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Protective effects of NMDA receptor antagonist, memantine, against senescence of PC12 cells: A possible role of nNOS and combined effects with donepezil

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ABSTRACT

Alzheimer disease (AD) is a neurodegenerative disorder characterized by cognitive dysfunction. The pathology of AD is mainly related to amyloid ß (Aß)-peptides, but glutamate-mediated toxicity is also one of the main processes of memory impairment in AD. Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) and is particularly involved in synaptic plasticity, memory, and learning. Memantine is a low-affinity voltage-dependent noncompetitive antagonist at glutamatergic NMDA receptors. Here, we investigated whether memantine protects against glutamate-induced senescence. In PC12 cells, treatment with glutamate induced senescent phenotypes as judged by the cell appearance and senescence-associated ß-galactosidase (SA-ßgal) in parallel with decreased SIRT1 and increased p53 expression. However, treatment with memantine decreased glutamate-induced senescent PC12 cells and reversed the changes in SIRT1 and p53 expression. Glutamate is known to stimulate the production of NO and O_2^- and has the capacity to generate $ONOO^-$ in the CNS. Therefore, we investigated whether glutamate activates nNOS and memantine reverses it. Treatment with glutamate increased nNOS expression, activity, and production of NO, whereas memantine blocked them. Next, the in vivo effects of memantine on cognitive function in senescence-accelerated mouse prone 8 (SAMP8), as a model of AD, were investigated. In the Morris water maze test, SAMP8 showed a marked decline in performance, but memantine administration improved it. Moreover, neuronal senescence and the level of oxidative stress in the hippocampus were decreased by memantine. Finally, the effects of combination treatment with memantine and donepezil, a cholinesterase inhibitor, were investigated. We observed additive effects of memantine and donepezil on the senescent phenotype of PC12 cells and the hippocampus of SAMP8. These results indicate that inhibition of the NMDA receptor by memantine leads to a decrease in nNOS activity and results in a reduction of glutamate-induced senescence. Thus, our present study suggests a critical role of memantine in the prevention of neuronal aging, and supports that donepezil has a combined effect with memantine.

1. Introduction

Alzheimer disease (AD) is a progressive, neurodegenerative disease characterized by a gradual decline in cognitive function. The etiology of AD is mainly attributable to Aß peptides or tau aggregates, and evidence also exists for both cholinergic and glutamatergic involvement in AD (Ingram et al., 1996). Acetylcholine (Ach), a neurotransmitter essential for processing memory and learning, is decreased in both concentration and function in patients with AD. Similarly, glutamate is the main excitatory neurotransmitter in the CNS and plays a pivotal role in learning and memory. Unlike Ach, glutamate leads to over-activation of N-methyl-D-aspartate (NMDA) receptors and results in neuronal damage. Since over-activation of NMDA receptors increases the amount of intracellular Ca^{2+}, glutamate activates neuronal nitric oxide synthase (nNOS), which produces nitric oxide (NO) and leads to production of reactive oxygen species (ROS; $ONOO^-$), which may trigger neuronal damage (Doucet et al., 2015).

Oxidative stress is known to be closely related to cellular senescence and age-related diseases associated with AD (Tacutu et al., 2011). An increase in oxidative stress has been suggested to be one of the earliest pathological changes in the brain in cognitive impairment due to AD (Mattson, 2004). Cellular senescence of neuronal cells, as well as of

Abbreviations: Aß, amyloid ß; Ach, acetylcholine; AD, Alzheimer disease; CNS, central nervous system; DAF-2, diaminofluorescein-2; DAPI, 4′, 6-diamidino-2-phenylindole; e, i, nNOS, endothelial, inducible, neuronal nitric oxide synthase; HUVEC, human umbilical vein endothelial cells; L-VNIO, N^5-(1-amino-3-butenyl)-L-ornithine; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; SAMP8, senescence-accelerated mouse prone 8; SAMR1, senescence-accelerated mice resistant 1; SA-ßgal, senescence-associated ß-galactosidase; ROS, reactive oxygen species.

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peripheral cells, has been described in AD and causes cellular dysfunction (Naylor et al., 2013).

Memantine (1-amino-3, 5-dimethyladamantane hydrochloride), a noncompetitive NMDA receptor antagonist, was approved about ten years ago for the treatment of moderately severe to severe AD (2002 EU, 2003 USA, since 2011 in Japan). Its effectiveness for cognition has been shown in randomized placebo-controlled trials in patients with AD (Areosa et al., 2005). Since memantine is able to prevent pathogenic Ca^{2+} influx caused by stimulation with glutamate, we considered that the effects of memantine may reduce oxidative damage to neuronal cells and inhibit cellular senescence. In the present study, we showed that glutamate induces senescence of PC12 cells and memantine inhibits nNOS activity, reduces oxidative damage and results in protection against glutamate-induced senescence.

Furthermore, donepezil, a cholinesterase inhibitor, has been used widely in combination with memantine for the treatment of AD patients. Thus, we also examined the combined effects of memantine and donepezil.

2. Materials and methods

2.1. Materials

Glutamate, MK-801, donepezil, and N^{ω}-propyl-L-arginine were purchased from Sigma (St. Louis, MO, USA). Memantine was provided by Daiichi Sankyo Company, Limited (Tokyo, Japan).

2.2. Cells

PC12 cells were purchased from ATCC (Manassas, VA, USA). They were cultured and treated with 2.5S nerve growth factor (NGF, 50 ng/ml, Alomone Labs Ltd., Jerusalem, Israel) to induce differentiation into neuronal cells (Ogawa et al., 1984). Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Walkersville, MD, USA).

2.3. NOS activation assay

NOS activity was determined using an NOS assay kit (Calbiochem) according to the manufacturer's instructions. NO production was observed using fluorescent dye diaminofluorescein-2 (DAF-2) (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Briefly, PC12 cells were loaded with DAF-2 (5 μM for 30 min at 37 °C) and then washed three times with phosphate-buffered saline (PBS). Green fluorescence intensity (DAF-2) was visualized with 4′, 6-diamidino-2-phenylindole (DAPI) (blue) (Dojindo Molecular Technologies, Inc., Tokyo, Japan) for nuclear staining. Fluorescent images were analyzed using a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

2.4. Animal experiments

Animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. Senescence-accelerated mice prone (SAMP) 8 and control senescence-accelerated mice resistant (SAMR) 1 male mice were all housed and maintained in a room at 22 ± 2 °C with automatic light cycles (12 h light/dark) and relative humidity of 40–60%. Mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Food and tap water were provided ad libitum throughout the study. In the water maze test of this study, groups of male SAMR1 (N = 5) and SAMP8 (N = 5) were first tested. Male mice of 12 weeks of age were treated daily for 3 weeks with memantine (10, 20 mg/kg), MK-801 (10 mg/kg), or donepezil (0.3 mg/kg) by subcutaneous injection (s.c) in the neck before the water maze test. Mice were anesthetized, and killed by cervical dislocation. The brain was removed for histological examination, after systemic perfusion with PBS.

2.5. Morris water maze test

The procedure of the Morris water maze test was described previously (Cao et al., 2007). Briefly, SAMR1 (N = 5) and SAMP8 mice (N = 5) were trained to find a visible platform with three trials on the first day, and then tested to find the hidden platform for 10 consecutive days. In each trial, the mice were allowed to swim until they found the hidden platform, or until 2 min had passed, and the mouse was then gently guided to the platform. On the test days, the platform was hidden 1 cm beneath the water. Probe tests were performed on the 10th day. The maze was conceptually divided into I, II, III, and IV, four equal quadrants by four poles along the perimeter of the pool. After the place navigation test was finished on the 10th day, the platform was removed from the water tank. The time was spent in the target quadrant where the platform located was recorded for analysis. Mice were started in a position opposite the location of the platform position and allowed to swim for 60 s. During the test for 10 days, mice were treated daily with memantine (10, 20 mg/kg), MK-801 (10 mg/kg), or donepezil (0.3 mg/kg).

2.6. Open field test

The open field test fear response to novel stimuli was used to assess locomotion, exploratory behavior, and anxiety. Open field test protocols were modified (Lukacs et al., 1995). The open field test consisted of a wooden box (50 × 50 × 50 cm). A 10 cm area near the surrounding wall was delimited and considered the periphery. The rest of the open field was considered the central area. The distance traveled, the ratio of the distance traveled in the central area/total distance traveled, and the time in the center of the open field were analyzed as measures of anxiety-like behavior. During the test, mice were allowed to move freely around the open field and to explore the environment for 15 min.

2.7. Senescence-Associated ß-Galactosidase (SA-ßgal) Staining

PC12 cells and HUVEC were grown in 60-mm collagen-coated dishes to 80% confluence. PC12 cells were pretreated with vehicle (0.05% DMSO), memantine (100 μM), MK-801 (100 μM), or N-propyl-L-arginine (100 μM) diluted in RPMI 1640 medium for 1 day. HUVEC were pretreated with vehicle (0.05% DMSO) or memantine (100, 200 μM) diluted in EGM-2 medium for 1 day. PC12 cells and HUVEC were washed three times with the medium and then treated for 10 h with 10 mM glutamate diluted in medium. After treatment, PC12 cells were cultured with medium containing these compounds for 10 days. At 10 days after the start of treatment with glutamate, PC12 cells and HUVEC were fixed, and the proportion of SA-ßgal-positive cells was determined as described (Dimri et al., 1995).

2.8. Antibodies and immunoblotting

Cells were lysed on ice for 1 h in buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 10 mM sodium fluoride). After blocking, the filters were incubated with the following antibodies: anti-nNOS (BD Biosciences, San Jose, CA, USA), anti-p53, anti-SIRT1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-NMDAR 2A, 2B (Abcam PLC, MA, USA), and anti-ß-actin (Sigma-Aldrich). After washing and incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for 1 h, the antigen-antibody complexes were visualized using an enhanced chemiluminescence system (GE Healthcare Life Sciences).

2.9. Measurement of Ach

The concentration of acetylcholine was measured with a choline/Ach quantification kit (BioVision, CA, USA) according to the manufacturer's instructions.

2.10. Detection for carbonylation of proteins

Carbonylation of proteins was detected using an Oxyblot protein oxidation detection kit (Millipore, MA, USA) according to the manufacturer's instructions.

2.11. Cell viability assays

Cell viability with glutamate (0–20 mM) for 5 h was assessed by using the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2 H-tetrazolium (MTS) assay (Promega, Madison, WI) in 96-well plates (20,000 cells/well) following the instructions of the manufacturer.

2.12. Data analysis

Values are shown as mean ± S.E.M in the text and figures. Differences between the groups were analyzed using one-way analysis of variance, followed by the Bonferroni test. Probability values less than 0.05 were considered significant.

3. Results

3.1. Glutamate-induced senescence of PC12 cells and memantine inhibited it

To investigate whether glutamate treatment induced cellular senescence, PC12 cells with neuronal differentiation induced by NGF were used. When PC12 cells were treated with 10 mM glutamate for 10 h, a large and flattened senescent appearance was observed and the number of SA-ßgal-positive cells was increased after 10 days of treatment (Fig. 1A and B). These results indicate that glutamate has the capacity to induce the senescence of PC12 cells. Next, when 200 μM memantine pretreatment for 1 day was performed before glutamate induction of senescent PC12 cells, the senescent appearance and the number of SA-ßgal-positive cells were decreased (Fig. 1A and B). To verify the changes in other proteins related to senescent phenotypes, SIRT1 and p53 expression were examined. Treatment with glutamate decreased SIRT1 expression and increased p53 expression; however, pretreatment with 100 or 200 μM memantine prevented these changes (Fig. 1C). These results indicate that pretreatment with memantine inhibited glutamate-induced the senescence of PC12 cells.

Memantine is a noncompetitive NMDA receptor antagonist. Therefore, to clarify the protective effect of memantine against cellular senescence via the NMDA receptor, PC12 cells were treated with another potent, selective noncompetitive NMDA receptor antagonist, MK801 (Benveniste et al., 1984). Similarly to memantine, pretreatment with 100 μM MK801 decreased the number of SA-ßgal-positive cells (Fig. 1D). These results indicate that memantine inhibited the senescence of PC12 cells through the NMDA receptor.

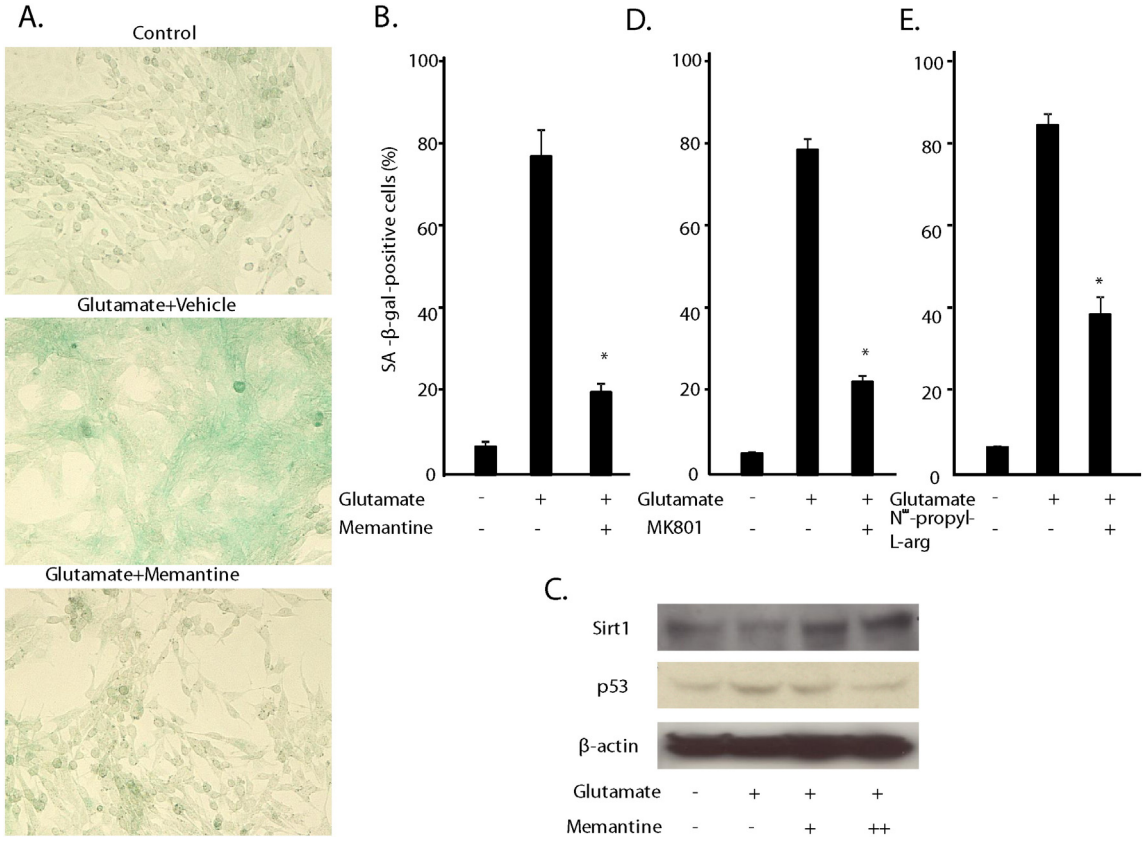

Fig. 1. A. Memantine (200 μM) inhibited SA-βgal activity and senescent morphological appearance induced by glutamate (10 mM). Percentage (%) of SA-βgal-positive PC12 cells with treatment with memantine (100 μM) (B), MK801 (100 μM) (D), or N^{ω}-propyl-L-arginine (100 μM) (E). ($^{*}p < 0.05$, N = 3). C. Expression of SIRT1 and p53 in glutamate (10 mM)-treated PC12 cells under treatment with memantine (+: 100 μM, ++: 200 μM). (N = 3, representative shown).

3.2. Glutamate increased production of NO and memantine decreased it

Glutamate leads to over-activation of NMDA receptors, and the excessive influx of Ca^{2+} into neuronal cells changes nNOS activity. Treatment with 10–40 mM glutamate for 10 h increased nNOS expression in a dose-dependent manner (Fig. 2A). Production of NO was also increased by treatment with 10 mM glutamate in the culture medium. However, pretreatment with 100 μM memantine, 100 μM MK801, or 100 μM N^{ω}-propyl-L-arginine, a potent selective inhibitor of nNOS, inhibited the production of NO (Fig. 2B, C, and D). To investigate whether memantine decreased the content of intracellular NO through nNOS, DAF-2 was used. Pretreatment with 100 or 200 μM memantine decreased the number of DAF-2-stained PC12 cells compared with glutamate-treated cells for 10 h (Fig. 3A and B). Furthermore, the number of SA-ßgal-positive PC12 cells was decreased by pretreatment with 100 μM N^{ω}-propyl-L-arginine (Fig. 1E). These results indicate that memantine inhibits the activation of nNOS and decreases NO induced by glutamate, leading to a reduction of senescence.

3.3. Memantine treatment improved cognitive decline in SAMP8

In order to assess the effects of memantine on cognitive function, we used an in vivo model of AD, SAMP8, and a control counterpart strain, SAMR1. SAMP8 was originally derived from the AKR/J strain, which is characterized by cognitive decline. SAMP8 showed a marked age-related deterioration of memory and learning as early as 2 months of age compared to SAMR1 (Yagi et al., 1988; Flood and Morley, 1998; Miyamoto et al., 1992). These mice exhibit age-related deficits in learning and memory at an early age, and are considered a suitable animal model to study the pathology of AD. By determining the time required to find the platform (escape latency) as a function of the number of days of training in the Morris water maze, we observed a marked decline in performance in SAMP8 (N = 5) compared with that in SAMR1 (N = 5) (Fig. 4A). SAMP8 treated with 20 mg/kg memantine for 3 weeks showed significantly reduced escape latency time compared with that in untreated SAMP8 on training day 10 (Fig. 4A). However, SAMP8 treated with 10 mg/kg MK801 did not show a change in escape latency time compared with untreated SAMP8 (Fig. 4A). The spatial probe trial helps to determine whether the mice would use a spatial learning strategy to locate the platform in the target quadrant. There was no difference in swim speed between the groups (Fig. 4B); the time was spent in the target quadrant where the platform was located was greatly reduced in SAMP8 compared to SAMR1 on day 10 (Fig. 4C). Memantine-treated SAMP8 showed a markedly longer time searching for the platform in the target quadrant (Fig. 4C). These results indicate that memantine, but not MK801, ameliorated spatial learning in SAMP8. The water maze is appropriate for hippocampus-dependent paradigms. However, memantine and MK801 administration may affect behavior and how animals respond to different stimuli. Therefore, we performed an open field test to examine locomotion, exploratory behavior, and anxiety. No significant effect of memantine (20 mg/kg) and MK801 (10 mg/kg) on locomotor performance was observed in SAMR1 and SAMP8, whereas SAMR1 showed significantly more movement compared with SAMP8 (Fig. 4D). The ratio of the distance traveled in the central area to that in the total area in the open field, an indirect measure of exploratory behavior and anxiety, was also determined. In SAMP8, memantine (20 mg/kg) and MK801 (10 mg/kg) increased this ratio (Fig. 4E), suggesting that both memantine and MK801 promoted exploratory behavior and diminished anxiety.

3.4. Memantine treatment inhibits senescence in hippocampus of SAMP8

Next, we assessed the number of SA-ßgal-positive cells in the CA1 and CA3 areas of the hippocampus in these mice. The number of SA-βgal-stained cells was significantly increased in SAMP8 compared with SAMR1, but treatment with memantine (10, 20 mg/kg) prevented this change in SAMP8 (Fig. 5A and B).

Glutamate stimulation increases the production of NO and O_2^- and has the capacity to generate $ONOO^-$ in the CNS (Eliasson et al., 1999). Therefore, we investigated the level of oxidative stress, using the SAMR1 and SAMP8 hippocampus at 12 weeks of age. SAMP8 hippocampus showed an increase in the level of oxidative stress compared with SAMR1 as judged by detection of carbonylated proteins. Memantine treatment (10 or 20 mg/kg) decreased carbonylated proteins in the

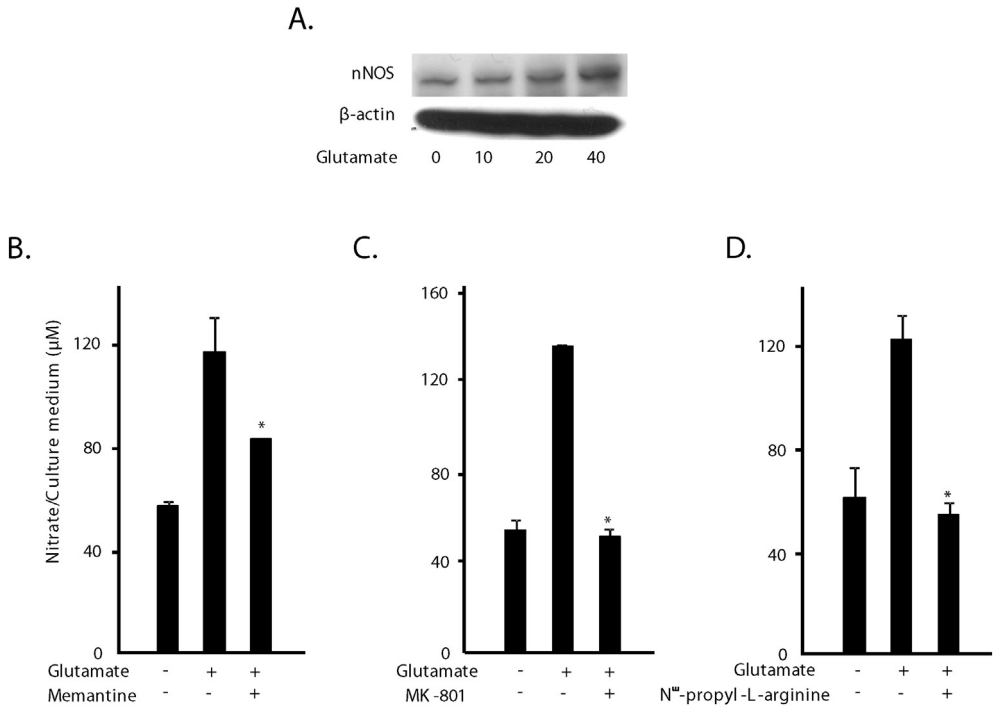

Fig. 2. A. Expression of nNOS in glutamate (10–40 mM)-treated PC12 cells. (N = 3, representative shown). Production of nitrate in PC12 cells treated with memantine (100 μM) (B), MK801 (100 μM) (C), or N^{ω}-propyl-L-arginine (100 μM) (D). ($^*p < 0.05$, N = 3).

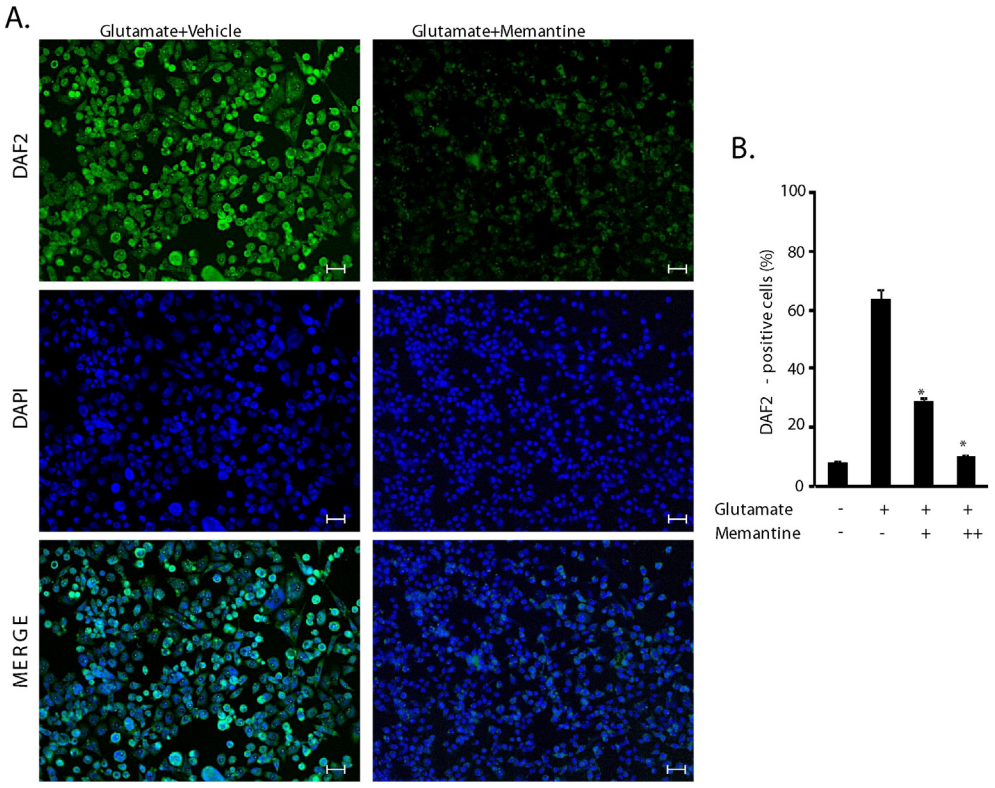

Fig. 3. A. NO production detected using DAF-2 (green) in glutamate (10 mM)-treated PC12 cells under treatment with or without memantine (200 μM). DAPI (blue) staining for nuclei. B. Number of DAF-2-stained PC12 cells treated with memantine (+: 100 μM, ++: 200 μM). ($^{*}p < 0.05$, N = 3).

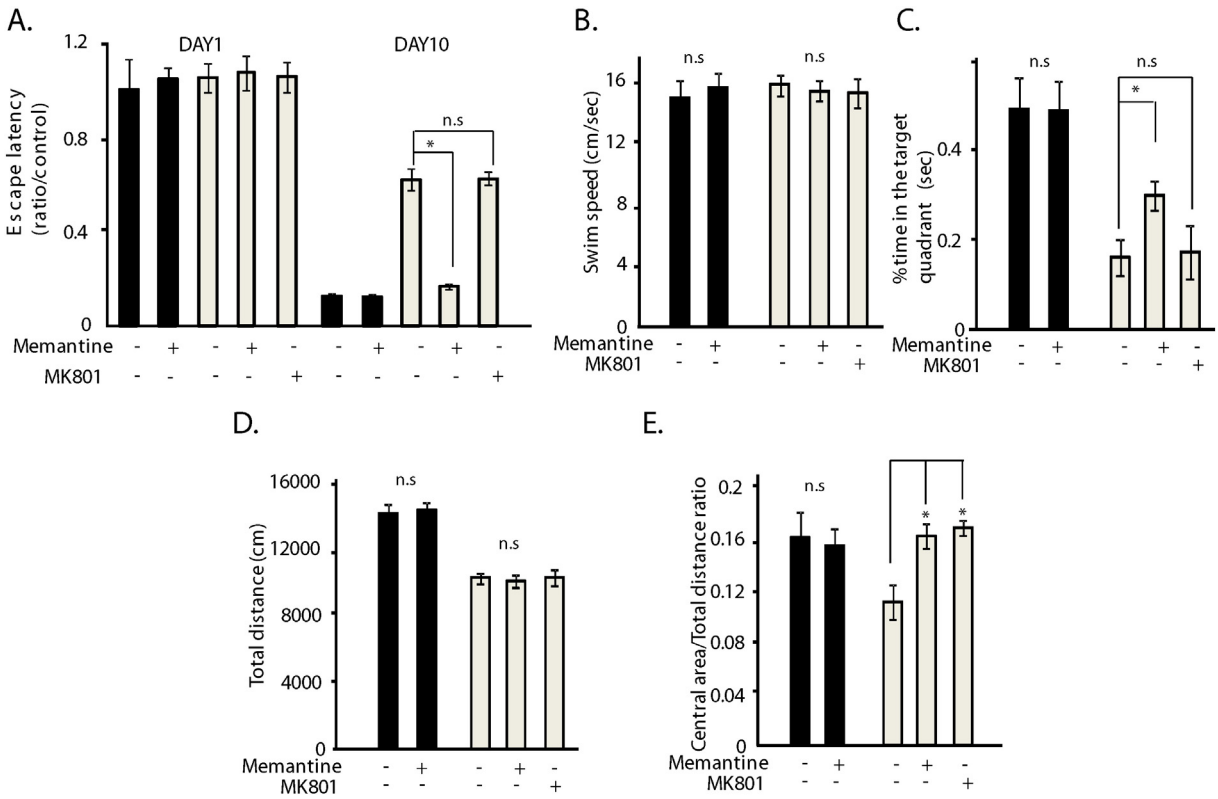

Fig. 4. A. Escape latency ratio on day 1 and 10 in SAMR1 (black bar) and SAMP8 (gray bar) mice (N = 5). Male mice were treated daily for 3 weeks with memantine (20 mg/kg s.c) or MK801 (10 mg/kg s.c) before trials. B. Swim speed during quadrant test on day 10. C. % time in target quadrant in memantine (20 mg/kg s.c)- and MK801 (10 mg/kg s.c)- treated mice on day 10. Total distance (D) and ratio of central/total distance (E) were measured in open field tests. ($^{*}p < 0.05$, n.s: not significant).

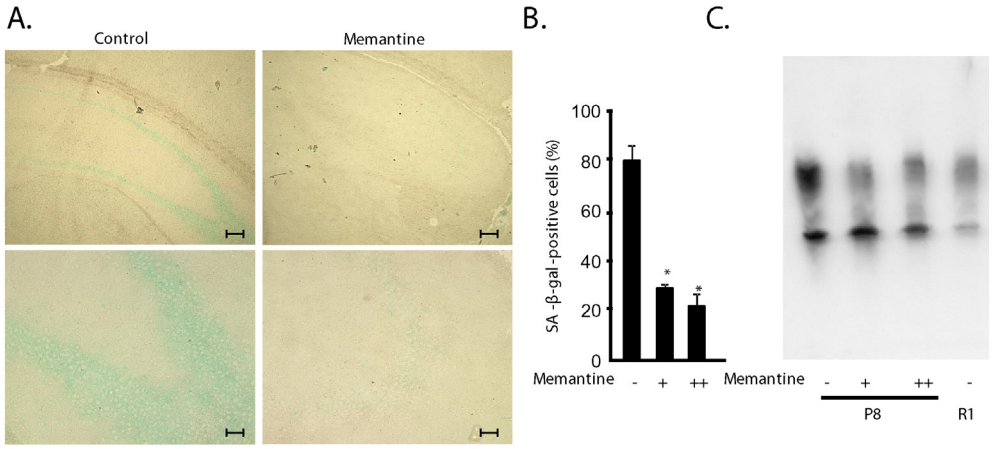

Fig. 5. A. B. SA-βgal-stained cells in CA1 and CA3 areas of hippocampus in SAMP8 with or without memantine (+: 10 mg/kg, ++: 20 mg/kg) treatment. (*$p < 0.05$, N = 3) (size scale 50 μm). C. Oxidative stress level was measured by detection of carbonyl groups introduced into proteins (R1: SAMR1, P8: SAMP8).

SAMP8 hippocampus (Fig. 5C). These results indicate that memantine inhibits senescence in the hippocampus of SAMP8 and suggest that reduction of oxidative stress involving NO may play an important role in the protective effect of memantine against senescence of the hippocampus.

3.5. Memantine and donepezil showed combined protective effect against senescent phenotype

Recently, the usefulness of combination therapy with memantine and donepezil has been investigated in many clinical studies. Because memantine and donepezil have different mechanisms of action, donepezil has been used together with memantine in many patients with AD. Therefore, we investigated the combined effect of memantine and donepezil. Combination treatment with memantine (50 μM) and donepezil (1 μM) significantly decreased the number of SA-ßgal-positive PC12 cells compared with only memantine or donepezil treatment (Fig. 6A). Moreover, SAMP8 treated with the combination showed significantly reduced escape latency at earlier days (Fig. 6B), and the time spent in the target quadrant was increased compared with that in memantine (10 mg/kg)- or donepezil (0.3 mg/kg)-treated SAMP8

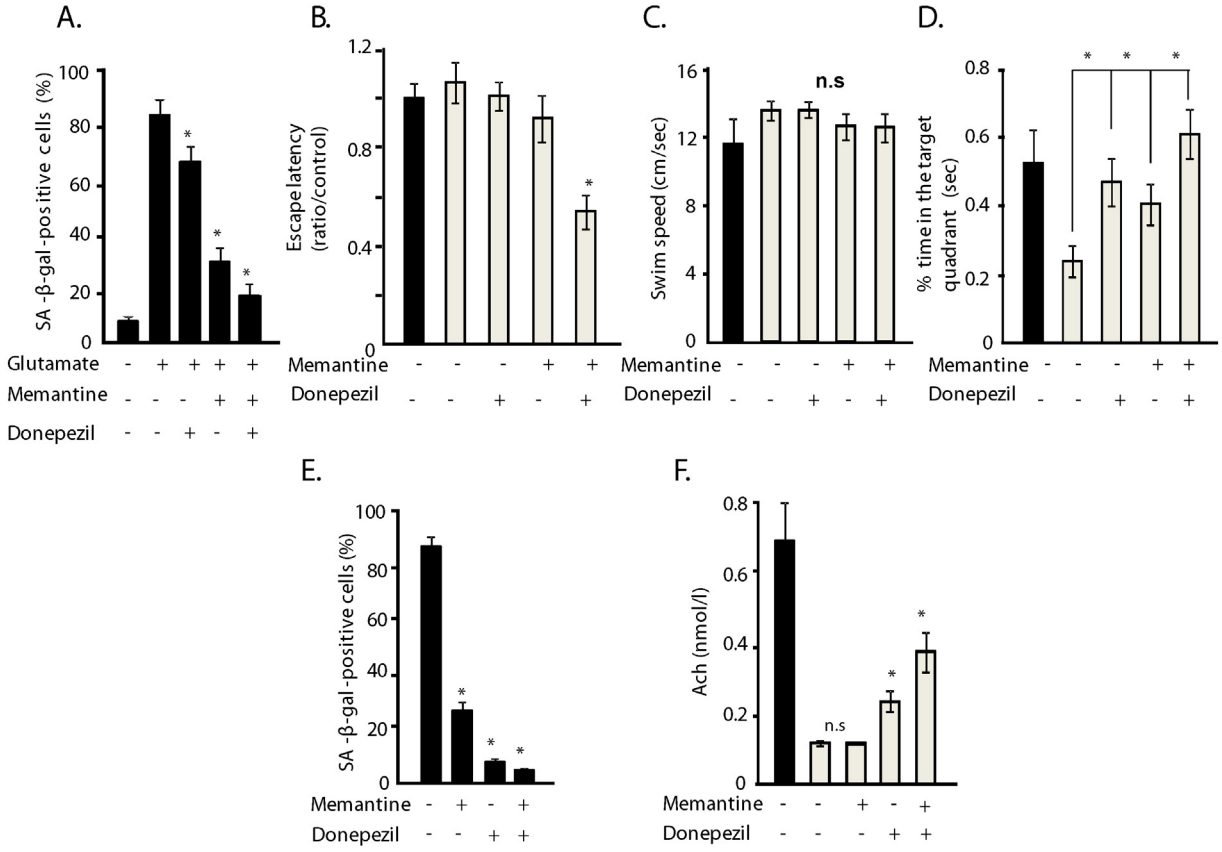

Fig. 6. A. A combination of memantine (50 μM) and donepezil (1 μM) more markedly reduced SA-βgal activity of PC12 cells compared with memantine or donepezil alone (*$p < 0.05$, N = 3). B. Escape latency ratio on day 1 in SAMR1 (black bar) (N = 5) and SAMP8 (gray bar) mice (N = 5) treated with donepezil (0.3 mg/kg), memantine (10 mg/kg), or donepezil + memantine. C. Swim speed during quadrant test on day 10. D. % time in target quadrant in donepezil, memantine, and donepezil + memantine-treated mice. E. Number of SA-βgal-stained cells in CA1 and CA3 areas of hippocampus (*$p < 0.05$, N = 3). F. Acetylcholine concentration was measured by a colorimetric method (*$p < 0.05$, N = 3).

(Fig. 6D). There was no difference in swim speed between these groups (Fig. 6C). The number of SA-βgal-stained cells in the CA1 and CA3 areas of the hippocampus was also significantly decreased in combination-treated compared with memantine- or donepezil-treated SAMP8 (Fig. 6E). Finally, the concentration of Ach in hippocampal lysates was increased in SAMP8 treated with donepezil but not memantine, but it was further increased by memantine in combination with donepezil (Fig. 6F).

4. Discussion

In this study, we showed that glutamate induced the senescence of PC12 cells and treatment with memantine inhibited it in accordance with restoration of the expression of SIRT1 and p53. Furthermore, memantine treatment reduced hippocampal senescence and led to improved spatial memory in SAMP8.

The Morris water maze results are often confounded by the fact that it is a potent anxiety/stress test (Morley et al., 2012). Therefore, there is a possibility that SAMP8 could show less anxiety than SAMR1, and the alteration in protein carbonyls could be due to stress.

Axonopathy observed in AD is considered to be the result of accumulation of Aß-peptide in glutamatergic synaptosomes, which leads to excessive release of glutamate with consequently more axonal degeneration in neurons through NMDA receptors (glutamate hypothesis) (Harrison, 1986). Indeed, recent findings indicate that increased levels of Aß-peptide and glutamate have a detrimental impact on neurons via interactions with NMDA receptors (Hiruma et al., 2003; Miguel-Hidalgo et al., 2002; Rammes et al., 2008), resulting in large Ca^{2+} entry into neurons and subsequent axonal and neuronal damage (Parsons et al., 2007). NMDA receptors are glutamate-gated Ca^{2+} channels that play pivotal roles in fundamental aspects of neuronal function. Glutamate treatment caused intracellular Ca^{2+} concentration to remain elevated up to 1 h in vitro (Dubinsky, 1993). Glutamate causes failure of the Ca^{2+} homeostasis early, and the degeneration primarily involves the activation of catabolic enzyme such as endonucleases, endopeptidases, and phospholipases which lead to production of ROS and $ONOO^-$ (Choi, 1992; Lazarewicz et al., 1990; Chan and Fishman, 1978). Therefore, we examined the cell viability in the course in early time. We found that when treated with 10 mM glutamate for 5 h, cell viability was not altered (Supplementary Fig. 1A). In accordance with this, it was reported that glutamate levels between 1 μM and 10 mM did not affect cell viability over a 24 h period in brain endothelial cells (Scott et al., 2007). In this study, we decided to use a glutamate concentration of 10 mM mainly because at this concentration it was easy to detect senescence phenotype at 10 days. However, glutamate, at mM concentrations, is known to exert toxic effect on CNS-derived preparation, including endothelial cells (Sharp et al., 2003; Parfenova et al., 2006). Because senescent cells were not detectable at an early time (24 h) and were detected at least 72 h after the start of treatment with glutamate, accumulation of the oxidative stress may occur, causing in cell damage, and it is thought that it gradually results in cellular senescence.

Moreover, it has been well documented that glutamate induces nNOS over-activation, secondary to elevation of extracellular glutamate, and leads to neuronal damage (Darra et al., 2009). Therefore, glutamate shows excitotoxicity at an early time, but treatment with memantine antagonized the NMDA receptor and resulted in attenuation of the senescence of PC12 cells via inhibition of nNOS activation. N^5-(1-amino-3-butenyl)-L-ornithine (L-VNIO), a selective nNOS inhibitor, was applied to examine the involvement of NOS in native testosterone-deficient SAMP8 with supplementation. L-VNIO did not show any effect on cognitive function (Ota et al., 2012). Moreover, we investigated cognitive function in aged endothelial NOS (eNOS) KO mice (96 weeks old). They did not show a cognitive decline compared with wild mice of the same age (data not shown). Unlike our in vitro experiments, these results suggest the possibility of no participation of NOS in vivo. However, it was reported that NOS inhibitors of nNOS, inducible NOS (iNOS), and eNOS had favorable effects on cognitive function in several AD mouse models (Maher et al., 2014; Santhanam et al., 2015). Given the differences observed in vivo and in vitro, because nNOS expression is naturally downregulated in the hippocampus of SAMP8 (Han et al., 2010), it could be difficult to evaluate the effect of nNOS as shown in vitro. Further studies are needed to clarify the participation of NOS.

In this study, similarly to memantine, treatment with MK-801 attenuated NO production and inhibited the senescence of PC12 cells. However, administration of MK-801 to SAMP8 did not alleviate learning deficit. Memantine has moderate affinity for NMDA receptors, with rapid blocking/unblocking kinetics and strong voltage dependency. In contrast, MK-801 has strong affinity for NMDA receptors and inhibits the induction of long-term potentiation at a concentration blocking NMDA receptors and worsens the impairment of spatial memory (Zajaczkowski et al., 1996). As described (Olney et al., 1989), noncompetitive NMDAR antagonists may by themselves induce excitotoxicity through inducing receptor hypoactivity. That is, blocking NMDA receptors on GABAergic/other inhibitory neurons leads to hypoactivity, resulting in overactivation/excitotoxicity of neurons downstream of these inhibitory neurons.

In another study, treatment of rats with memantine at the same concentrations as those used in this study (10, 20 mg/kg/day) showed serum levels of 52–64 ng/ml and 150–199 ng/ml respectively (Nakamura et al., 2006). These serum concentrations of memantine are similar to the levels seen in the serum of dementia patients treated with memantine, and the concentration of memantine in mice in our study may have been higher, assuring inhibition of the NMDA receptor based on in vitro data.

Memantine has been approved for the treatment of moderate to severe AD in Europe since 2002, in USA since 2003, and in Japan since 2011 (Allgaier and Allgaier, 2014), and has been shown in clinical trials to be a safe and effective treatment for vascular dementia (Orgogozo et al., 2002). Because memantine showed a protective effect on PC12 cells, we then examined its effect on vascular endothelial cells. Although the effect was weaker than that on neuronal cells, memantine inhibited glutamine-induced endothelial senescence (Supplementary Fig. 1B), and NMDA receptors were found in endothelial cells (Supplementary Fig. 1C). These results suggest that memantine may have a protective effect on not only neuronal function, but also vascular function.

In this study, memantine and donepezil showed an additive protective effect against neuronal senescence and improved cognitive function at an earlier time compared with monotherapy. Clinically, the combination of memantine and donepezil has demonstrated efficacy for treating the symptoms of AD (Gareri et al., 2014). Consistent with our results, several preclinical studies also have indicated cognition-enhancing effects of memantine and donepezil with repeated administration in other mouse models of AD (Nagakura et al., 2013). Because memantine and donepezil have different and complementary mechanisms of action, together they potentially offer additional benefits in relation to the etiology of AD. The detailed mechanism of the combined effect needs further study.

Memantine combined with environmental enrichment improves spatial memory in SAMP8 (Dong et al., 2012). Moreover, it has been recently reported that memantine can prevent Aß-peptide production (Miguel-Hidalgo et al., 2002). Although other mechanism may exist, the findings of the present study indicate that neuronal senescence may constitute an important target for AD and memantine may prevent it. Our results appear to support arguments justifying the presence of a “glutamate-associated excitotoxic insult” as a mechanism of the neurodegenerative changes observed in AD. We believe that the application of memantine as anti-excitotoxic treatment chronically may act as a defense against brain/neuronal aging and senescence causing AD.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2015.09.016.

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