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Novel Neuroprotective Mechanisms of Memantine: Increase in Neurotrophic Factor Release from Astroglia and Anti-Inflammation by Preventing Microglial Over-Activation

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Abstract

Memantine provides clinically relevant efficacy in patients with Alzheimer's disease and Parkinson's diseases. In addition to blockade of N-methyl-D-aspartate receptor on neurons, memantine has neurotrophic and neuroprotective effects in *in vivo* and *in vitro* studies, however, the mechanism underlying these effects remains unclear. To address this question, primary midbrain neuron-glia cultures and reconstituted cultures were used, and lipopolysaccharide (LPS), an endotoxin from bacteria, was used to produce inflammation-mediated dopaminegic neuronal death. Here, we show that memantine exerted both potent neurotrophic and neuroprotective effects on dopaminergic neurons in rat neuron-glia cultures. The neurotrophic effect of memantine was glia-dependent, since memantine failed to show any positive effect on dopaminergic neurons in neuron-enriched cultures. More specifically, it appears that astroglia, not microglia, are the source of the memantine-elicited neurotrophic effects through the increased production of GDNF. Mechanistic studies revealed that GDNF upregulaton was associated with histone hyperacetylation by inhibiting the cellular histone deacetylase activity. In addition, memantine also displays neuroprotective effects against LPS-induced dopaminergic neuronal damage through its inhibition

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of microglia over-activation revealed by both OX-42 immunostaining and by the reduction of pro-inflammatory factors production such as extracelluar superoxide anion, intracellular reactive oxygen species, nitric oxide, prostaglandin E_2, and tumor necrosis factor-α. These results suggest that memantine therapy for neurodegenerative diseases acts in part through alternative novel mechanisms by reducing microglia-associated inflammation and stimulating the release of neurotrophic factors from astroglia.

Keywords

GDNF; neuroinflammation; neuroprotection; neurodegenerative disease; HDAC

INTRODUCTION

Memantine has been extensively investigated in recent years mainly because of its clinical efficacy in Alzheimer's disease (AD) and neuroprotective effects in laboratory studies. *In vitro* studies using neuronal cell cultures demonstrated that neuronal damage induced by glutamate (Weller *et al*, 1993b), N-methyl-D-aspartate (NMDA) (Weller *et al*, 1993a), prion protein (Muller *et al*, 1993), and gp120 (Nath *et al*, 2000; Ushijima *et al*, 1993) were inhibited by memantine. In addition, *in vivo* studies show a protective effect of memantine against ischemiareperfusion injury in spontaneously hypertensive rats (Dogan *et al*, 1999). Recently, several clinical trials also revealed the beneficial effects of memantine on the treatment of AD (Reisberg *et al*, 2003), vascular dementia (Wilcock *et al*, 2002), and Parkinson's disease (PD) (Merello *et al*, 1999). Since it is known that memantine is a NMDA receptor blocker, most of the reports attributed neuroprotective effects or clinical therapeutic benefits of memantine to its inhibitory effects on NMDA-receptor mediated excitotoxicity (Lipton, 2006).

Recent advances in research of the central nervous system (CNS) strongly suggest that glia (astroglia and microglia) are key players in neurodegenerative diseases and prime targets for therapy (Block *et al*, 2007; Ralay Ranaivo *et al*, 2006). The differential roles of astroglia and microglia in neuron survival/degeneration have been reported in our laboratory and others (Block and Hong, 2005; Liu and Hong, 2003b; Teismann *et al*, 2003). On one hand, astroglia have been shown to be a major source of a variety of neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), which are beneficial for the survival of neurons (Chen *et al*, 2006b; Lin *et al*, 1993) Chen et al, 2006). On the other hand, microglia mediate an inflammatory response, which is a critical component underlying the pathogenesis of a diverse range of neurodegenerative diseases, such as AD and PD (Rogers *et al*, 2007; Wyss-Coray, 2006). It has 6 been reported that many drugs exert neuroprotective effects by modulating functions of glial cells. Some of them are neuroprotectvie through their anti-inflammatory effects by reducing the release of pro-inflammatory factors from over-activated microglia (Liu *et al*, 2002; Ralay Ranaivo *et al*, 2006), while others enhance the production of neurotrophic factors from astroglia (Chen *et al*, 2006b; Darlington, 2005).

Current mechanistic studies of memantine focus mainly on its effect(s) on neurons (Lipton, 2007; Zhao *et al*, 2006), in contrast, its possible effects on other cell types in the CNS have not been investigated. Given the crucial role of glia in the pathogenesis of neurodegenerative diseases, it is important to examine these roles in the neuroprotective effect of memantine. A recent report from Wenk and his colleagues proposed a possible role of memantine on microglial activation (Rosi *et al*, 2006). Here, using a series of midbrain primary cultures, we report that memantine showed potent efficacy in protecting dopaminergic (DA) neurons against LPS-induced damage. Mechanistic studies revealed two novel mechanisms underling the neuroprotective effects of memantine: 1) increase the release of GDNF from

astroglia through histone hyperacetylation on *gdnf* promoter region by inhibiting activity of the cellular histone deacetylase (HDAC), and 2) anti-inflammatory action by inhibiting the over-activation of microglia, which is independent of NMDA receptors.

MATERIALS and METHODS

Animals

Timed-pregnant adult female Fisher 344 rats were purchased from Charles River Laboratories (Raleigh, NC, USA). Experimental use of the animals was performed in strict accordance with the National Institutes of Health guidelines.

Mesencephalic neuron-glia cultures

Rat primary mesencephalic neuron-glia cultures were prepared using a previously described protocol (Liu and Hong, 2003a). Briefly, ventral mesencephalic tissues were dissected from day 14 embryos. Cells were dissociated by gentle mechanical trituration and immediately seeded at 5×10^5/well in 24-well plates pre-coated with poly D-lysine (20 μg/ml). Plates were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cultures were treated 7 days after plating. At the time of treatment, the composition of the cultures was approximately 48% astroglia, 12% microglia, 40% neurons, of which about 2% of cells represent tyrosine hydroxylase (TH)-immunoreactive neurons, based on immunocytochemical (ICC) analysis.

Neuron-Enriched Cultures and Microglia-Depleted Cultures

Forty-eight hours after seeding, cytosine β-D-arabinofuranoside (10 μM) and l-leucine methyl ester (LME) (1mM) were added to the mesencephalic neuron-glia cultures cultures. Cytosine β-D-arabinofuranoside and LME were removed 48 h later and replaced with fresh media. Neuronenriched cultures were 98% pure and microglia-depleted cultures were 95% pure, as indicated by ICC staining with OX-42 and GFAP antibodies.

Microglia-Enriched Cultures

Primary enriched-microglia cultures were prepared from the whole brains of 1-day-old Fisher 344 rat pups, using a previously described protocol (Liu *et al*, 2003a). Two weeks after seeding, 8 microglia were shaken off and either replated at 5×10^4 in a 96-well plate for superoxide and Intracellular reactive oxygen species (iROS) assays, or reseeded on top of a neuron-enriched culture in a 24-well plate at 7.5×10^4/well for a neuron-microglia co-culture.

Astroglia-Enriched Cultures

Primary enriched astroglia cultures, with a purity of 98%, were prepared, using the previously described protocol (Liu *et al*, 2003a). Astroglia-enriched cultures were treated with memantine 24 h after seeding. The conditioned medium was aspirated from the cells, centrifuged, filtered through 0.22-μm-pore-diameter Millipore filters, and then dialyzed overnight using the Slide-A-Lyzer Dialysis Cassette (Pierce Biotechnology Inc., Rockford, IL) to remove memantine. Conditioned media were stored at -80°C until use.

[^{3}H] DA Uptake Assay

The [^{3}H] DA uptake assay was performed as described previously (Liu *et al*, 2002). Radioactivity was determined by liquid scintillation counting with a Beckman Tri-carb 2900 TR liquid scintillation counter (Fullerton, CA). The nonspecific DA uptake observed in the presence of mazindol (10 μM) was subtracted.

Immunostaining

DA neurons were recognized with the polyclonal antibody against TH (kindly gift from Dr. John Reinhard of GlaxoSmithKline, Research Triangle Park, NC), and microglia were detected with the OX-42 antibody (PharMingen, San Diego, CA) against CR3 receptor, using previously described protocol (Liu *et al*, 2003a). For visual counting of TH-positive neurons, nine representative areas per well of the 24-well plate were counted under the microscope at 100 magnifications. Counting was performed in a double-blind manner by two individuals, and conclusions were drawn only when the difference was within 5%.

Superoxide Assay

Extracellular superoxide production from microglia was determined by measuring the superoxide dismutase inhibitable reduction of tetrazolium salt as previously described (Qin *et al*, 2002).

Intracellular ROS (iROS) Assay

The production of iROS was measured by DCFH oxidation as previously described (Qin *et al*, 2002).

TNF-α and PGE_2 Assays

The production of TNF-α and PGE_2 was measured with a commercial ELISA kit from R&D Systems (Minneapolis, MN), and from Cayman Chemical Company (Ann Arbor, MI), respectively.

Nitrite Assay

As an indicator of nitric oxide production, the amount of nitrite accumulated in culture supernatant was determined with a colorimetric assay using Griess reagent [1% sulfanilamide, 2.5% H_3PO_4, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride] as previously described (Qin *et al*, 2002).

HDAC Activity Assay

HDAC activity was measured by using a fluorescence activity assay kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions. Assays were performed in 96-well plates in a final volume of 210 μl. Various concentrations of memantine (1 to 1000 μM) were incubated in the presence of 3 μg of HeLa nuclear extract HDACs in the 96-well plate. In this assay, valproic acid (VPA) (0.5 and 1 mM), being a well-known HDAC inhibitor, was included for comparison purpose. The reaction was initiated by adding acetylated substrate (100 μM) to all the wells. Plates were incubated on a shaker for 30 min at 37°C. After adding the developer incubating for 15 min at room temperature, The intensity of fluorescence was determined with the Spectra Max Plus microplate spectrophotometer (Molecular Devices), with an excitation wavelength of 340–360 nm and an emission wavelength of 440–465 nm.

HDAC Activity Assay of Astroglial Lysate

Primary astroglia cultures were treated with memanitne (1 to 1000 μM) and valproic acid (1 mM) for various time points, then total lysate was prepared following the previously described method (Wei et al, 2004). Ten μg of astroglial total lysates were used for the assay of HDAC activity.

RT-PCR Analysis

Total RNA was extracted from rat primary astroglia-enriched cultures according to the acidphenol method followed by isopropanol precipitation (Wei *et al*, 2004). The forward (F) and reverse (R) primers for PCR reaction were used below: rat GDNF gene (F), 5'-CACCATGAA GTTATGGGATGTCGTGGCT-3' and (R), 5'-TCAGATACATCCACACCGTTTAG CGGA-3'; rat β-actin gene (F), TTGTAACCAACTGGGACGATATGG and (R), GAT CTTGATCTTCAT GGTGCTAGG. The protocol for touchdown PCR was as follows: denaturation (95°C, 15 sec), annealing (60°C, 30 sec) to (50°C, 30 sec), and extension (72°C, 2 min) for 20 cycles and denaturation (95°C, 15 sec), annealing (50°C, 30 sec), extension (72°C, 2 min) for 10 cycles. Reaction was carried out in a PerkinElmer 9700 thermal cycler (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA), and PCR products were analyzed using 2% agarose gels.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed on the basis of a protocol from Upstate Biotechnology ChIP kit (Upstate Biotechnology, Lake Placid, NY) with slight modifications. Briefly, astrocytes treated for indicated times were crosslinked with 1% (v/v) formaldehyde in culture medium at room temperature for 10 min. Cells were then washed, lysed, and sonicated to shear chromatin to about 200–1000 bp in length for further application to ChIP with anti-acetyl histone H4 antibodies or nonimmune rabbit IgG followed by RT-PCR as previously described (Wei *et al*, 2004). Primers were selected upstream of respective initiator site of gene trascription (Caumont *et al*, 2006b) to amplify from mononucleosomal DNA. Three primers were designed to amplify sequences proximal to the GDNF promoter region (GenBank accession no. AJ011432) as fellows: GDNF primer a (Pa), forward, 5'-CATGCTGACCTGGAAATGGGTACATTAAGC-3'; reverse, 5'-CA TCACTGTGAATGAGAGATTACACTG AGGGC-3'(−1148/−956 bp from respective initiator site). GDNF primer b (Pb), forward, 5'-AAATCCACGCCTATGTGGATGGATCG-3'; reverse, 5'-TTTGGGGAA ACCTAA GCAAGGACAGGACT-3' (−598/--390 bp from respective initiator site). GDNF primer c (Pc), forward, 5'-CATGGAAATGGAGCCTAAGTCTGAGAAG-3'; reverse, 5'-CGCTGCAAGTGGGATGCATTTATAGAG-3' (−251/−14 bp from respective initiator site) (all the primers obtained from Sigma-Genosys (St. Louis, MO)). Levels of histone modifications at each pair set of GDNF gene promoter were determined by measuring the amount of that gene in ChIP by use of RT-PCR. Input or total DNA (nonimmunoprecipitated) and immunoprecipitated DNA were run in triplicate for each sample, and was repeated at least twice independently. The values of the ChIP DNA were normalized to the input DNA.

Western Blot Analysis

Cells were lysed in ice-cold modified radioimmunoprecipitation assay (RIPA) buffer consisting of 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 150mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF. Immunobloting analysis was performed as previously described (Wei *et al*, 2004). Rabbit anti-GAPDH (1:1000, Abcam, Cambridge, MA) was used as a control to confirm equal loading. Protein quantitation was determined by ImageQuant software version 5.1 (Amersham Biosciences).

High Pressure Liquid Chromatography (HPLC)

Amino acids were detected and quantified by HPLC as previously described (Rawls and McGinty, 1997). Glutamate and aspartate were identified by overlaying absorption spectra at 420 and 440 nm and quantifying at 420 nm using HP Chemstation software (Hewlett

Packard Company) based on peak area by comparison with an external standard calibration curve ranging from 0.1 to 5 M. The detection limit was 100 nM, based on signal to noise ratio.

Statistical Analysis

The data were expressed as the mean ± S.E.M. Statistical significance was assessed with an analysis of variance followed by Bonferroni's t test using the Statview program (Abacus concepts, Berkeley, Ca). A value of $p<0.05$ was considered statistically significant. Data are expressed as mean ± S.E.M. 13

RESULTS

Memantine Enhanced Survival of DA Neurons in Rat Primary Midbrain Cultures

To assess the viability of DA neurons in rat primary midbrain neuron-glia cultures, [^{3}H] DA uptake assay was used as a functional index, and immuno-cytochemical staining for TH-positive (a marker for DA neurons) neurons was used for both morphometric analysis and cell count. Various concentrations of memantine (0.1–30 μM) or vehicle were added to cultures seven days after seeding. One week later, [^{3}H] DA uptake assay was performed. As shown in Figure 1A, memantine in the range of 3–10 μM enhanced the capacity of DA uptake in a dose-dependent manner. A higher concentration of memantine (30 μM) showed neurotoxicity (data not shown). Thus, a range of memantine concentration (0.1–10 μM) was used for the rest of the study. The increased DA uptake after memantine treatment was confirmed by the results from ICC studies (Figure 1B and 1C). Cell count analysis revealed that memantine increased the number of TH-positive neurons (DA neurons) in a dose-related manner. Furthermore, morphometric analysis indicated that memantine treatment promoted arborization of TH neurons, in both length and numbers of neurites per neuron, compared with vehicle controls.

Neurotrophic Effects of Memantine Were Astroglia-Dependent

In an effort to understand the cellular mechanism underlying the enhanced number and activity of DA neurons after memantine treatment, a series of experiments using different types of cell cultures were conducted. First, neuron-enriched cultures (>98% purity) were used to investigate whether memantine has a direct effect on DA neurons. As shown in Figure 2A, while memantine (10 μM) enhanced [^{3}H] DA uptake capacity in neuron-glia cultures, it failed to increase DA uptake capacity in neuron-enriched cultures, indicating that the observed neurotrophic effect of memantine was not due to a direct effect on DA neurons.

To determine the possibility that glial cells (microglia or astroglia) mediated the memantineinduced neurotrophic effect, we used neuron-microglia co-cultures and microglia-depleted cultures (>95% purity). Neuron-enriched cultures supplemented with 7.5×10^4/well of microglia (neuron-microglia co-cultures) failed to increase DA uptake after memantine treatment (Figure 2A). On the other hand, microglia-depleted cultures that contain only neurons and astroglia, exhibited a [^{3}H] DA uptake capacity with mematine that was comparable to that of neuron-glia cultures (Figure 2A). These three experiments suggest that astroglia, but not microglia, are potential targets for memantine-induced neurotrophic effects on DA neurons.

Conditioned Media from Memantine-Treated Astroglia Promoted Survival of DA Neurons in Neuron-Enriched Cultures

To confirm the role of astroglia in the memantine-induced survival-promoting effect and further investigate the underlying mechanism, conditioned media from astroglia-enriched cultures in the absence (ACM) or presence of 10 μM memantine (ACM-MMT) were

prepared. Astroglia were incubated with or without memantine for 48 h, and conditioned media was collected and dialyzed to remove memantine. This conditioned media was then added to neuron-enriched cultures and incubated for 7 days before assays. Astroglia conditioned media significantly increased [^{3}H] DA uptake compared with both non-conditioned media and memantine-treated cultures (Figure 2B). Menantine-treated astroglia conditioned media displayed a significant increase in DA uptake capacity, compared with astroglia conditioned media. Immunostaining analysis with anti-TH antibody showed higher levels of TH-positive neurons with more neurite configurations in enriched neuron cultures treated with menantine-treated astroglia conditioned media than those in control cultures (Figure 2C).

Memantine-Increased the Release of GDNF from Astroglia

GDNF, and other growth factors have been shown to promote DA neurons survival, induce neurite outgrowth and sprouting, upregulate TH expression, and enhance synaptic efficacy (Baquet *et al*, 2005; Murer *et al*, 2001). GDNF is one of major neurotrophic factors in astroglia (Lin *et al*, 1993). Here, experiments were designed to test the possible involvement of GDNF in the neurotrophic effect of memantine. First, quantitative RT-PCR analysis showed that memantine treatment caused a time-dependent increase in GDNF mRNA levels in astroglial cultures. This increase reached two fold at 0.5 h, three fold at 1 h and returned to the control value at 24 h posttreatment (Figure 3A and 3B). Next, Western blot analysis revealed that memantine treatment increased the expression GDNF at 6 h and 12 h after treatment compared with 0 min (Figure 3C and 3D). A second experiment was conducted in order to provide evidence indicating GDNF was associated with the trophic effect of memantine. When the goat anti-GDNF antibody was mixed with memantine in neuron-glia cultures, the GDNF-neutralizing antibody significantly reduced memantine-enhanced DA uptake capacity; whereas treatment with goat isotype IgG control antibody has no effect (Figure 3E). Taken together, these two experiments strongly indicated a critical role of GDNF in mediating the neurotrophic effect of memantine.

Memantine-Induced Suppression of Astroglia Cellular HDAC Activity Was Associated with Chromatin Remodeling at GDNF Promoter Region

We had previously reported that VPA increased the expression of neurotrophic factors through the inhibition of HDAC activity which was associated with the increase of histone hyperacetylation in astroglia (Wu *et al*, 2008). Thus, changes in histone acetylation were examined in order to further understand the molecular mechanism underlying memantine-elicited increase in the expression of GDNF. We first compared the mechanism by which these two compounds inhibit HDAC activity. HeLa nuclear extract was used as a source of HDAC enzymes for the inhibition of HDAC activity assay. As shown in Figure 4A, memantine failed to show any effect in inhibiting the HDAC activity. In contrast, VPA at clinically relevant concentrations, greatly inhibited the HDAC activity in a dose-dependent manner (Figure 4A). These findings indicate, despite their similar effects in the expression of GDNF, these two compounds are different in their effect on HDAC enzymes. Thus, we determined whether the HDAC activity is affected in the cellular levels of astroglia treated with memantine. The cellular HDAC activity in memantine-treated astroglia was significantly suppressed in a time-dependent manner and maximally at 4 hr, compared with the controls (Figure 4B). We also compared the effects of VAP (1 mM), a widely used HDAC inhibitor, and memantine (10 μM) on the HDAC activity of astrocglia at 4 hr after administration. The activity was almost equi-potently inhibited about 30% by either memantine or VPA. These results indicate that memantine and VPA inhibited HDAC activity in different mechanisms. Therefore, we examined if changes of histone acetylation levels occur at the GDNF promoter region by ChIP study. The result showed that markedly enhanced association of acetylated H4 for one (Pc) out of these three primer set regions was

observed in the memantine-treated astroglia, indicating that memantine triggered recruitment of acetylated histone, specifically to the proximally close to the initiator site of GDNF gene (Figure 4C).

Neuroprotective Effects of Memantine against LPS-Induced Neurotoxicity Were Mediated through Microglia

Neuroprotective effects of memantine on DA neurons in midbrain neuron-glia cultures were determined by using LPS, an endotoxin from bacteria, as pro-inflammatory stimulus to induce neurotoxicity. Neuron-glia cultures were treated with various concentrations of memantine for 30 min prior to LPS (5 ng/ml). After 7-day incubation, LPS decreased the [^{3}H] DA uptake capacity by 60%, whereas memantine reduced LPS-induced loss of DA uptake in a dose-dependent fashion (Figure 5A). Morphological observation also revealed that DA neurons exhibited less damage in memantine-treated LPS cultures when compared with cells treated with LPS alone (Figure 5C; upper panel). Specifically, DA neurons treated with LPS in the presence of memantine displayed much longer and more elaborate TH-positive neurites compared with those from cultures treated with LPS alone (Figure 5C; upper panel).

Memantine alone produced astroglia-dependent neurotrophic effect in neuron-glia cultures (Figure 1). Therefore, astroglia will contribute to the neuroprotective effect of memantine against LPS-induced neurotoxicity. Since LPS is a potent inducer of microglial activation, the involvement of microglia would also be expected. Thus, neuron-microglia reconstituted cultures were used to determine whether microglia play a role in memantine-elicited neuroprotection. In the absence of astroglia in this culture, the role of microglia in memantine-elicited neuroprotection can be precisely evaluated. Cultures were pre-treated with various concentrations of memantine (1–10 μM) or vehicle for 30 min prior to LPS treatment (2.5 ng/ml). Lower concentration of LPS was used in neuron-microglia co-cultures because this type of culture is more sensitive to LPS treatment without the presumed protection of astroglia. Seven days later, the neurotoxic effects of LPS on DA neurons were assessed by [^{3}H] DA uptake. In the absence of astroglia, memantine alone (10 μM) failed to increase the capacity of DA uptake in neuron-microglia co-cultures, indicating no trophic effect was observed under this condition (Figure 5B). However, memantine showed a pronounced neuroprotective effect on DA neurons against the LPS-induced neurotoxicity, strongly suggesting that microglia play a role in mediating the neuroprotective effect of memantine. The morphological changes elicited in TH-positive neurons in neuron-microglia co-cultures after LPS and/or memantine treatment were shown in figure 5C (lower panel).

The morphology of microglia in the primary midbrain neuron-glia cultures and in neuronmicroglia co-cultures was assessed by ICC staining with an OX42 antibody against the CR3 receptor, a marker for microglia. After LPS treatment, in both types of cultures, microglia dramatically changed the morphology from resting round and small shape to rod- and/or amoeboid shape with a significant enlargement of cell size (Figure 5D). Pre-treatment with memantine attenuated the LPS-induced change in morphology seen in microglial cells, indicating that memantine inhibits the activation of microglia.

Memantine Reduced LPS-Induced Production of Reactive Oxygen Species and Pro-Inflammatory Factors from Microglia

Activated microglia produces an array of pro-inflammatory factors that are key mediators underling LPS-induced DA neurotoxicity (Block *et al*, 2004). To provide more evidence of the anti-inflammatory effect of memantine, we determined several major inflammation-related factors released from microglia after LPS treatment. Because each factor released from microglia is different in terms of time and quantities, specific cultures and time points

were tailored for each pro-inflammatory factor. Enriched microglia cultures were used for the determination of LPS-induced reactive oxygen species generation including extracellular superoxide and iROS. Memantine attenuated production of superoxide anion (Figure 6A) and iROS (Figure 6B) in a dose-dependent fashion compared with vehicle controls. In addition, in neuron-glia cultures, LPS-induced increase in TNF-α (4 h after LPS treatment) was significantly reduced by memantine (Figure 6C). The increased release of nitric oxide (NO, measured as nitrite) and PGE_2 24 h after LPS stimulation was also significantly reduced in memantine-treated samples (Figure 6D and 6E).

Supernatant Levels of Glutamate and Aspartate Remained Unchanged after LPS Treatment in Primary Midbrain Neuron-Glia Cultures

Since memantine is a NMDA receptor blocker, most of the reports attributed neuroprotective effects or clinical therapeutic benefits of this compound to its inhibitory effects on NMDA-receptor mediated excitotoxicity (Lipton, 2006). To determine whether NMDA receptors play a role in the neuroprotective effect of memantine in LPS-induced DA neurotoxicity, we measured the concentrations of two excitatory amino acids, glutamate and aspartate, in the supernatants of primary neuron-glia cultures treated with LPS and/or memantine (Figure 7). Basal levels of these two excitatory amino acids were low, around 12 μM for glutamate and 10 μM for aspartate. The levels remained unchanged for the entire time course (0.5 to 48 h) determined after LPS treatment. Supernatant concentrations of both glutamate and aspartate in cultures treated with memantine (10 μM) alone or added together with LPS did not differ from the levels of vehicle-or LPS-treated groups. These results suggested an NMDA receptor independent mechanism for glia-mediated neurotrophic and anti-inflammatory effects of memantine.

DISCUSSION

This study is the first report describing a novel mechanism whereby glia mediated the neuroprotective effect of memantine. Our study showed that increase in the GDNF production from astroglia through histone remodeling enhanced the number and activity of DA neurons in the midbrain neuron-glia cultures. This action, plus a microglia-directed anti-inflammatory effect, likely underlies the neuroprotective effect of memantine against LPS-induced neurotoxicity. Furthermore, our study suggests that these glia-dependent actions of memantine are mechanistically remote from NMDA receptor.

Many reports have demonstrated potent neuroprotection by memantine in excitotoxin (such as glutamate, NMDA or gp 120)-induced neurodegeneration (Lipton, 2007; Weller *et al*, 1993a; Weller M, 1993). However, the majority of these *in vitro* studies use neuron cultures, which eliminate the opportunity to investigate the role of glial cells in the neuroprotective effect of this compound. In our study, we used a combination of various cultures including mixed neuron-glia cultures, neuron-microglia cocultures, and microglia-depleted cultures, which allow us to investigate the interaction between neurons and glial cells. With these *in vitro* models, we showed the main sites of action for memantine in protecting LPS-induced DA damage are the glial cells, but not the neurons.

Memantine Prolonged DA Neuron Survival in Neuron-Glia Cultures via Astroglia-Mediated GDNF Production

An important observation in this study was that memantine enhanced the release of GDNF from astroglia, which may account for its neuron survival-enhancing effect in primary neuron-glia cultures. Our data show that memantine increases DA uptake by about 70% in neuron-glia cultures (Figure 2A), compared to about only 10% increase in neuron-enriched cultures (Figure 2A). Further experiments using neuron-microglia coculture and microglia-

depleted culture (Figure 2A) suggest astroglia as a critical component of memantine-mediated protection. Additionally, conditioned medium from memantine-treated astroglia significantly enhances the function of DA neurons (Figure 2B).

It has been demonstrated that neurotrophic factors play an important role in the development, maintenance, and survival of neurons, glia, and oligodendrocytes (Huang *et al*, 2004). Deficiency in the release of neurotrophic factors can lead to neuronal death and contribute to the pathogenesis of neurodegenerative diseases such as PD and AD (Phillips *et al*, 1991; Schindowski *et al*, 2008). Astroglia have been shown to be a major source of neurotrophic factors, especially GDNF (Darlington, 2005). Studies from both RT-PCR and Western blot analysis clearly showed elevated expression of GDNF in the memantine-treated astroglia (Figure 3). This finding is consistent with results from a previous report indicating an increase in GDNF production from C6 glioma cell line after memantine treatment (Caumont *et al*, 2006a). Moreover, we found that the neurotrophic effect of memantine was dramatically blocked by GDNF neutralizing antibodies (Figure 3E), suggesting that this neurotrophic factor is critical for memantine-enhanced neuronal survival.

Mechanistic studies showed a link between the inhibition of HDAC activity and the increase of GDNF transcripts in astroglia treated with memantine (Figure 4) and VPA (Wu *et al*, 2008), based on the enzyme activity and ChIP assays. Despite their similarity in producing neurotrophic effects, there is a critical difference in how HDAC activity was affected by these two compounds. Memantine failed to inhibit the HeLa nuclear HDAC activity, which is different from the typical HDAC inhibitors, such as valproate and Trichostain A (Di Gennaro *et al*, 2004; Finnin *et al*, 1999). Thus, we further examined the exact mechanism how memantine enhanced the epigenetic expression of neurotrophic factors. Our data showed that at the cellular level, memantine was capable of reducing the activity of astroglia HDAC in a potency similar to that of VPA. Therefore, it is likely that an indirect mechanism mediates the inhibitory effect of memantine on HDACs. We further postulated that the epigenetic effect on the expression of GDNF of memantine resulted from the inhibition of HDAC activity. This possibility was supported by a ChIP assay, which showed that memantine enhanced the association of acetylated H4 at gdnf promoter region close to the initiator site of gene transcription. This promoter-restricted localization of histone acetylation indicated that the local chromatin environment at the gdnf gene promoter region could be changed by memantine to facilitate gene transcription (Figure 4C)

Inhibition of Microglial Over-activation Underlies the Anti-inflammatory Effect of Memantine

It was recently reported that memantine treatment enhanced functional recovery and antiinflammatory effects in rat models of intracerebral hemorrhage and LPS-induced neuroinflammtion (Lee *et al*, 2006). Results from our study demonstrated the involvement of microglia in memantine-mediated DA neuroprotection against LPS-induced neuro-inflammation. We have previously reported that the release of microglail pro-inflammatory factors, such as superoxide production, TNF-α, nitric oxide, and prostaglandin E_2 underlies LPS-induced DA neuronal toxicity in culture and animal studies (Qin *et al*, 2004; Qin *et al*, 2007). In this study, biochemical measurements of pro-inflammatory factors showed that the anti-inflammatory property of memantine is mediated through the inhibition of microglial over-activation. Release of pro-inflammatory factors, such as superoxide production, ROS (Figure 6A and 6B), TNF-α, NO, and PGE^2 (Figure 6C–6E) was reduced by memantine.

Since memantine is a well-known low affinity uncompetitive antagonist of the NMDA receptor, the prevailing view as to how memantine is neuroprotective and beneficial to AD patients has focused on the blockade of NMDA receptors (Chen and Lipton, 2006a; Lipton,

2006). In excitotoxin-induced neurotoxicity models, memantine has been clearly shown to be a potent neuroprotective agent by inhibiting the opening of NMDA receptor-gated calcium channels on neurons. Several groups reported the release of excitatory amino acid from microglia by high concentration of LPS (100 ng/ml). The concentration of released glutamate, however, was limited to 10–15 μM, which may not be in sufficient concentrations to produce significant neuronal death (Obrenovitch *et al*, 2000). We measured the concentrations of excitatory amino acids, glutamate and aspartate, in the supernatant of neuron-glia cultures after LPS treatment by HPLC (Figure 7). Interestingly, with neurotoxic concentration of LPS (5 ng/ml), we could not detect any change in both glutamate and aspartate levels in the supernatant from 0.5 to 48 h after LPS (Figure 7). The discrapancy between our results and previous reports could come from the difference in LPS concentration. In our experiments, much lower concentration (5 ng/ml) of LPS was applied. In addition, previous reports have used microglial cultures, whereas, we used neuron-glia cultures. In our culture system, even if there was an increase in the release of glutamate or aspartate, their level would remain low due to the quick and efficient uptake by astroglia. Nevertheless, in agreement with a recent report by Wenk and colleagues who demonstrated a lack of NMDA receptors (NMDAR1) in microglia (Rosi *et al*, 2006), our results suggest that the anti-inflammatory effect of memantine is mediated through a novel mechanism, which is independent of NMDA receptors.

Finally it is interesting to compare the neuroprotective effects of memantine and valproate, a HDAC inhibitor. Although both compounds show anti-inflammatory effects by dampening microglial release of pro-inflamamtory factors, and increasing neurotrophic factor release from astroglia, there are major differences in their mode of actions: 1) memantine elicited its antiinflammatory effect by preventing the over-activation of microglia and reducing the release of proinflammatory factors (Figure 5 and 6). In contrast, we reported that the anti-inflammatory effect of valproate resulted from the decease in microglia number, since valproate induced apoptoic death of microglia (Chen *et al*, 2007) As mentioned above, despite their similar effects on the release of neurotrophic fectors from astroglia by both memantine and valproate, the mode of action mechanisms of these two compounds on HDAC inhibition was different. Unlikely the typical HDAC inhibitors, memantine does not directly inhibit HDAC (Figure 4A). Further detailed comparisons on molecular actions of these two drugs may provide insights to a possible choice of drug which can be used clinically for the neuroprotective purpose.

In conclusion, this study illustrates alternative mechanisms for neuroprotective effects of memantine by acting on glia: a neurotrophic effect mediated by astroglia through histone hyperacetylation, and an anti-inflammatory effect mediated by attenuation of microglia overactivation during inflammation. Our results also underline the emerging role of glia as active participants in neuronal survival, and further support the concept that astroglial dysfunction and aberrant activation of microglia contribute to the pathogenesis of various neurodegenerative disorders.

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ABBREVIATIONS

ACM Astrocyte conditioned media

AD	Alzheimer's disease
GDNF	Glial cell line-derived neurotrophic factor
HDAC	histone deacetylase
LME	l-leucine methylester
MMT	memantine
LPS	lipopolysaccharide
NMDA	N-methyl-D-aspartate
PGE_2	prostaglandin E_2
iROS	intracellular reactive oxygen species
PD	Parkinson's disease
SOD	superoxide dismutase
TH	tyrosine hydroxylase
TNF-α	tumor necrosis factor-alpha
VPA	valproic acid

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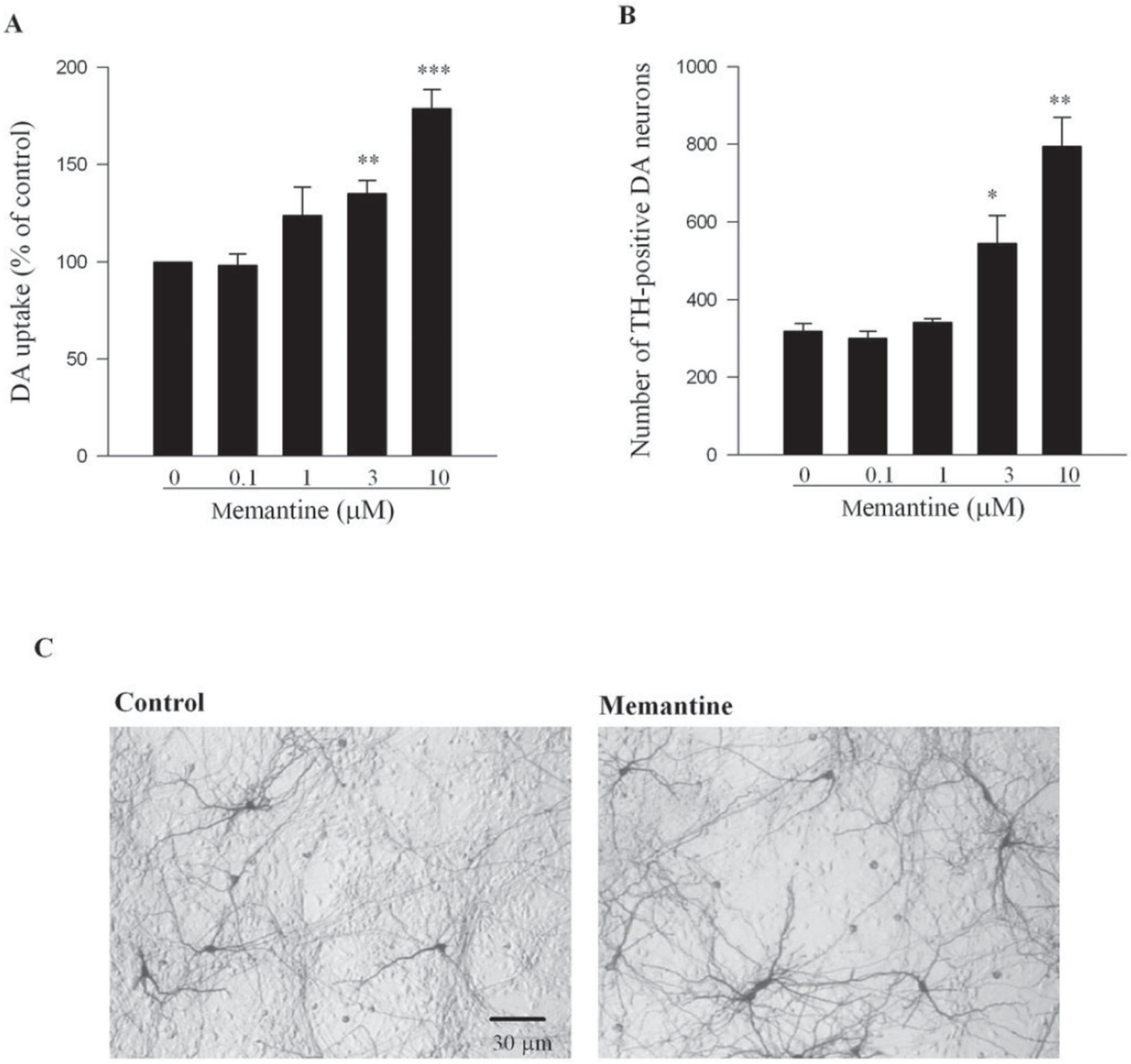

Figure 1. Memantine enhanced the survival of DA neurons and its functional DA uptake capacity

Rat primary midbrain neuron-glia cultures were treated with memantine (0.1– 10 μM) for 7 days. (A) The functional status of DA neurons was quantified by the [^{3}H] DA uptake assay. Results were expressed as a percentage of the vehicle-treated control cultures. (B) The numeration of TH-positive neurons was shown and presented as TH-positive cellnumbers per well. (C) Examples of control and memantine treated neurons stained by TH antibody were shown. All results were the mean ± S.E.M. from three independent experiments in triplicate. *, $p<0.05$, **, $p<0.01$, and ***, $p<0.001$ compared with the vehicle-treated control cultures.

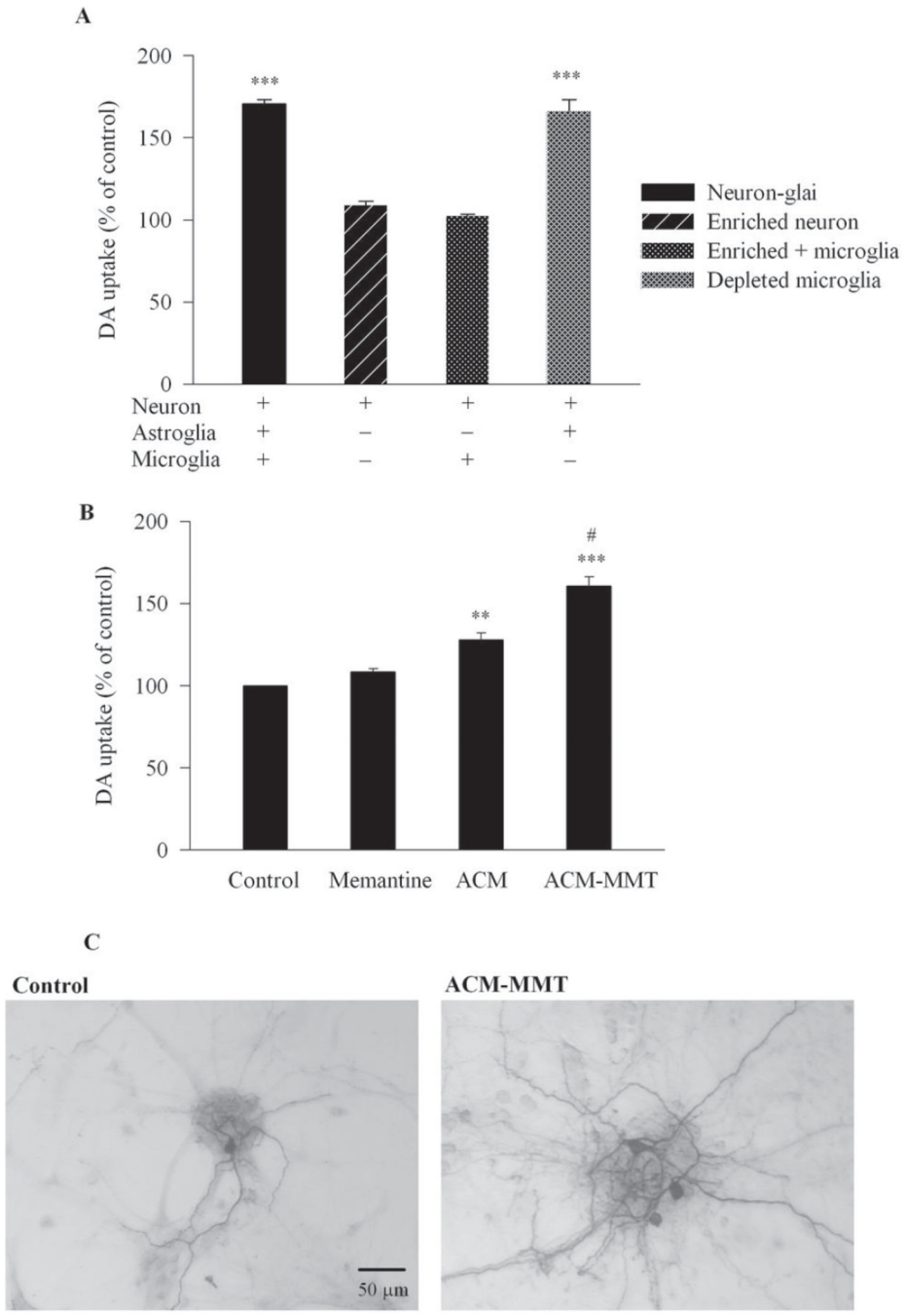

Figure 2. The neurotrophic effect of memanatine was astroglia-dependent

(A) Memantine (10 μM) or vehicle was added to the following different cell cultures: neuron-glia cultures, neuron-enriched cultures, neuron-microglia co-cultures by adding 7.5 × 10^4/well of enriched microglia to the neuron-enriched cultures, and microglia-depleted cultures. DA neuronal function was quantified by the [^{3}H] DA uptake assay. Results were expressed as a percentage of corresponding control cultures and were the mean ± S.E.M. from three independent experiments in triplicate. ***, $p< 0.001$ compared with corresponding control cultures. (B, C) The astrogliaconditioned media was prepared from astroglia-enriched cultures treated for 48 h with memantine (10 μM) (ACM-MMT) or vehicle (ACM). Seven days after adding the conditioned media to the neuron-enriched

cultures, DA neuronal function was quantified by the [^{3}H] DA uptake assay (B) and the cultures were stained with TH antibody (C). Results were expressed as a percentage of the vehicle-treated non-conditioned control cultures and were the mean ± S.E.M. from three independent experiments in triplicate. **, $p<0.01$, ***, $p<0.001$ compared with the control cultures; $^{\#}$, $p<0.05$ compared with the ACM-treated cultures.

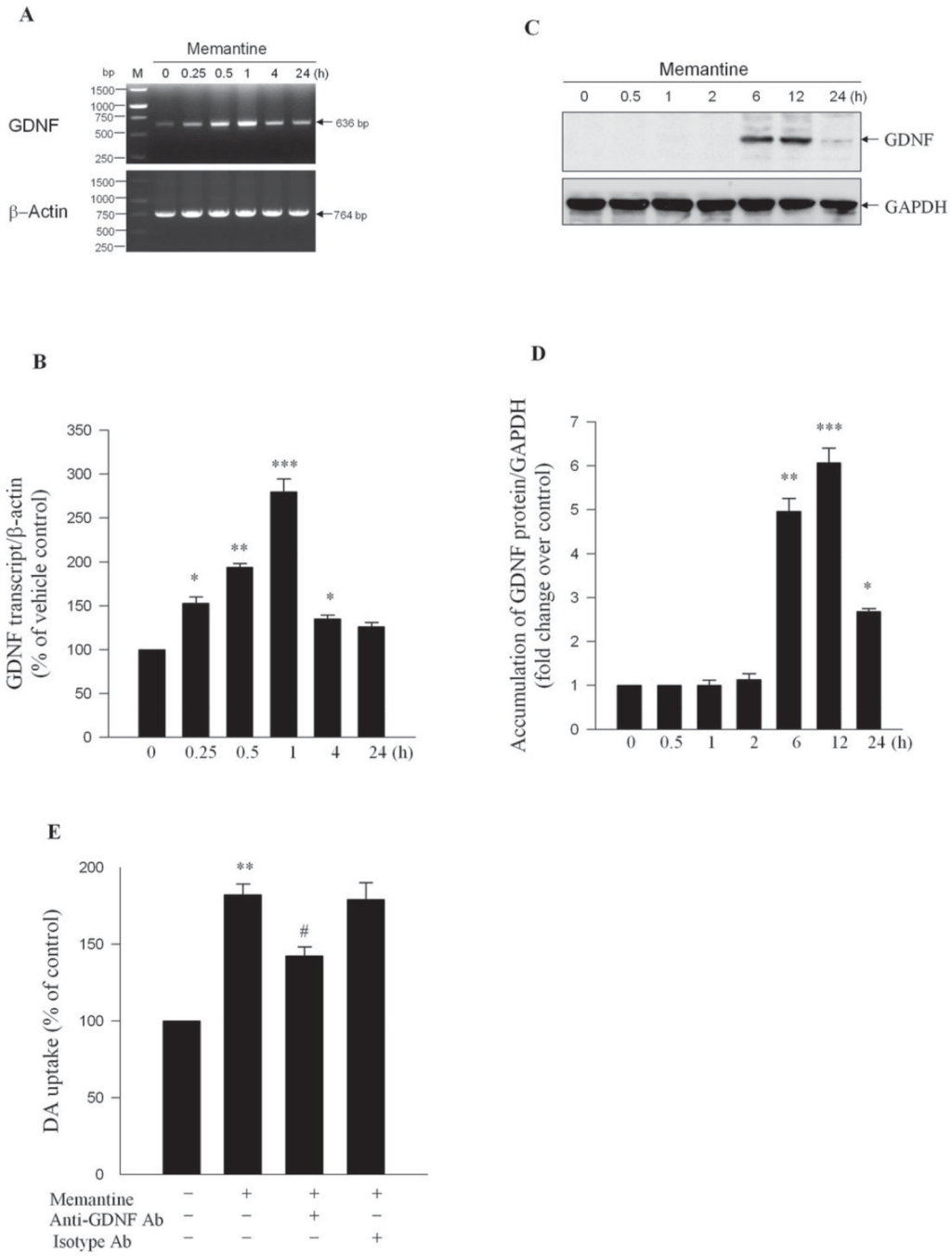

Figure 3. GDNF mediated memantine-induced neurotrophic effects

Rat primary astrocytes were exposed to 10 μM memantine for various time points ranging from 0 minute to 24 h. (A) Total RNA was extracted. Results of semiquantitative real-time PCR displayed the detection of a 635 bp band of GDNF. β-actin was used as loading control. (B) The ratio of densitometry values of GDNF and β-actin was analyzed and normalized to 0 min value. (C) Total protein of astroglial cells was extracted. Western blot analyses were performed with antibodies to GDNF. GAPDH was used as loading control. (D) The ratio of densitometry values of GDNF and GAPDH was analyzed and normalized to 0 min value. Results were expressed as mean ± S.E.M. from three experiments performed in triplicate. *, $p<0.05$ and **, $p<0.01$ ***, $p<0.001$ versus 0 min; (E) Neuron-glia cultures

were treated with either control goat IgG (isotype Ab), or goat anti-GDNF, combined with memantine (10 μM) treatment. DA uptake capacity was measured 7 days later. **, $p<0.01$ compared with the vehicle-treated control cultures; $^{\#}$, $p<0.05$ compared with memantine or isotype Ab-treated cultures.

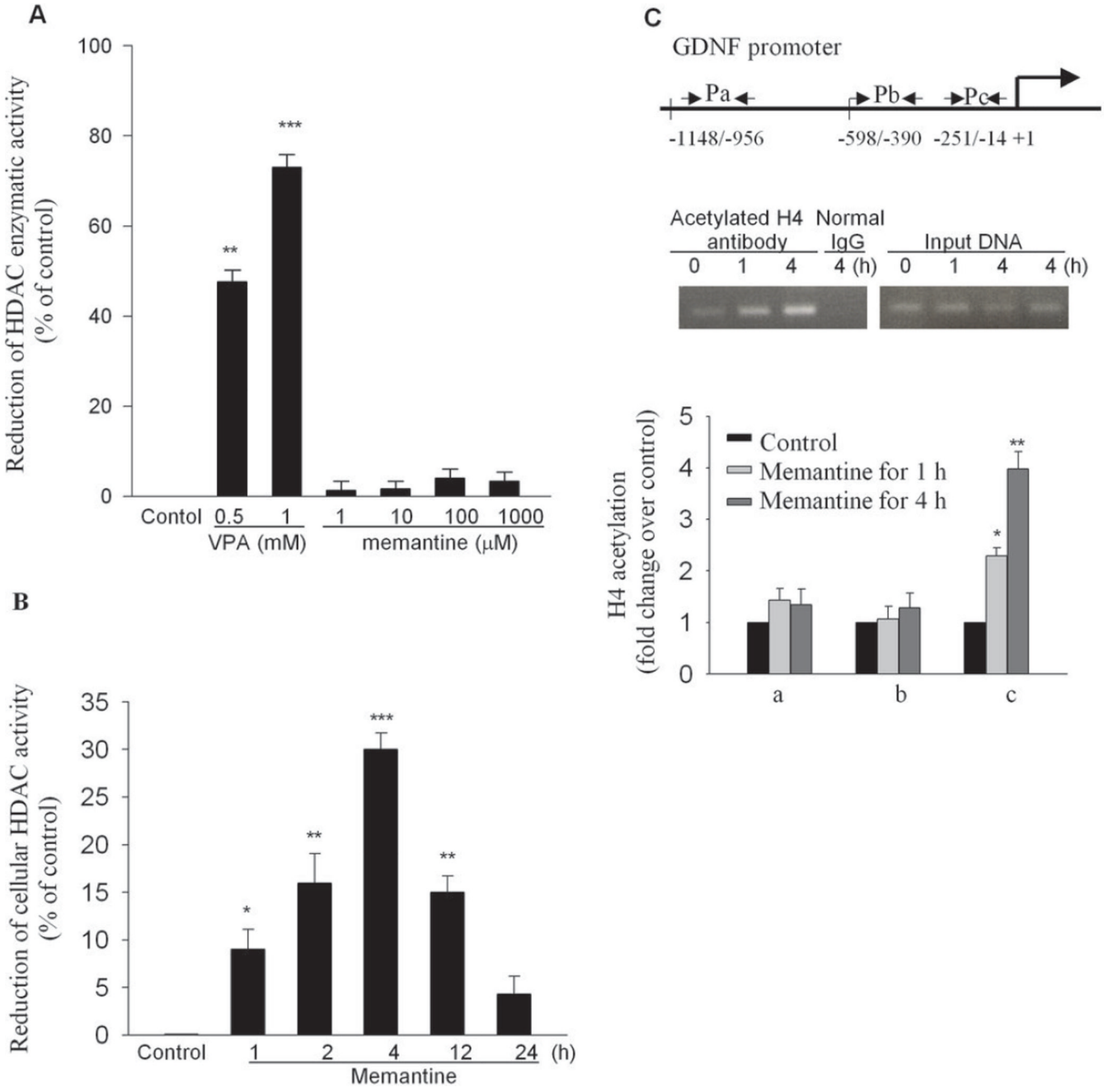

Figure 4. Inhibition of Cellular HDAC activity was associated with histone modification of GDNF promoter in memantine-treated astrglia

(A) Various concentrations of memantine (1 to 1000 μM) and valproic acid (0.5 to 1 mM) were tested for inhibition of HeLa nuclear extract HDAC activity. (B) Rat primary astroglia were exposed to memantine (10 μM) for various time points. Total lysates were extracted for the cellular HDAC activity assay. (C) Chromatin was prepared from primary astroglia treated with memantine for 0, 1, and 4 h, using antibodies against acetylated H4 or normal rabbit IgG. Three pairs of primers referring to regions of GDNF promoter (Pa, Pb, and Pc) were used for ChIP study (upper panel). The levels of the GDNF promoter in the immunoprecipiates were measured by RT-PCR with these 3 primer sets (lower panel). The

values of the ChIP DNA were normalized to the input DNA. Data are expressed as fold-change over the control. *, $p<0.05$, **, $p< 0.01$, and ***, $p< 0.001$ compared with controls.

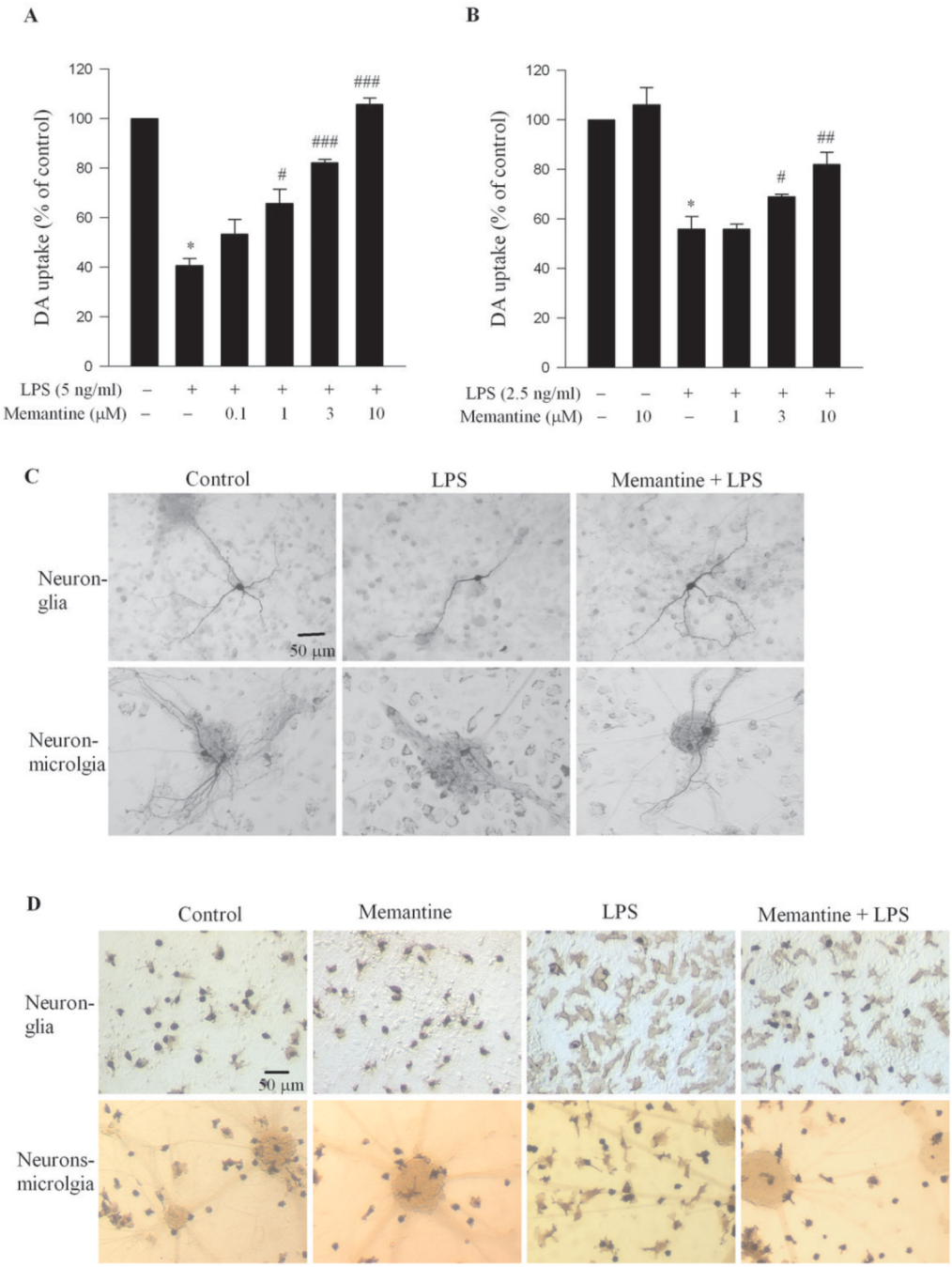

Figure 5. Memantine was neuroprotective against LPS-induced neurotoxcity

(A) Rat primary neuron-glia cultures were pretreated with memantine (0.1–10 μM) for 30 min before LPS (5 ng/ml) administration. [^{3}H] DA uptake was determined seven days after LPS stimulation. (B) Neuron-enriched cultures were supplemented with 7.5×10^4/well of enriched microglia. Twenty-four hrs later, this neuron-microglia co-cultures were pre-treated with memantine (10 μM) for 30 min before LPS (2.5 ng/ml) administration. [^{3}H] DA uptake was determined seven days after LPS stimulation. Results were expressed as a percentage of the vehicle-treated control cultures and were the mean ± S.E.M. from four independent experiments in triplicate. *, $p<0.05$, versus control; $^\#$, $p<0.05$ and $^{\#\#}$, $p<0.01$, $^{\#\#\#}$, $p<0.001$ versus LPS-treated. (C) Examples of control, LPS without or with memantine-treated

neurons stained by TH antibody. Upper panel: neuron-glia cultures; lower panel: neuron-microglia co-cultures. (D) Examples of OX42 staining for activated microglia in neuron-glia cultures. (E) Examples of OX42 staining for activated microglia in neuron-microglia co-cultures.

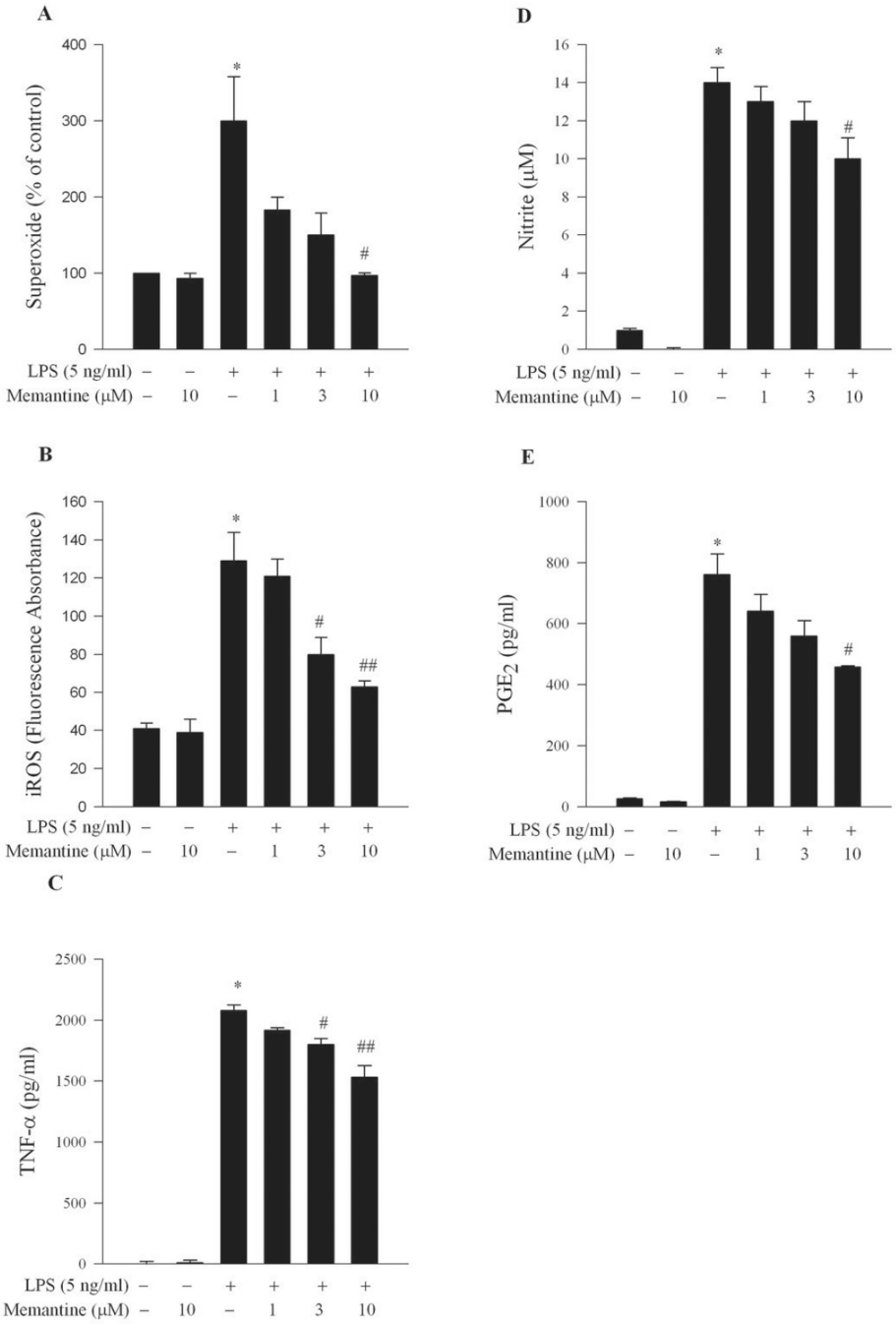

Figure 6. Memantine inhibited LPS-induced production of reactive oxygen species (ROS), and proinflammatory factors

(A, B) Microglia-enriched cultures seeded at 5×10^4/well in 96-well plates were pre-treated with memantine (10 μM) for 30 min before LPS (5 ng/ml) administration. (A) Production of extracellular superoxide was measured as SOD-inhibitable reduction of WST-1. Results were normalized to percentage of control level. (B) iROS production was determined by a fluorescence probe DCFH-DA. (C–E) Rat primary midbrain neuron-glia cultures were pre-treated with vehicle and memantine (1–10 μM) for 30 min before the LPS (5 ng/ml) stimulation. Supernatant was collected at 4 h for TNF-α assay (C), at 24 h for nitrite assay (D) and PGE_2 assay (E). Results were expressed as mean ± S.E.M. from three 36

independent experiments in triplicate. #, $p<0.05$ and ##, $p<0.01$ versus LPS-treated cultures. *, $p<0.05$, versus control.

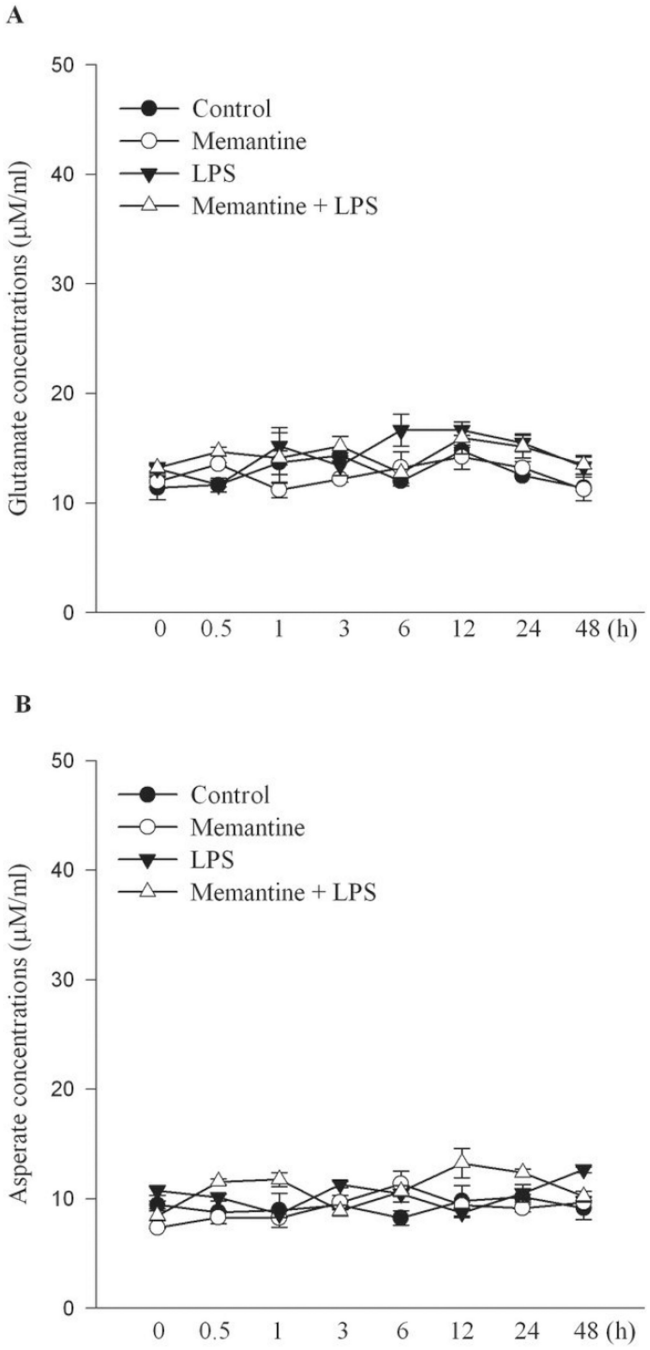

Figure 7. Concentrations of glutamate and asperate in supernatants of primary midbrain cultures

Supernatants taken at indicated time points from primary neuron-glia cultures treated with LPS (5 ng/ml) with/without memantine (10 μM) were measured with HPLC for glutamate and asperate concentrations. The glutamate and asperate concentrations were remained in low and consistent concentrations during treatment, and not robustly fluctuated during LPS and/or memantine treatment.