Neuropsychopharmacology (2009) 34, 2344–2357

www.neuropsychopharmacology.org

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

Hung-Ming Wu[1,2,3,9], Nian-Sheng Tzeng[3,4,9], Li Qian[3,5], Sung-Jen Wei[6,7], Xiaoming Hu[3], Shih-Heng Chen[1,2,3], Scott M Rawls[8], Patrick Flood[5], Jau-Shyong Hong[3] and Ru-Band Lu*[,1,2]

[1]College of Medicine, Institute of Behavioral Medicine, National Cheng-Kung University, Tainan, Taiwan; [2]Department of Psychiatry, National Cheng-Kung University Hospital, Tainan, Taiwan; [3]Laboratory of Pharmacology, Neuropharmacology Section, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, Durham, NC, USA; [4]Department of Psychiatry, National Defense Medical Center, Tri-Service General Hospital, Taipei, Taiwan; [5]Comprehensive Center for Inflammatory Disorders, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [6]Cancer Biology Group, Laboratory of Molecular Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, Durham, NC, USA; [7]Medical Research Division, Department of Pharmacology, Edinburg Regional Academic Health Center, The University of Texas Health Science Center at San Antonio, Edinburg, TX, USA; [8]Department of Pharmaceutical Sciences and Center for Substance Abuse Research, Temple University School of Pharmacy, Philadelphia, PA, USA

Memantine shows clinically relevant efficacy in patients with Alzheimer's disease and Parkinson's disease. Most *in vivo* and *in vitro* studies attribute the neuroprotective effects of memantine to the blockade of *N*-methyl-D-aspartate (NMDA) receptor on neurons. However, it cannot be excluded that mechanisms other than NMDA receptor blockade may contribute to the neuroprotective effects of this compound. 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 dopaminergic (DA) neuronal death. Here, we show that memantine exerted both potent neurotrophic and neuroprotective effects on DA neurons in rat neuron–glia cultures. The neurotrophic effect of memantine was glia dependent, as memantine failed to show any positive effect on DA neurons in neuron-enriched cultures. More specifically, it seems to be that astroglia, not microglia, are the source of the memantine-elicited neurotrophic effects through the increased production of glial cell line-derived neurotrophic factor (GDNF). Mechanistic studies showed that GDNF upregulation was associated with histone hyperacetylation by inhibiting the cellular histone deacetylase activity. In addition, memantine also displays neuroprotective effects against LPS-induced DA neuronal damage through its inhibition of microglia activation showed by both OX-42 immunostaining and reduction of pro-inflammatory factor production, such as extracellular superoxide anion, intracellular reactive oxygen species, nitric oxide, prostaglandin E_2, and tumor necrosis factor-α. These results suggest that the neuroprotective effects of memantine shown in our cell culture studies are mediated in part through alternative novel mechanisms by reducing microglia-associated inflammation and by stimulating neurotrophic factor release from astroglia.
Neuropsychopharmacology (2009) **34,** 2344–2357; doi:10.1038/npp.2009.64; published online 17 June 2009

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 showed 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 showed that memantine has neuroprotective effects against human immunodeficiency virus (HIV) and β-amyloid peptide-induced neurodegeneration, as well as prevents the progression of neuronal loss in amyotrophic lateral sclerosis and multiple sclerosis disease models (Wenk *et al*, 2006). Recently, several clinical trials also showed the beneficial effects of memantine for the treatment of AD (Reisberg *et al*, 2003), vascular dementia

*Correspondence: Professor R-B Lu, Department of Psychiatry, College of Medicine & Hospital, National Cheng Kung University, No. 138, Sheng-Li Road, 70428 Tainan, Taiwan, ROC,
Tel: +886 6 235 3535 ext. 5108, Fax: +886 6 302 8012,
E-mails: rblu@mail.ncku.edu.tw or rubandlu@gmail.com
[9]These authors contributed equally to this work.
Received 26 December 2008; revised 11 May 2009; accepted 11 May 2009

(Wilcock *et al*, 2002), and Parkinson's disease (PD) (Merello *et al*, 1999). As it is known that memantine is a NMDA receptor blocker, most of the reports attributed the clinical therapeutic benefits and pre-clinically neuroprotective effects of memantine to its protective activity against NMDA receptor-mediated excitotoxicity (Danysz and Parsons, 2003; Wenk *et al*, 2006).

Recent advances in research of the central nervous system (CNS) strongly suggest that glia (astroglia and microglia) play an important role in neurodegenerative diseases and are the 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 by 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*, 2006; Lin *et al*, 1993). 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 been reported that many drugs exert neuroprotective effects by modulating functions of glial cells. Some of them are neuroprotective through their anti-inflammatory effects by reducing the release of pro-inflammatory factors from activated microglia (Liu *et al*, 2002; Ralay Ranaivo *et al*, 2006), whereas others enhance the production of neurotrophic factors from astroglia (Chen *et al*, 2006; 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 only been reported in a few studies (Wenk *et al*, 2006; Willard *et al*, 2000). Given the crucial role of glia in the pathogenesis of neurodegenerative diseases, it is important to examine these roles in the neuroprotective effects of memantine. Recent reports from Wenk *et al* (Rosi *et al*, 2006; Willard *et al*, 2000) proposed a possible role of memantine on microglial activation. Here, using a series of midbrain primary cultures, we report that memantine showed potent efficacy in protecting dopaminergic (DA) neurons against lipopolysaccharide (LPS)-induced damage. Mechanistic studies showed two novel mechanisms underlying 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 activation of microglia, which is remote from NMDA receptors.

MATERIALS AND METHODS

Animals

Timed-pregnant adult female Fisher 344 rats were purchased from Charles River Laboratories (Raleigh, NC). 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 an earlier described protocol (Liu and Hong, 2003a). Briefly, ventral mesencephalic tissues were dissected from the 14-day-old 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 ~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

At 48 h after seeding, cytosine β-D-arabinofuranoside (10 μM) and l-leucine methyl ester (LME) (1 mM) were added to the mesencephalic neuron–glia cultures. Cytosine β-D-arabinofuranoside and LME were removed 48 h later and replaced with fresh media. Neuron-enriched cultures were 98% pure and microglia-depleted cultures were 95% pure (Gao *et al*, 2002).

Microglia-Enriched Cultures

Primary enriched-microglia cultures were prepared from the whole brains of 1-day-old Fisher 344 rat pups, using an earlier described protocol (Liu and Hong, 2003a). At 2 weeks after seeding, microglia were shaken off and either re-plated 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 earlier described protocol (Liu and Hong, 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 earlier (Liu *et al*, 2002). Radioactivity was determined by liquid scintillation counting with a Beckman Tri-carb 2900 TR liquid scintillation counter (Fullerton, CA). The non-specific DA uptake observed in the presence of mazindol (10 μM) was subtracted.

Immunostaining

Single immunocytochemical staining of cells was performed as described earlier (Liu and Hong, 2003a). DA neurons were recognized with the polyclonal antibody against TH (kind gift from Dr John Reinhard of GlaxoSmithKline, Research Triangle Park, Durham, NC), and microglia were detected with the OX-42 antibody (PharMingen, San Diego, CA) against CR3 receptor. Briefly, the cells were fixed for 20 min at room temperature in 3.7% paraformaldehyde in phosphate-buffered saline (PBS). After washing (two times) with PBS, the cultures were treated with 1% hydrogen peroxide for 10 min. The cultures were again washed (three times) with PBS and then incubated for 40 min with blocking solution (PBS containing 1% bovine serum albumin, 0.4% Triton X-100, and 4% appropriate serum: normal horse serum for OX-42, and normal goat serum for TH staining). The cultures were incubated overnight at 4°C with the primary antibody diluted in antibody diluents (Dako), and then the cells were washed (three times) for 10 min each time in PBS. The cultures were next incubated for 1 h with PBS containing 0.3% Triton X-100 and the appropriate biotinylated secondary antibody (OX-42, horse anti-mouse antibody, 1 : 227; TH, goat anti-rabbit antibody, 1 : 227). After washing (three times) with PBS, the cultures were incubated for 1 h with the Vectastain ABC reagents (Vector Laboratory, Burlingame, CA) diluted in PBS containing 0.3% Triton X-100. After washing (two times) with PBS, the bound complex was visualized by incubating cultures with 3,3′-diaminobenzidine and urea-hydrogen peroxide tablets from Sigma dissolved in water. For visual counting of TH-positive neurons, all wells of the 24-well plates from three independent experiments in triplicate were counted under the microscope at $\times 100$ magnification using a Nikon Diaphot microscope. Three vertical and horizontal lines were drawn in equal intervals in each well of the 24-well plate. For quantification of primary neurites (the segments of neurites originating directly from the cell body of neurons), branching points of outgrowth neurites (Arantes and Andrews, 2006), and microglia, nine representative areas ($0.9 \times 0.7\ mm^2$/field) per well at the line intersections were focused, and 81 fields from three independent experiment in triplicate were selected at $\times 100$ magnification by using Nikon Diaphot equipped with Dage-MTI (DC 330) camera with MetaMorph software. The number of primary neurites and branching points of TH-positive neurons, and OX-42-positive microglia were counted. For each microscopic field, the number of neuronal cell bodies was also counted, and 100–180 TH-positive neurons from three independent experiments were analyzed. 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 described earlier (Qin *et al*, 2002).

Intracellular ROS Assay

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

Tumor Necrosis Factor-*alpha* and Prostaglandin E_2 Assays

The production of tumor necrosis factor-*alpha* (TNF-α) and prostaglandin E_2 (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 supernatants was determined with a colorimetric assay using Griess reagent (1% sulfanilamide, 2.5% H_3PO_4, 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride) as described earlier (Qin *et al*, 2002).

HDAC Activity Assay

Histone deacetylase 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 (1–1000 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 and 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 memantine (1–1000 μM) and VPA (1 mM) for various time points, and then total lysate was prepared following the earlier described method (Wei *et al*, 2004). A total of 10 μg of astroglial total lysate were used for the assay of HDAC activity.

RT-PCR Analysis

Total RNA was extracted from rat primary astroglia-enriched cultures according to the acid–phenol 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′-CACCATGAAGTTATG GGATGTCGTGGCT-3′ and (R), 5′-TCAGATACATCCACA CCGTTTAGCGGA-3′; rat *β*-actin gene (F), 5′-TTGTAACCA ACTGGGACGATATGG-3′ and (R), 5′-GATCTTGATCTTC ATGGTGCTAGG-3′. The protocol for touchdown PCR was as follows: denaturation (95°C, 15 s), annealing (60°C, 30 s) to (50°C, 30 s), and extension (72°C, 2 min) for 20 cycles and denaturation (95°C, 15 s), annealing (50°C, 30 s), 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 Assay

Chromatin immunoprecipitation (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 non-immune rabbit IgG followed by RT-PCR as described earlier (Wei *et al*, 2004). Primers were selected upstream of respective initiator site of gene transcription (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 follows: GDNF primer a (Pa), forward, 5′-CATGCTGACC TGGAAATGGGTACATTAAGC-3′; reverse, 5′-CATCACTG TGAATGAGAGATTACACTGAGGGC-3′ (−1148/−956 bp from respective initiator site). GDNF primer b (Pb), forward, 5′-AAATCCACGCCTATGTGGATGGATCG-3′; reverse, 5′-TTTGGGGAAACCTAAGCAAGGACAGGACT-3′ (−598/−390 bp from respective initiator site). GDNF primer c (Pc), forward, 5′-CATGGAAATGGAGCCTAAGTCTGAGA AG-3′; reverse, 5′-CGCTGCAAGTGGGATGCATTTATAG AG-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 (non-immunoprecipitated) 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, 150 mM 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 described earlier (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).

Statistical Analysis

Values were presented as mean ± SEM. Statistical analysis for experiments with two groups was performed by Student's *t*-test or Mann–Whitney *U*-test. Comparison of more than two groups was performed by one-way ANOVA followed by Bonferroni's *t*-test for *post hoc* test using the StatView software (Abacus concepts, Berkeley, CA). Differences were considered significant at the $p<0.05$ level.

RESULTS

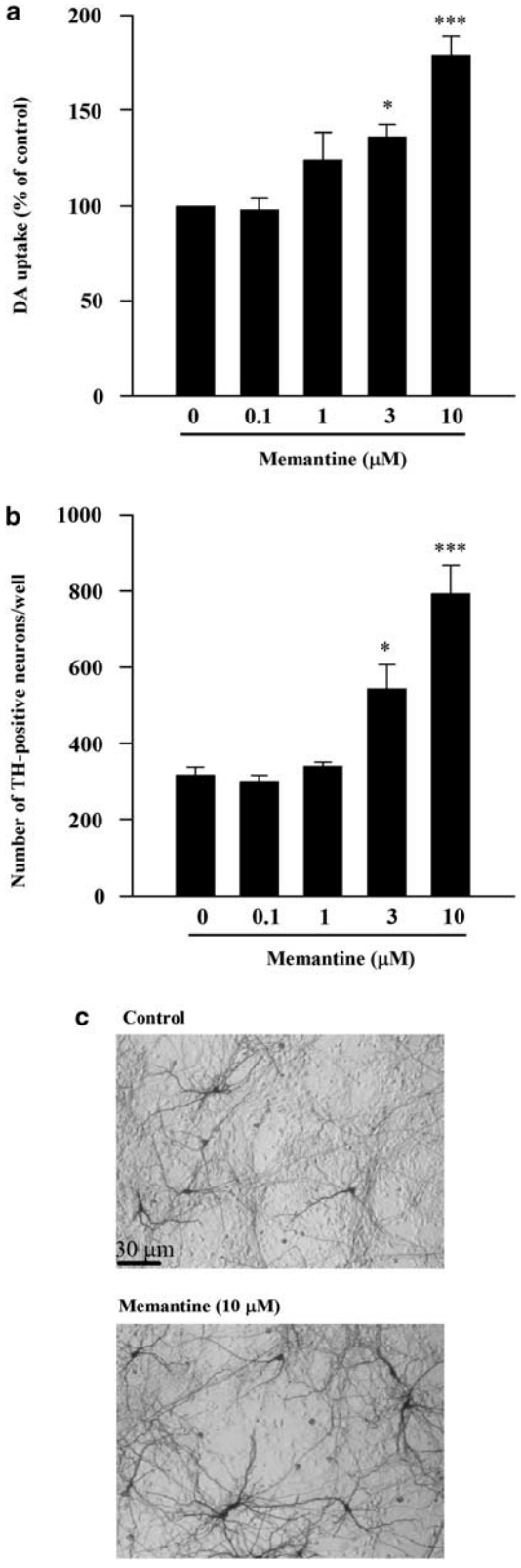
Memantine Enhanced Survival of DA Neurons in Rat Primary Midbrain Cultures

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 ICC 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 7 days after seeding. At 1 week later, [^{3}H] DA uptake assay was performed. As shown in Figure 1a, memantine showed a significant treatment effect ($F_{(4, 10)}=14.29$, $p<0.001$) in a dose-dependent manner, and subsequently *post hoc* analysis showed that memantine significantly enhanced the capacity of DA uptake at 3 μM ($t=3.06$, $p<0.05$) and 10 μM ($t=6.37$, $p<0.001$), compared with vehicle-treated control. A higher concentration (30 μM) of memantine showed neurotoxicity (data not shown). Thus, a range of memantine concentrations (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 c). Cell count analysis showed that memantine significantly prolonged the survival of TH-positive neurons (DA neurons) ($F_{(4, 10)}=22.31$, $p<0.001$) in a dose-related manner 7 days after treatment; *post hoc* test showed the increase was statistically significant at 3 μM ($t=3.59$, $p<0.05$), and 10 μM ($t=7.53$, $p<0.001$), compared with vehicle-treated control (Figure 1b). Furthermore, the morphological analysis showed that the primary neurite number per TH-positive neuron was significantly increased in memantine (10 μM)-treated cultures compared with vehicle-treated control cultures (median: 4 *vs* 3, $p<0.01$, Mann–Whitney *U*-test), and the number of branch points per TH-positive neuron was larger than that in controls (median: 3 *vs* 2, $p<0.001$, Mann–Whitney *U*-test). Memantine at 3 μM did not significantly increase neurite outgrowth of TH-positive neurons, suggesting that the enhanced DA uptake capacity at this concentration was associated with the increased TH-positive neuron number.

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 neuron–glia, enriched neuron, enriched neuron plus microglia, and microglia-depleted cultures were conducted. ANOVA analysis showed a significant effect of memantine on [^{3}H] DA uptake capacity among these cultures ($F_{(4, 10)}=108.94$, $p<0.001$) (Figure 2a). Neuron-enriched cultures (>98% purity) were used to investigate whether memantine has a direct effect on DA neurons. *Post hoc* analysis showed that although memantine (10 μM) enhanced [^{3}H] DA uptake capacity in neuron–glia cultures ($t=14.67$, $p<0.001$), it failed to increase DA uptake capacity in neuron-enriched cultures ($t=1.89$, $p=0.88$) (Figure 2a), indicating that the observed neurotrophic effect of memantine was not because of a direct effect

on DA neurons. To determine the possibility that glial cells (microglia or astroglia) mediated the memantine-induced neurotrophic effect, we used neuron–microglia co-cultures and microglia-depleted cultures (>95% purity).

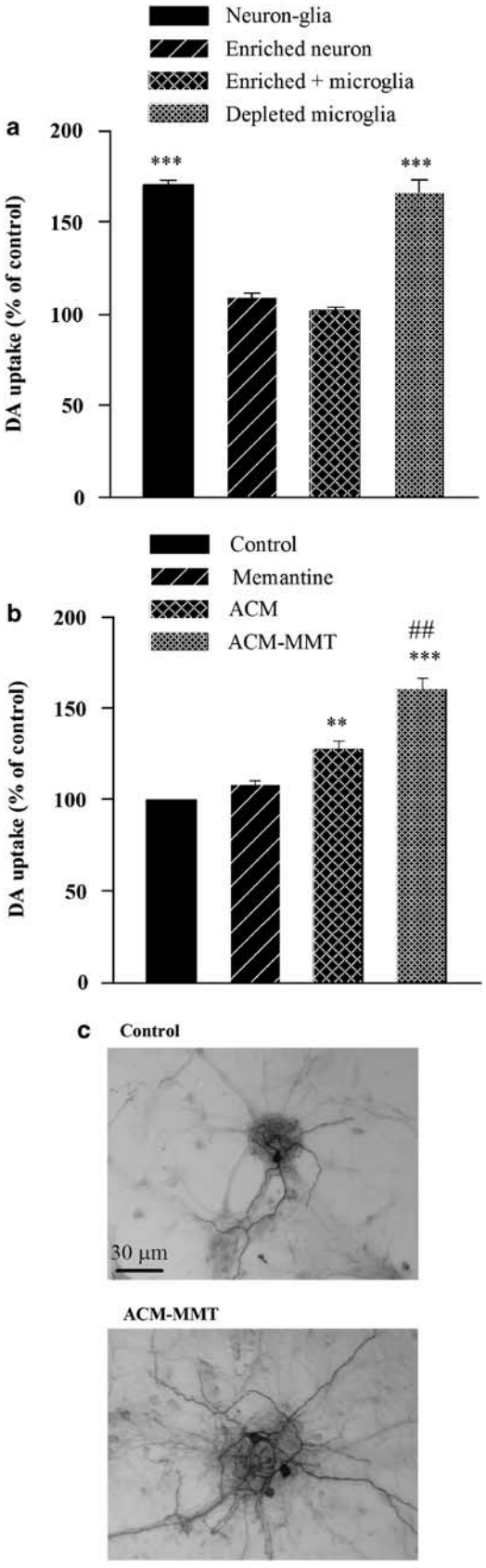


Neuron-enriched cultures supplemented with 7.5×10^4/well of microglia (neuron–microglia co-cultures) failed to increase DA uptake after memantine treatment ($t = 0.56$, $p = 1$) (Figure 2a). On the other hand, microglia-depleted cultures that contain only neurons and astroglia, exhibited a significantly increased [^{3}H] DA uptake capacity with memantine treatment ($t = 13.77$, $p < 0.001$), which was comparable to that of neuron–glia cultures ($t = 0.89$, $p = 1$) (Figure 2a). These experiments suggest that astroglia, but not microglia, are potential targets for memantine-induced neurotrophic effects on DA neurons.

Conditioned Medium 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 medium was collected and dialyzed to remove memantine. This conditioned medium was then added to neuron-enriched cultures and incubated for 7 days before assays. ANOVA analysis showed a significant difference among the groups treated with non-conditioned medium, memantine (10 μM), ACM, and ACM-MMT ($F_{(3,8)} = 53.98$, $p < 0.001$), and *post hoc* test showed that ACM and ACM-MMT significantly increased [^{3}H] DA uptake, compared with both non-conditioned medium (ACM, $t = 5.39$, $p < 0.01$, and ACM-MMT, $t = 11.69$, $p < 0.001$) and memantine-treated cultures (ACM, $t = 3.79$, $p < 0.05$, and ACM-MMT, $t = 10.08$, $p < 0.001$)(Figure 2b). In addition, ACM-MMT displayed a significant increase in DA uptake capacity when compared with ACM ($t = 6.29$, $p < 0.01$) (Figure 2b). Similarly, immunostaining and cell count analysis showed a main effect of astroglia conditioned medium for group ($F_{(3,8)} = 53.98$, $p < 0.001$). *Post hoc* test showed that the number of TH-positive neurons per well was significantly increased in the enriched neuron cultures treated with ACM (365 ± 12: $t = 4.23$, $p < 0.05$; $t = 5.08$, $p < 0.01$), and ACM-MMT (542 ± 23: $t = 12.18$, $p < 0.001$; $t = 13.04$, $p < 0.001$), compared with that treated with memantine (10 μM) (271 ± 14) and vehicle alone (252 ± 11), respectively. ACM-MMT displayed a significant increase in TH-positive neurons when compared with ACM ($t = 7.96$, $p < 0.001$). Furthermore, those TH-positive neurons had more neurite

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 cell numbers per well. Values represented mean ± SEM of three independent experiments performed in triplicate. *$p < 0.05$, ***$p < 0.001$, Bonferroni's *t*-test *vs* control for (a) and (b). (c) Examples of vehicle- and memantine-treated neurons stained by TH antibody were shown.



configurations in the treatment of ACM-MMT than those in controls (Figure 2c).

Memantine Increased the Release of GDNF from Astroglia

Glial cell line-derived neurotrophic factor, and other growth factors have been shown to promote DA neuron 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, RT-PCR analysis showed that memantine (10 μM) treatment caused a significant main effects ($F_{(5, 12)} = 76.31$, $p < 0.001$) on GDNF mRNA levels in a time-dependent manner in astroglial cultures (Figure 3a and b). *Post hoc* analysis showed that memantine significantly increased GDNF transcripts within 0.25 h ($t = 5.07$, $p < 0.01$), peaked at 1 h ($t = 17.22$, $p < 0.001$), and returned to the baseline level at 24 h ($t = 2.49$, $p > 0.05$) post-treatment of memantine (Figure 3b). Next, western blot analysis showed that memantine treatment had a significant effect on the expression of GDNF protein ($F_{(6, 14)} = 130.27$, $p < 0.001$), and *post hoc* analysis showed there was a significantly increased level of GDNF protein at 6 h ($t = 14.97$, $p < 0.001$), peaked at 12 h ($t = 19.12$, $p < 0.001$), and still expressed at 24 h ($t = 6.24$, $p < 0.001$) after memantine treatment, compared with vehicle-treated control (Figure 3c and d). To provide evidence indicating GDNF was associated with the trophic effect of memantine, the neutralization experiment was performed in neuron–glia cultures. ANOVA analysis showed a significant main effect ($F_{(3, 8)} = 20.6$, $p < 0.001$) among these cultures treated with vehicle, memantine (10 μM), anti-GDNF antibody plus memantine (10 μM), and goat IgG isotype antibody plus memantine (10 μM). *Post hoc* test showed the GDNF-neutralizing antibody significantly reduced memantine-enhanced DA uptake capacity ($t = 3.5$, $p < 0.05$) (Figure 3e), whereas the goat IgG isotype antibody had no effect ($t = 0.57$, $p = 1$ *vs* memantine) (Figure 3e). Taken together, these experiments strongly indicated a critical role of GDNF in mediating the neurotrophic effect of memantine.

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 mean ± SEM of three independent experiments performed in triplicate. ***$p < 0.001$, Bonferroni's *t*-test *vs* control. (b, c) The astroglia-conditioned media was prepared from astroglia-enriched cultures treated for 48 h with memantine (10 μM) (ACM-MMT) or vehicle (ACM). At 7 days after adding the conditioned medium 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 represented mean ± SEM of three independent experiments performed in triplicate. **$p < 0.01$, ***$p < 0.001$, Bonferroni's *t*-test *vs* control, and ##$p < 0.01$, Bonferroni's *t*-test *vs* ACM-treated cultures.

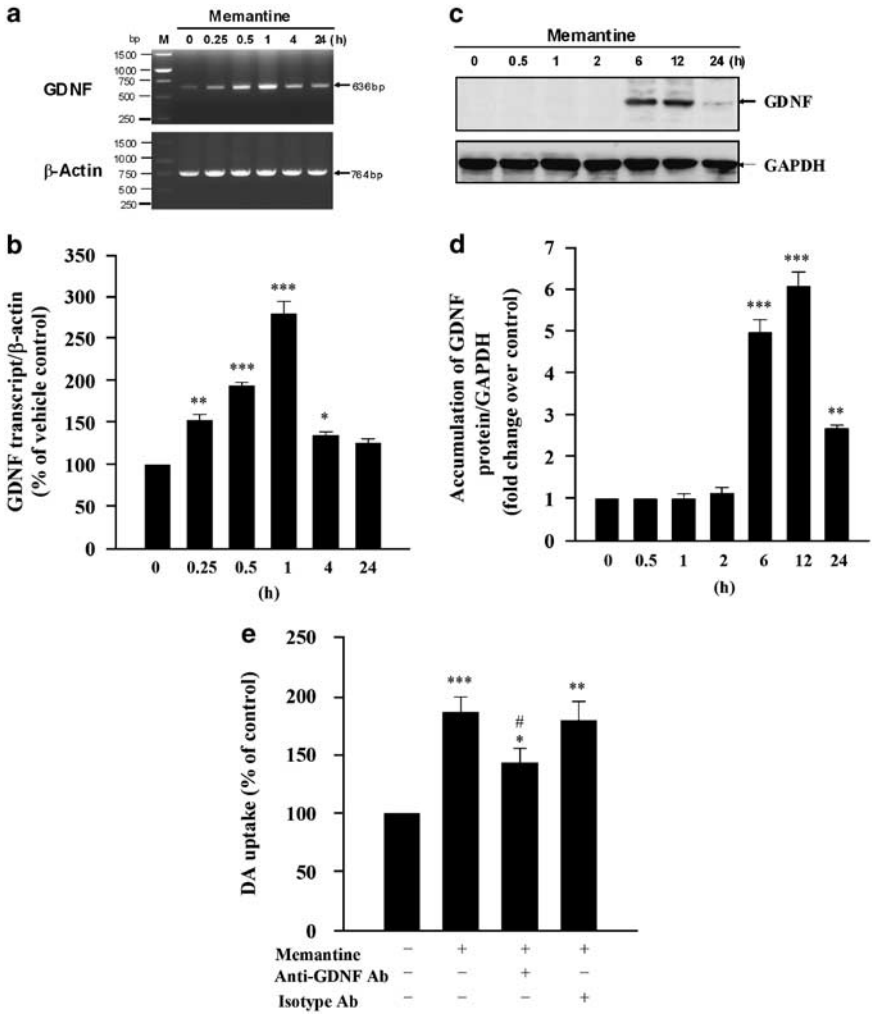


Figure 3 GDNF-mediated memantine-induced neurotrophic effects. Rat primary astroglia were exposed to 10 μM memantine for various time points ranging from 0 min to 24 h. (a) Total RNA was extracted. Results of semiquantitative real-time PCR displayed the detection of a 635 bp band of GDNF. β-actin was 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 the antibody 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. Values were expressed as mean ± SEM of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, Bonferroni's *t*-test *vs* 0 min for (b) and (d); (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. Results were expressed as a percentage of the vehicle-treated control cultures and represented mean ± SEM of three independent experiments performed in triplicate. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, Bonferroni's test *vs* control; $^{\#}p<0.05$, Bonferroni's *t*-test *vs* memantine-treated cultures.

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

We had earlier 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 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. ANOVA analysis showed that there was a significant difference between the treated groups ($F_{(6,14)}=239.04$, $p<0.001$) (Figure 4a). *Post hoc* analysis indicated that memantine (1–1000 μM) failed to show any effect in inhibiting the HDAC activity (all *p*-values >0.5). In contrast, VPA at clinically relevant concentrations greatly inhibited the HDAC activity (0.5 mM: $t=17.46$, $p<0.001$; 1 mM: $t=27.27$, $p<0.001$) in a dose-dependent manner (Figure 4a). These findings indicate, despite their similar effects in the expression of GDNF, these two compounds were 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 (10 μM). ANOVA analysis showed a significant main effect among these groups ($F_{(5,12)}=28.95$, $p<0.001$) (Figure 4b). The cellular HDAC activity in memantine-treated astroglia was significantly suppressed at 2 h ($t=5.73$, $p<0.01$), maximally at 4 h ($t=10.77$, $p<0.001$), and at 12 h ($t=5.37$, $p<0.01$), compared with the controls with *post hoc* analysis (Figure 4b). We also compared the effects of VAP (1 mM),

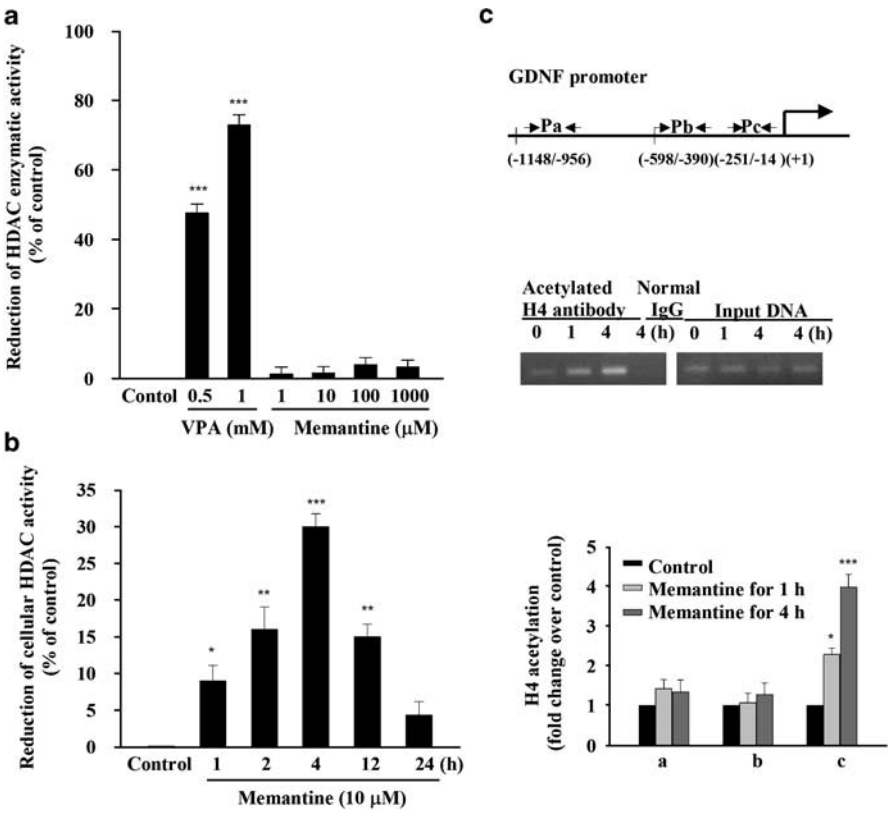


Figure 4 Inhibition of cellular HDAC activity was associated with histone modification of GDNF promoter in memantine-treated astrglia. (a) Various concentrations of memantine (1–1000 μM) and valproic acid (0.5–1 mM) were tested for inhibition of HeLa nuclear extract HDAC activity. Values were expressed as a percentage of the vehicle-treated control cultures and represented mean ± SEM of three independent experiments performed in triplicate. ***$p<0.001$, Bonferroni's test *vs* control. (b) Rat primary astroglia were exposed to memantine (10 μM) for various time points. Total lysates were extracted for the cellular HDAC activity assay. Values were expressed as a percentage of the vehicle-treated control cultures and represented mean ± SEM of three independent experiments performed in triplicate. *$p<0.05$, **$p<0.01$, ***$p<0.001$, Bonferroni's *t*-test *vs* control. (c) Chromatin was prepared from primary astroglia treated with memantine (10 μM) 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 immunoprecipitates were measured by RT-PCR with these three primer sets (lower panel). The values of the ChIP DNA were normalized to the input DNA. Values were presented as mean ± SEM of three independent experiments. *$p<0.05$, ***$p<0.001$, Bonferroni's *t*-test *vs* control.

a widely used HDAC inhibitor, and memantine (10 μM) on the HDAC activity of astroglia at 4 h after administration. The activity was almost equipotently inhibited about 30% by either memantine or VPA ($t=0.43$, $p>0.5$, Student's *t*-test). These results indicate that memantine and VPA inhibited HDAC activity in different mechanisms. Therefore, we examined whether 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) ($F_{(2,6)}=66.33$, $p<0.001$, Figure 4c) out of these three primer set regions was observed in the memantine-treated astroglia. *Post hoc* analysis showed that the levels of acetylation of Pc region were significantly increased at 1 h ($t=6.48$, $p<0.05$), and 4 h ($t=11.49$, $p<0.001$) (Figure 4c), indicating that memantine triggered recruitment of acetylated histone, specifically to the proximally close to the initiator site of GDNF gene.

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 before LPS (5 ng/ml). Significant effects of memantine treatment against LPS-induced neurontoxicity on [^{3}H] DA uptake were found ($F_{(5,12)}=46.52$, $p<0.001$) (Figure 5a). *Post hoc* analysis showed that LPS decreased the [^{3}H] DA uptake capacity by 60% of vehicle-treated control ($t=12.11$, $p<0.001$ *vs* control), whereas memantine significantly reduced LPS-induced loss of DA uptake at 1 μM ($t=4.66$, $p<0.01$), 3 μM ($t=7.75$, $p<0.001$), and 10 μM ($t=12.11$, $p<0.001$) (Figure 5a). Similarly, cell count analysis showed a significant effect of memantine pretreament in LPS-stimulated cultures ($F_{(5,12)}=31.93$, $p<0.001$), and *post hoc* analysis showed memantine significantly prolonged survival of the DA neurons at 3 μM (132 ± 7.8% of vehicle-treated control, $t=6.28$, $p<0.01$), and 10 μM (187 ± 7.2%, $t=10.5$, $p<0.001$) against neurotoxicity induced by LPS (50 ± 11% of vehicle-treated control). In addition, morphological observation showed that 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).

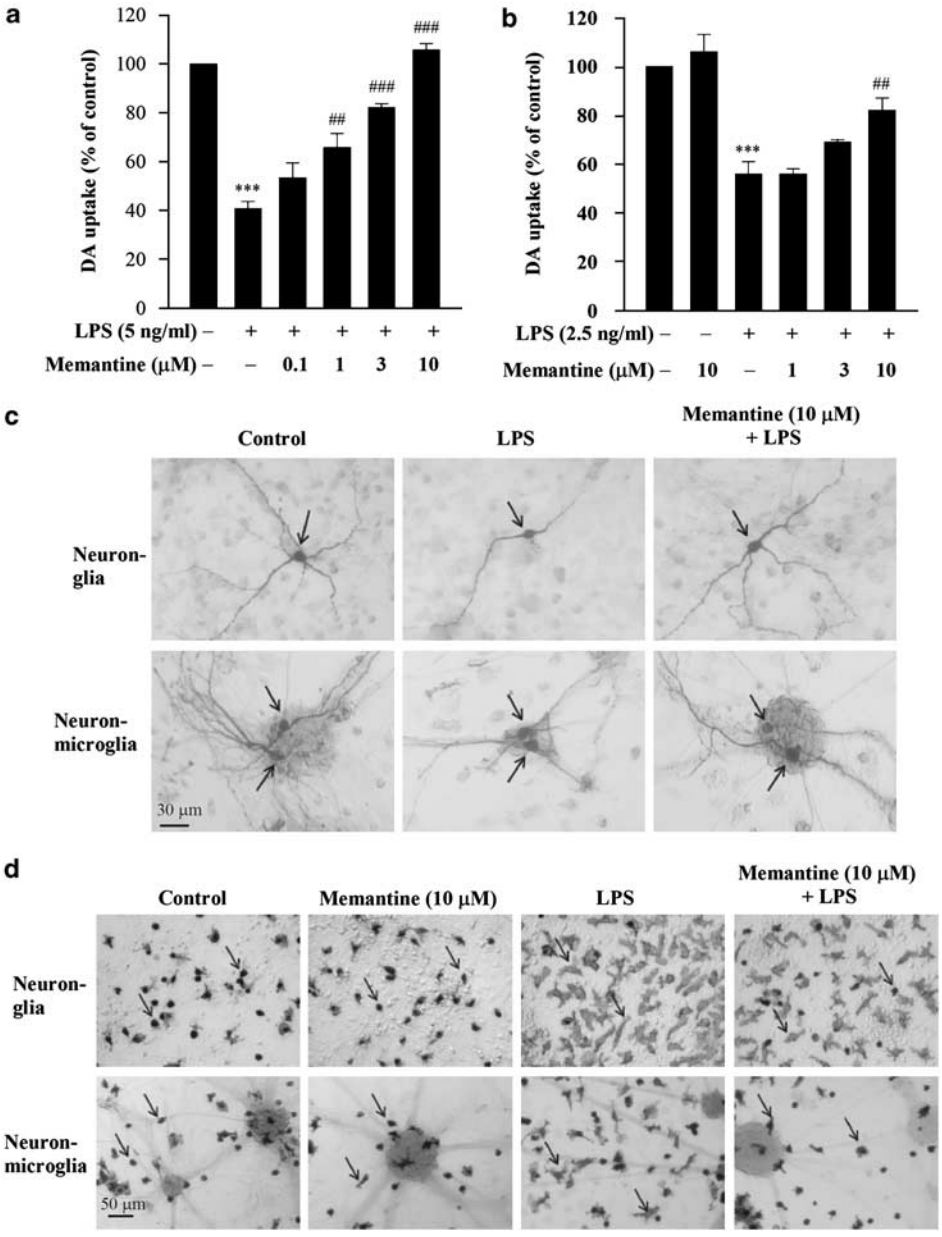


Figure 5 Memantine was neuroprotective against LPS-induced neurotoxicity. (a) Rat primary neuron–glia cultures were pre-treated with memantine (0.1–10 μM) for 30 min before LPS (5 ng/ml) administration. [^{3}H] DA uptake was determined 7 days after LPS stimulation. (b) Neuron-enriched cultures were supplemented with 7.5×10^4/well of enriched microglia. At 24 h 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 7 days after LPS stimulation. Results were expressed as a percentage of the vehicle-treated control cultures and were the mean ± SEM of four independent experiments performed in triplicate. ***$p<0.001$, Bonferroni's *t*-test *vs* control; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, Bonferroni's *t*-test *vs* LPS-treated cultures for (a) and (b). (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. Arrows indicate the TH-positive neurons. (d) Examples of OX-42 staining for microglia (arrows) in neuron–glia cultures and in neuron–microglia co-cultures.

Memantine alone produced astroglia-dependent neurotrophic effects in neuron–glia cultures (Figure 1). Therefore, astroglia will contribute to the neuroprotective effect of memantine against LPS-induced neurotoxicity. As 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 before 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. ANOVA analysis showed that memantine had a significant treatment effect on the loss of LPS-induced [^{3}H] DA uptake capacity ($F_{(5,12)} = 29.47$, $p<0.001$) (Figure 5b). In the absence of astroglia, memantine alone (10 μM) failed to

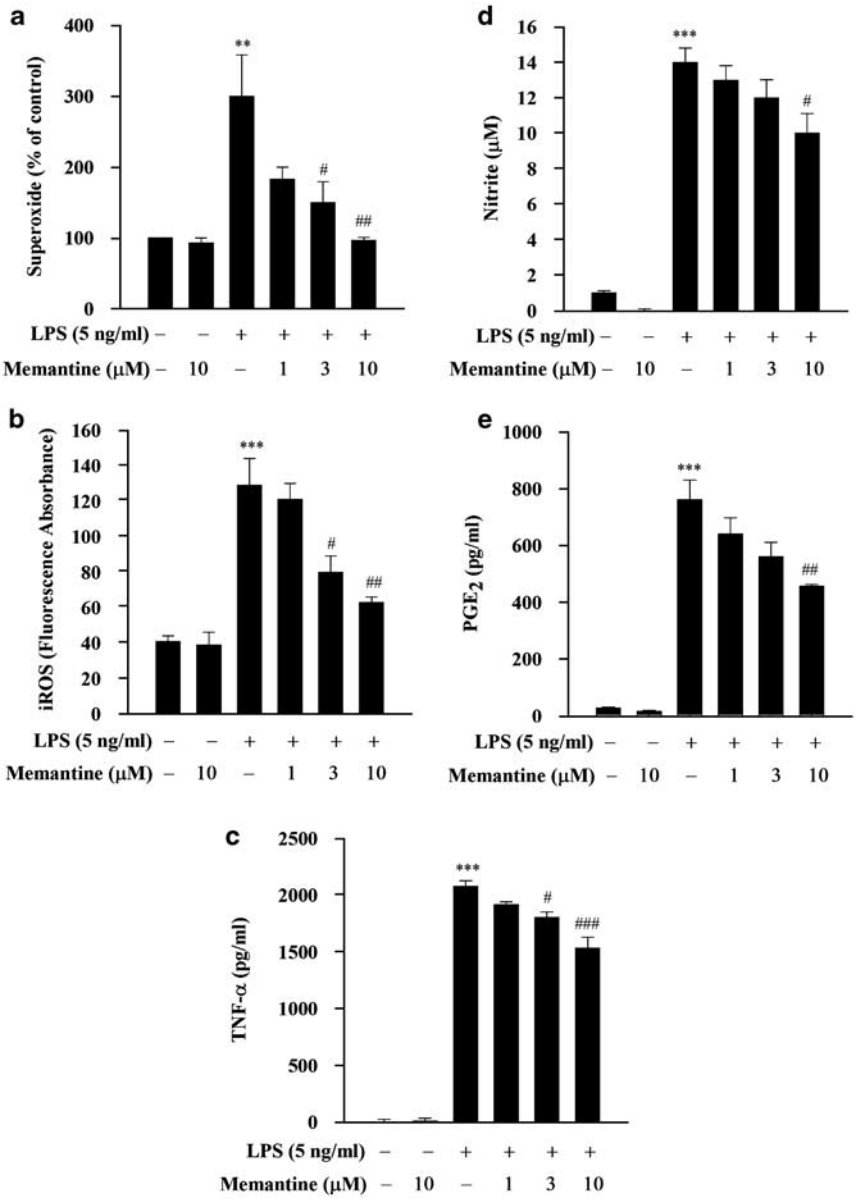


Figure 6 Memantine attenuated LPS-induced production of reactive oxygen species (ROS), and proinflammatory factors. (a, b) Microglia-enriched cultures seeded at 5×10^4/well in 96-well plates were pre-treated with memantine (10 μM) for 30 min before LPS (5 ng/ml) administration. (a) Production of extracellular superoxide was measured as SOD-inhibitable reduction of WST-1. Results were normalized to a percentage of control level. (b) iROS production was determined by a fluorescence probe DCFH-DA. Results were normalized to a percentage of control level. (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). All values were presented as mean ± SEM of three independent experiments performed in triplicate. $^{**}p<0.01$, $^{***}p<0.001$, Bonferroni's *t*-test *vs* control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.01$, Bonferroni's *t*-test *vs* LPS-treated cultures.

increase the capacity of DA uptake in neuron–microglia co-cultures ($t=1.07$, $p=1$ *vs* vehicle-treated control) with *post hoc* test (Figure 5b), indicating no trophic effect was observed under this condition. Furthermore, memantine significantly reduced LPS-induced loss of DA uptake capacity at 10 μM ($t=4.62$, $p<0.01$) (Figure 5b). Similarly, cell counting analysis showed that memantine had a significant effect on TH-positive neuronal survival against LPS ($F_{(5,12)}=24.2$, $p<0.001$), and *post hoc* test showed that memantine had pronounced neuroprotection on TH-positive neuron number at 10 μM against the LPS-induced neurotoxicity (79 ± 6.6 *vs* 53 ± 4.8% of vehicle-treated control, $t=3.84$, $p<0.05$). The result suggests that inhibition of microglia activation by memantine was associated with its neuroprotective effect. The morphological observation showed that TH-positive neurons exhibited less loss of the neurites in the treatment of LPS and memantine, compared with those with LPS alone (Figure 5c; lower panel).

The morphology of microglia in the primary midbrain neuron–glia cultures and in neuron–microglia co-cultures was assessed by ICC staining with an OX-42 antibody against the CR3 receptors. LPS treatment dramatically changed the morphology of microglia from resting round

and small shape (Figure 5d) to rod and/or amoeboid shape with a significant enlargement of cell size (Figure 5d) in both types of cultures. However, pre-treatment with memantine (10 μM) significantly reduced 18 ± 3% of activated microglia, compared with LPS-treated cultures ($p<0.05$, Student's *t*-test), and modestly attenuated the morphological changes (Figure 5d), 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. As 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. After LPS exposure in neuron–glia cultures, TNF-α production increase was peaked at 3–6 h, and both NO and PGE_2 at 24 h. Enriched microglia cultures were used for the determination of LPS-induced reactive oxygen species generation, including extracellular superoxide and iROS. Memantine treatment had significant effects on superoxide production ($F_{(5,12)}=8.55$, $p=0.001$) and iROS ($F_{(5,12)}=19.99$, $p<0.001$). *Post hoc* analysis showed memantine significantly attenuated the LPS-induced superoxide anion level at 3 μM ($t=3.87$, $p<0.05$), and 10 μM ($t=5.24$, $p<0.01$) (Figure 6a); iROS at 3 μM ($t=3.98$, $p<0.05$), and 10 μM ($t=5.37$, $p<0.01$) (Figure 6b). In neuron–glia cultures, memantine treatment also had main effects on the LPS-induced pro-inflammatory factors (TNF-α: $F_{(5,12)}=384.47$, $p<0.001$; nitric oxide: $F_{(5,12)}=66.9$, $p<0.001$; PGE_2: $F_{(5,12)}=57.39$, $p<0.001$) (Figure 6c–e). As indicated by *post hoc* test, LPS-induced increase in TNF-α (4 h after LPS treatment) was significantly reduced by memantine at 3 μM ($t=4.02$, $p<0.05$), and 10 μM ($t=7.89$, $p<0.001$) (Figure 6c); the increased release of nitric oxide (NO, measured as nitrite), and PGE_2 24 h after LPS stimulation was also significantly attenuated in memantine-treated samples at 10 μM ($t=3.7$, $p<0.05$, and $t=5.12$, $p<0.01$, respectively) (Figure 6d and e).

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

As 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 (Parsons *et al*, 2007). 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 vehicle, memantine (10 μM), and LPS (5 ng/ml) with/without memantine pre-treatment at the indicated time points (Supplementary Materials and Methods). Basal levels of these two excitatory amino acids were low, around 12 μM for glutamate and 10 μM for aspartate in the cultures (Supplementary Figure S1). Two-way ANOVA analysis showed that there were no significant main effects of either treatment or time on glutamate ($F_{(3,64)}=0.836$, $p>0.05$; $F_{(7,64)}=0.55$, $p>0.05$) and aspartate concentrations ($F_{(3,64)}=2.57$, $p>0.05$; $F_{(7,64)}=2.58$, $p>0.05$), and no significant interaction between treatment and time for the glutamate ($F_{(21,64)}=0.81$, $p>0.05$) and aspartate levels ($F_{(21,64)}=1.57$, $p>0.05$) in these cultures. In primary neuron–glia cultures, memantine and/or LPS failed to cause significant changes of both glutamate and aspartate concentrations for the entire course (0.5–48 h) (Supplementary Figure S1). These results suggest that a mechanism for glia-mediated neuroprotective effects of memantine may be remote from the NMDA receptor antagonism.

DISCUSSION

In addition to NMDA antagonism, the present results provide an alternative mechanism, whereby glia mediated the neuroprotective effects 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 effects 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 shown potent neuroprotection by memantine against excitotoxin (such as glutamate, NMDA, or gp120)-induced neurodegeneration (Lipton, 2007; Weller *et al*, 1993a). 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 co-cultures, 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 neuronal damage are glial cells, not the neurons.

Memantine Prolonged DA Neuron Survival in Neuron–Glia Cultures through 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 with about only 10% increase in neuron-enriched cultures (Figure 2a). Further experiments using neuron–microglia co-culture and microglia-depleted culture (Figure 2a) suggest astroglia as a critical component of memantine-mediated protection. In addition, conditioned

medium from memantine-treated astroglia significantly enhances the function of DA neurons (Figure 2b).

It has also been shown 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 an earlier 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 effects of memantine were significantly, but partially blocked by the GDNF neutralizing antibody (Figure 3e), suggesting that this neurotrophic factor is critical for memantine-enhanced neuronal survival. However, a partial blockade by the antibody suggests that there may be other neurotrophic factors, such as BDNF and TGF-β family members, released from astroglia, also involved in memantine-elicited trophic effects. Earlier, GDNF has been clinically tested for its therapeutic effects by direct infusion into the brain of PD patients. Unfortunately, the trial was terminated because of the occurrence of severe adverse effects (Kordower *et al*, 1999; Nutt *et al*, 2003; Peterson and Nutt, 2008). This study offers an alternative approach for the treatment of neurodegenerative diseases by administration of small molecules such as memantine to trigger physiological release of GDNF as well as other neurotrophic factors, which may synergistically provide neuroprotection in patients, particularly with PD.

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 VPA 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 might be changed by memantine to facilitate gene transcription (Figure 4c).

Inhibition of Microglial Activation Underlies the Anti-Inflammatory Effect of Memantine

It was recently reported that memantine treatment-enhanced functional recovery and anti-inflammatory effects in rat models of intracerebral hemorrhage and LPS-induced neuroinflammation (Lee *et al*, 2006; Willard *et al*, 2000). The results from our study showed the involvement of microglia in memantine-mediated DA neuroprotection against LPS-induced neuroinflammation. We have reported earlier that the release of microglial pro-inflammatory factors, such as superoxide production, TNF-α, nitric oxide, and PGE_2 underlies LPS-induced DA neuronal toxicity in culture and animal studies (Qin *et al*, 2004, 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 activation. Release of pro-inflammatory factors, such as superoxide production, ROS (Figure 6a and b), TNF-α, NO, and PGE_2 (Figure 6c–e) was reduced by memantine. It is interesting to note that LPS-elicited increase in the levels of superoxide and iROS was reduced differentially by memantine. One possible explanation for these differential effects is that superoxide is exclusively produced by the activation of microglial PHOX and we speculate that this superoxide-producing enzyme is a possible target of memantine. Thus, LPS-induced increase in superoxide was reduced to the control level by memantine. In contrast, iROS may come from different sources, besides the contribution from superoxide, which may enter the cells through the formation of H_2O_2, iROS can be generated from other intracellular sources, such as mitochondria or other peroxidases, which may not be affected by memantine.

As memantine is a well-known low affinity uncompetitive antagonist of the NMDA receptor, the prevailing view as to how memantine is beneficial to AD patients and is neuroprotective in pre-clinical studies has focused on blockade of NMDA receptors (Danysz and Parsons, 2003; Parsons *et al*, 2007). 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) (Barger *et al*, 2007; Takeuchi *et al*, 2005). The occurrence of excitoxicity depends on the concentrations of glutamate, the compositions of cell cultures (eg, enriched neurons, or neuron-glia cultures) and status of neurons (eg, dysfunction) (Obrenovitch *et al*, 2000). Although it has been reported that in certain conditions, low concentrations of glutamate may affect the neuronal function through NMDA receptors (Obrenovitch *et al*, 2000), glutamate at around 10 μM concentrations may be not neurotoxic in neuron–glia cultures. We measured the concentrations of excitatory amino acids, glutamate and aspartate, in the supernatants of neuron–glia cultures after LPS treatment by HPLC (Supplementary Figure S1). Interestingly, with neurotoxic concentration of LPS (5 ng/ml), we could not detect any change in both glutamate and aspartate levels in the supernatants from 0.5 to 48 h after LPS (Supplementary Figure S1). The discrepancy between our results and earlier reports could come from the difference in LPS concentration (Barger *et al*,

2007; Takeuchi *et al*, 2005). In our experiments, much lower concentration (5 ng/ml) of LPS was applied. In addition, the earlier 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 levels would remain low because of the quick and efficient reuptake by astroglia (Hertz and Zielke, 2004; Tanaka *et al*, 1997). Nevertheless, in agreement with a recent report by Wenk *et al* (Rosi *et al*, 2006) showed a lack of NMDA receptors (NMDAR1) in microglia, our results suggest that the anti-inflammatory effect of memantine is mediated through a novel mechanism far from the NMDA receptors.

Finally, it is interesting to compare the neuroprotective effects of memantine and VPA, 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 anti-inflammatory effect by preventing the activation of microglia and reducing the release of pro-inflammatory factors (Figures 5 and 6). In contrast, we reported that the anti-inflammatory effect of VPA resulted from the decrease in microglia number, as VPA induced apoptotic death of microglia (Chen *et al*, 2007). As mentioned above, despite their similar effects on the release of neurotrophic factors from astroglia by both memantine and VPA, the mode of action mechanisms of these two compounds on HDAC inhibition was different. Unlikely, the typical HDAC inhibitors, memantine did 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 that 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 activation 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.

ACKNOWLEDGEMENTS

This project was supported by a grant from the National Cheng Kung University, Project of Promoting Academic Excellence & Developing World Class Research Centers, Taiwan, Republic of China. This research was also supported in part by the Intramural Research Program of the NIH/NIEHS. We gratefully acknowledge the technical assistance of Belinda Wilson and Dr Hui-Ling Chen, and the statistical analysis of Dr Zongli Xu.

DISCLOSURE/CONFLICT OF INTEREST

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

REFERENCES

Arantes RM, Andrews NW (2006). A role for synaptotagmin VII-regulated exocytosis of lysosomes in neurite outgrowth from primary sympathetic neurons. *J Neurosci* **26**: 4630–4637.

Baquet ZC, Bickford PC, Jones KR (2005). Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. *J Neurosci* **25**: 6251–6259.

Barger SW, Goodwin ME, Porter MM, Beggs ML (2007). Glutamate release from activated microglia requires the oxidative burst and lipid peroxidation. *J Neurochem* **101**: 1205–1213.

Block ML, Hong JS (2005). Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Prog Neurobiol* **76**: 77–98.

Block ML, Wu X, Pei Z, Li G, Wang T, Qin L *et al* (2004). Nanometer size diesel exhaust particles are selectively toxic to dopaminergic neurons: the role of microglia, phagocytosis, and NADPH oxidase. *FASEB J* **18**: 1618–1620.

Block ML, Zecca L, Hong JS (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci* **8**: 57–69.

Caumont AS, Octave JN, Hermans E (2006a). Amantadine and memantine induce the expression of the glial cell line-derived neurotrophic factor in C6 glioma cells. *Neurosci Lett* **394**: 196–201.

Caumont AS, Octave JN, Hermans E (2006b). Specific regulation of rat glial cell line-derived neurotrophic factor gene expression by riluzole in C6 glioma cells. *J Neurochem* **97**: 128–139.

Chen PS, Peng GS, Li G, Yang S, Wu X, Wang CC *et al* (2006). Valproate protects dopaminergic neurons in midbrain neuron/glia cultures by stimulating the release of neurotrophic factors from astrocytes. *Mol Psychiatry* **11**: 1116–1125.

Chen PS, Wang CC, Bortner CD, Peng GS, Wu X, Pang H *et al* (2007). Valproic acid and other histone deacetylase inhibitors induce microglial apoptosis and attenuate lipopolysaccharide-induced dopaminergic neurotoxicity. *Neuroscience* **149**: 203–212.

Danysz W, Parsons CG (2003). The NMDA receptor antagonist memantine as a symptomatological and neuroprotective treatment for Alzheimer's disease: preclinical evidence. *Int J Geriatr Psychiatry* **18**(Suppl 1): S23–S32.

Darlington CL (2005). Astrocytes as targets for neuroprotective drugs. *Curr Opin Investig Drugs* **6**: 700–703.

Di Gennaro E, Bruzzese F, Caraglia M, Abruzzese A, Budillon A (2004). Acetylation of proteins as novel target for antitumor therapy: review article. *Amino Acids* **26**: 435–441.

Finnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA *et al* (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* **401**: 188–193.

Gao HM, Hong JS, Zhang W, Liu B (2002). Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. *J Neurosci* **22**: 782–790.

Hertz L, Zielke HR (2004). Astrocytic control of glutamatergic activity: astrocytes as stars of the show. *Trends Neurosci* **27**: 735–743.

Huang Y, Cheung L, Rowe D, Halliday G (2004). Genetic contributions to Parkinson's disease. *Brain Res Brain Res Rev* **46**: 44–70.

Kordower JH, Palfi S, Chen EY, Ma SY, Sendera T, Cochran EJ *et al* (1999). Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. *Ann Neurol* **46**: 419–424.

Lee ST, Chu K, Jung KH, Kim J, Kim EH, Kim SJ *et al* (2006). Memantine reduces hematoma expansion in experimental intracerebral hemorrhage, resulting in functional improvement. *J Cereb Blood Flow Metab* **26**: 536–544.

Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**: 1130–1132.

Lipton SA (2007). Pathologically-activated therapeutics for neuroprotection: mechanism of NMDA receptor block by memantine and S-nitrosylation. *Curr Drug Targets* **8**: 621–632.

Liu B, Hong JS (2003a). Primary rat mesencephalic neuron–glia, neuron-enriched, microglia-enriched, and astroglia-enriched cultures. *Methods Mol Med* **79**: 387–395.

Liu B, Hong JS (2003b). Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J Pharmacol Exp Ther* **304**: 1–7.

Liu Y, Qin L, Wilson BC, An L, Hong JS, Liu B (2002). Inhibition by naloxone stereoisomers of beta-amyloid peptide (1–42)-induced superoxide production in microglia and degeneration of cortical and mesencephalic neurons. *J Pharmacol Exp Ther* **302**: 1212–1219.

Merello M, Nouzeilles MI, Cammarota A, Leiguarda R (1999). Effect of memantine (NMDA antagonist) on Parkinson's disease: a double-blind crossover randomized study. *Clin Neuropharmacol* **22**: 273–276.

Muller WE, Ushijima H, Schroder HC, Forrest JM, Schatton WF, Rytik PG *et al* (1993). Cytoprotective effect of NMDA receptor antagonists on prion protein (PrionSc)-induced toxicity in rat cortical cell cultures. *Eur J Pharmacol* **246**: 261–267.

Murer MG, Yan Q, Raisman-Vozari R (2001). Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Prog Neurobiol* **63**: 71–124.

Nath A, Haughey NJ, Jones M, Anderson C, Bell JE, Geiger JD (2000). Synergistic neurotoxicity by human immunodeficiency virus proteins Tat and gp120: protection by memantine. *Ann Neurol* **47**: 186–194.

Nutt JG, Burchiel KJ, Comella CL, Jankovic J, Lang AE, Laws Jr ER *et al* (2003). Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. *Neurology* **60**: 69–73.

Obrenovitch TP, Urenjak J, Zilkha E, Jay TM (2000). Excitotoxicity in neurological disorders—the glutamate paradox. *Int J Dev Neurosci* **18**: 281–287.

Parsons CG, Stoffler A, Danysz W (2007). Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system—too little activation is bad, too much is even worse. *Neuropharmacology* **53**: 699–723.

Peterson AL, Nutt JG (2008). Treatment of Parkinson's disease with trophic factors. *Neurotherapeutics* **5**: 270–280.

Phillips HS, Hains JM, Armanini M, Laramee GR, Johnson SA, Winslow JW (1991). BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. *Neuron* **7**: 695–702.

Qin L, Liu Y, Cooper C, Liu B, Wilson B, Hong JS (2002). Microglia enhance beta-amyloid peptide-induced toxicity in cortical and mesencephalic neurons by producing reactive oxygen species. *J Neurochem* **83**: 973–983.

Qin L, Liu Y, Wang T, Wei SJ, Block ML, Wilson B *et al* (2004). NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia. *J Biol Chem* **279**: 1415–1421.

Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS *et al* (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* **55**: 453–462.

Ralay Ranaivo H, Craft JM, Hu W, Guo L, Wing LK, Van Eldik LJ *et al* (2006). Glia as a therapeutic target: selective suppression of human amyloid-beta-induced upregulation of brain proinflammatory cytokine production attenuates neurodegeneration. *J Neurosci* **26**: 662–670.

Reisberg B, Doody R, Stoffler A, Schmitt F, Ferris S, Mobius HJ (2003). Memantine in moderate-to-severe Alzheimer's disease. *N Engl J Med* **348**: 1333–1341.

Rogers J, Mastroeni D, Leonard B, Joyce J, Grover A (2007). Neuroinflammation in Alzheimer's disease and Parkinson's disease: are microglia pathogenic in either disorder? *Int Rev Neurobiol* **82**: 235–246.

Rosi S, Vazdarjanova A, Ramirez-Amaya V, Worley PF, Barnes CA, Wenk GL (2006). Memantine protects against LPS-induced neuroinflammation, restores behaviorally-induced gene expression and spatial learning in the rat. *Neuroscience* **142**: 1303–1315.

Schindowski K, Belarbi K, Buee L (2008). Neurotrophic factors in Alzheimer's disease: role of axonal transport. *Genes Brain Behav* **7**(Suppl 1): 43–56.

Takeuchi H, Mizuno T, Zhang G, Wang J, Kawanokuchi J, Kuno R *et al* (2005). Neuritic beading induced by activated microglia is an early feature of neuronal dysfunction toward neuronal death by inhibition of mitochondrial respiration and axonal transport. *J Biol Chem* **280**: 10444–10454.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K *et al* (1997). Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* **276**: 1699–1702.

Teismann P, Tieu K, Cohen O, Choi DK, Wu DC, Marks D *et al* (2003). Pathogenic role of glial cells in Parkinson's disease. *Mov Disord* **18**: 121–129.

Ushijima H, Ando S, Kunisada T, Schroder HC, Klocking HP, Kijjoa A *et al* (1993). HIV-1 gp120 and NMDA induce protein kinase C translocation differentially in rat primary neuronal cultures. *J Acquir Immune Defic Syndr* **6**: 339–343.

Wei SJ, Trempus CS, Ali RC, Hansen LA, Tennant RW (2004). 12-O-tetradecanoylphorbol-13-acetate and UV radiation-induced nucleoside diphosphate protein kinase B mediates neoplastic transformation of epidermal cells. *J Biol Chem* **279**: 5993–6004.

Weller M, Finiels-Marlier F, Paul SM (1993a). NMDA receptor-mediated glutamate toxicity of cultured cerebellar, cortical and mesencephalic neurons: neuroprotective properties of amantadine and memantine. *Brain Res* **613**: 143–148.

Weller M, Marini AM, Finiels-Marlier F, Martin B, Paul SM (1993b). MK-801 and memantine protect cultured neurons from glutamate toxicity induced by glutamate carboxypeptidase-mediated cleavage of methotrexate. *Eur J Pharmacol* **248**: 303–312.

Wenk GL, Parsons CG, Danysz W (2006). Potential role of *N*-methyl-D-aspartate receptors as executors of neurodegeneration resulting from diverse insults: focus on memantine. *Behav Pharmacol* **17**: 411–424.

Wilcock G, Mobius HJ, Stoffler A (2002). A double-blind, placebo-controlled multicentre study of memantine in mild to moderate vascular dementia (MMM500). *Int Clin Psychopharmacol* **17**: 297–305.

Willard LB, Hauss-Wegrzyniak B, Danysz W, Wenk GL (2000). The cytotoxicity of chronic neuroinflammation upon basal forebrain cholinergic neurons of rats can be attenuated by glutamatergic antagonism or cyclooxygenase-2 inhibition. *Exp Brain Res* **134**: 58–65.

Wu X, Chen PS, Dallas S, Wilson B, Block ML, Wang CC *et al* (2008). Histone deacetylase inhibitors up-regulate astrocyte GDNF and BDNF gene transcription and protect dopaminergic neurons. *Int J Neuropsychopharmacol* **11**: 1123–1134.

Wyss-Coray T (2006). Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nat Med* **12**: 1005–1015.

Zhao X, Marszalec W, Toth PT, Huang J, Yeh JZ, Narahashi T (2006). *In vitro* galantamine–memantine co-application: mechanism of beneficial action. *Neuropharmacology* **51**: 1181–1191.

Supplementary information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)