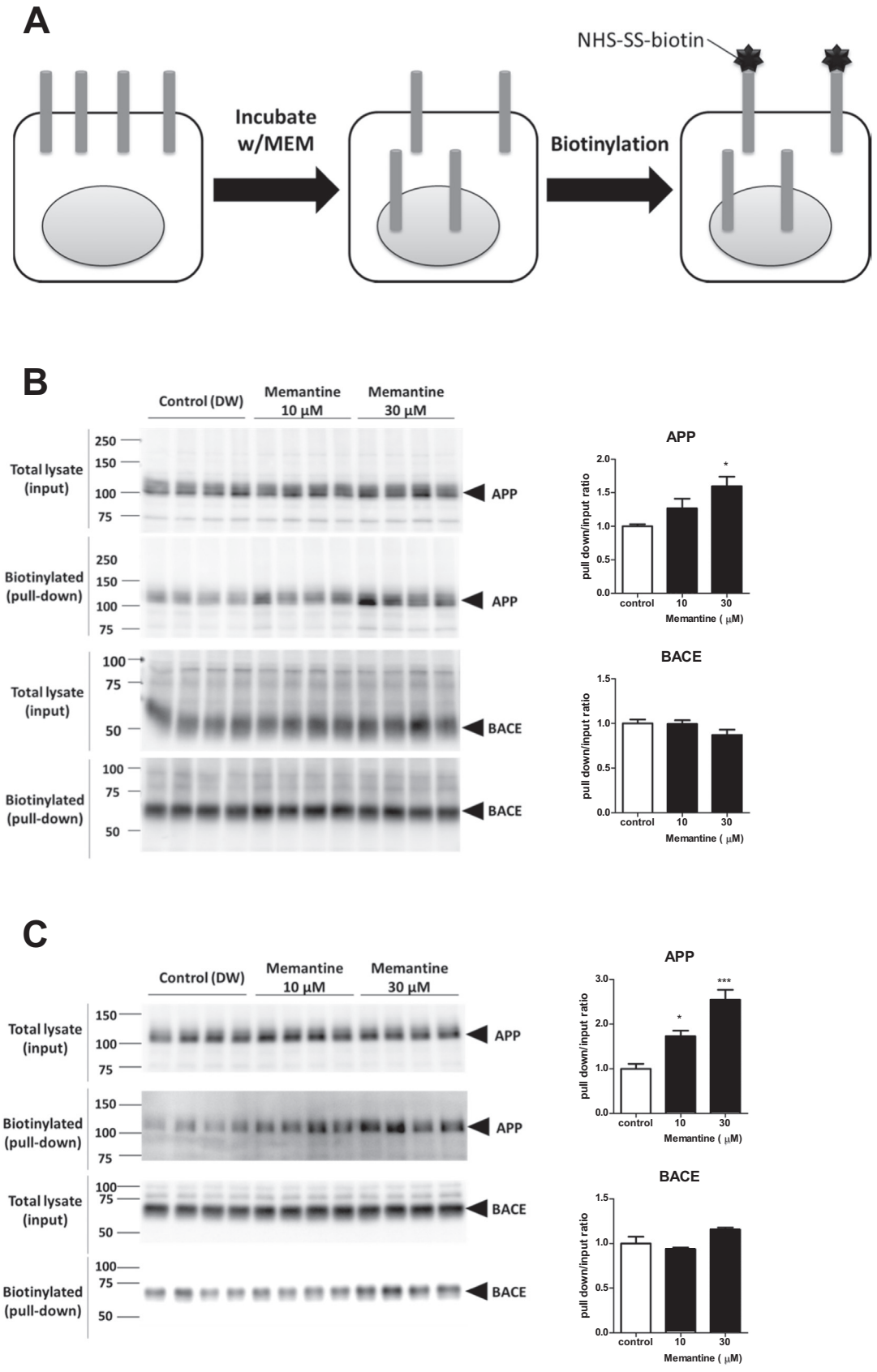

Fig. 6. Effects of memantine on APP and BACE endocytosis. (A) Schematic diagram of the biotinylation assay using NHS-SS-biotin. Immunoblot analysis of cell lysates from rat primary cortical cultures at DIV 8 (B) or DIV 14 (C) using anti-APP and anti-BACE1 antibodies. Graphs show the pull-down/input ratios (n =4, mean ± S.E.M.). * $P < 0.05$, *** $P < 0.001$, compared to the control group by Dunnett's test.

potential as an attractive therapeutic drug for not only disease modification but also symptomatic amelioration in patients with AD.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2017.02.001.

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