Neuropharmacology 61 (2011) 574–582

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Amantadine protects dopamine neurons by a dual action: Reducing activation of microglia and inducing expression of GNDF in astroglia

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ARTICLE INFO

Article history:
Received 3 March 2011
Received in revised form
18 April 2011
Accepted 26 April 2011

Keywords:
Parkinson's disease
In vitro model
Neurodegenerative disease
Neuroinflammation
Neuroprotection
MPP^+
LPS

ABSTRACT

Amantadine is commonly given to alleviate L-DOPA-induced dyskinesia of Parkinson's disease (PD) patients. Animal and human evidence showed that amantadine may also exert neuroprotection in several neurological disorders. Additionally, it is generally believed that this neuroprotection results from the ability of amantadine to inhibit glutamatergic NMDA receptor. However, several lines of evidence questioned the neuroprotective capacity of NMDA receptor antagonists in animal models of PD. Thus the cellular and molecular mechanism of neuroprotection of amantadine remains unclear. Using primary cultures with different composition of neurons, microglia, and astroglia we investigated the direct role of these glial cell types in the neuroprotective effect of amantadine. First, amantadine protected rat midbrain cultures from either MPP^+ or lipopolysaccharide (LPS), two toxins commonly used as PD models. Second, our studies revealed that amantadine reduced both LPS- and MPP^+-induced toxicity of dopamine neurons through 1) the inhibition of the release of microglial pro-inflammatory factors, 2) an increase in expression of neurotrophic factors such as GDNF from astroglia. Lastly, differently from the general view on amantadine's action, we provided evidence suggesting that NMDA receptor inhibition was not crucial for the neuroprotective effect of amantadine. In conclusion, we report that amantadine protected dopamine neurons in two PD models through a novel dual mechanism, namely reducing the release of pro-inflammatory factors from activated microglia and increasing the expression of GNDF in astroglia.

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1. Introduction

The unexpected motor symptoms improvement in a Parkinson's disease (PD) patient treated for influenza with amantadine led to the first clinical trial that revealed the potential benefit of this drug in PD (Schwab et al., 1969). Nowadays, amantadine is commonly used in combination with levodopamine (L-DOPA) to reduce the motor disorders of PD patients (Diaz and Waters, 2009). The American Academy of Neurology recommends amantadine to alleviate the L-DOPA-induced dyskinesia due to its long-lasting efficacy (Pahwa et al., 2006; Wolf et al., 2010). In addition, evidence suggests that amantadine may delay the onset and severity of dementia related to PD (Inzelberg et al., 2006; Vale, 2008) similarly to its analogue memantine, which is regularly used for the treatment of Alzheimer's disease (Robinson and Keating, 2006). Despite the structural similarity between these two analogs, memantine fails to improve the motor symptoms of PD and L-DOPA-induced dyskinesia as amantadine does. Compared with the other anti-parkinsonian drugs, amantadine displays fewer adverse effects (Danielczyk, 1995), while Merims and colleagues claimed that amantadine causes no hallucinations in PD patients (Merims et al., 2004). Apart from PD, amantadine may be beneficial in other neurological conditions such as brain trauma (Leone and Polsonetti, 2005) and depression (Rogoz et al., 2007).

The beneficial effect on different neurological disorders suggests that amantadine, in addition to symptoms relieving, may also exert

Abbreviations: PD, Parkinson's disease; DA, dopamine; TH, tyrosine hydroxylase; NMDAr, N-methyl-D-aspartate receptor; LPS, lipopolysaccharide; GDNF, glial cell line-derived neurotrophic factor; TNFα, tumor necrosis factor α; PGE_2, prostaglandine 2; NO, nitric oxide; ChIP, Chromatin Immunoprecipitation; ICC, Immunocytochemistry.

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0028-3908/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.neuropharm.2011.04.030

neuroprotection. For example an indirect evidence of neuroprotection is a retrospective study reporting that parkinsonian patients treated with amantadine lived longer compared to non-treated ones (Uitti et al., 1996). In addition, several *in vitro* and *in vivo* studies revealed that amantadine prevents neuronal death induced by various toxins. For instance, Wenk and colleagues (Wenk et al., 1995) showed that rats treated with amantadine are less susceptible to NMDA-induced neuronal loss in the nucleus basalis magnocellularis. Moreover, amantadine protects retinal ganglion, cortical, and mesencephalic neurons from NMDA-induced toxicity (Chen et al., 1992; Lustig et al., 1992; Weller et al., 1993). Lastly, Rojas and colleagues (Rojas et al., 1992) demonstrated that amantadine prevents the degeneration of the terminals of dopamine (DA) neurons in striatum of MPTP-treated mice. However, it was recently described that amantadine inhibits the brain entry of MPTP (Lin et al., 2010), thus confounding the interpretation of neuroprotective effect of amantadine in the MPTP model.

Early studies suggested that amantadine may be an indirect DA agonist by augmenting the synthesis and reducing the uptake of DA (Lang and Blair, 1989). However, nowadays it is generally believed that amantadine exerts its beneficial effects through uncompetitive inhibition of NMDA receptor (NMDAr) (Danysz et al., 1997). In disagreement, more recent evidence questioned the notion that NMDAr activation is involved in the DA neurodegeneration in PD (Luquin et al., 2006; Matarredona et al., 1997). Accordingly, MK-801, a potent uncompetitive NMDAr blocker, fails to protect mice from MPTP-induced parkinsonian behaviours and DA neuron degeneration (Chan et al., 1997; Sonsalla et al., 1992). Whereas, Wang and colleagues (Wang et al., 2010) demonstrated that pharmacological activation of NMDAr with an agonist (D-cycloserine) protects rodents from MPTP-induced behavioural impairment, neurodegeneration, and neuroinflammation.

Accumulating evidence strongly highlights the role of glia in neurodegenerative disorders. For instance, over-activated microglia may exert a pivotal role in the progression of neurodegenerative disorders (Block et al., 2007), whereas astroglia may serve as the main source of growth factors (Darlington, 2005). Interestingly, Caumont and colleagues (Caumont et al., 2006) reported that amantadine releases GDNF from glioma cells, while Rogŏz and colleagues (Rogoz et al., 2008) demonstrated that amantadine increases the mRNA of BNDF in the cerebral cortex of rats.

Altogether these reports highlight the fact that despite the evidence of neuroprotection in animal and human studies, the underlying cellular and molecular mechanism remains unclear.

In this study we used various *in vitro* midbrain cultures to investigate the direct role of the different glial cell types and their cross-talk with neurons, which would not be possible in an *in vivo* paradigm, in the neuroprotective properties of amantadine. Firstly, we showed that amantadine protected different midbrain cultures challenged with either MPP^+ or lipopolysaccharide (LPS), both of which cause a selective and progressive degeneration of dopamine neurons. Secondly and more importantly, we presented evidence indicating critical roles of microglia and astroglia in mediating the beneficial effects of amantadine. Lastly, we provided evidence suggesting that the neuroprotection produced by amantadine in our culture system is likely NMDAr-independent.

2. Materials and methods

2.1. Chemicals

Amantadine hydrochloride, MPP^+, arabinofuranosyl cytidine (Ara-C), L-leucine methyl ester (LME), mazindol and dopamine (DA), NMDA, and every primer used for RT-PCR were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dihyphenylethylamine, 3, 4-[7-^{3}H] (^{3}H dopamine) was purchased from Perkin Elmer (Boston, MA, USA). Lipopolysaccharide (LPS) was bought from Calbiochem (Darmstadt, Germany). The production of tumor necrosis factor-alpha (TNF-α) was measured using a commercially available ELISA kit from R&D Systems (Minneapolis, MN, USA). Prostaglandin E_2 (PGE2) release was measured using a commercially available ELISA kit from Cayman Chemical Company (Ann Arbor, MI, USA). Chromatin Immunoprecipitation (ChIP) assay kit, anti-acetyl-histone H3, anti-$p47^{phox}$, and anti-mouse antibody were obtained from Millipore (Billerica, MA, USA). Rabbit anti-tyrosine hydroxylase polyclonal antibody was from Chemicon International Inc. (Billerica, MA, USA), while peroxidase or biotinylated labeled anti-rabbit IgG (H + L), and Vectastain ABC kit were bought from Vector Laboratories, Inc. (Burlingame, CA, USA). Antibodies against $p47^{phox}$ and gp91 were bought from BD Bioscience (Franklin Lakes, NJ, USA). SYBR Green PCR Master Mix was purchased from Applied Biosystems, (Carlsbad, CA, USA). Fluo-4 AM was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Animals

Timed-pregnant adult female Fischer 344 rats were obtained from Charles River Laboratories (Raleigh, NC, USA). The use of the animals was in strict accordance with the National Institutes of Health. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Mesencephalic neuron-glia cultures

Rat primary mesencephalic neuron-glia cultures were prepared as described earlier (Gao et al., 2002b). In brief, mesencephalic tissues were dissected from 14-day old embryos and dissociated by gentle mechanical trituration. Cells were suspended in maintenance medium containing minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 10% heat-inactivated horse serum (HS), 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential aminoacids, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were immediately seeded at 5×10^5 cells/well in 24-well plates pre-coated with poly-D-lysine (20 μg/ml) or 1×10^5 cells/well in 96-well plate pre-coated with poly-D-lysine. For treatment, 7-day-old cultures were exposed to various concentrations of amantadine dissolved in treatment medium composed by MEM containing 2% FBS, 2% HS, 2 mM L-glutamine, and 1 mM sodium pyruvate. At the time of the treatment the composition of the cultures was approximately 11% microglia, 48% astroglia, and 41% neurons of which 2.8–3.8% were TH^+ neurons (Gao et al., 2002b).

2.4. Neuron-enriched cultures

Neuron-enriched cultures were prepared by adding 10 μM Ara-C to mesencephalic neuron-glia cultures 55 h after seeding. Cultures were incubated with Ara-C for 48 h after which they were switched to treatment medium containing 1 μM Ara-c and amantadine (30 μM) for 48 h prior to MPP^+ treatment. At the time of the treatment the neuron-enriched culture was composed of at least 92% of neurons (Gao et al., 2002a).

2.5. Microglia-depleted cultures

Neuron-astroglia cultures were prepared by adding 1.5 mM LME, a microglia toxin, to mesencephalic neuron-glia cultures 48 h after seeding. Cultures were incubated with LME for 4 days after which they were switched to treatment medium containing amantadine (30 μM) for 48 h prior to MPP^+ treatment.

2.6. Microglia-enriched cultures

Primary microglia-enriched cultures were prepared from the whole brains of 1–3-day-old rat pups using an earlier described protocol (Gao et al., 2002b). Two weeks after seeding, microglia were shaken off for 30 min at 180 rpm at +37 °C resulting in purity greater than 98% (Gao et al., 2002b). Microglia were seeded at 1×10^5 cells/well in 96-well plates overnight in Dulbecco's modified eagle medium/F12 (DMEM/F12) (1:1) supplemented with 10% FBS. Amantadine was pre-incubated 30 min before the application of LPS in treatment medium.

2.7. Neuron-microglia cultures

Reconstituted neuron-microglia cultures were prepared by adding 10 μM Ara-c to mesencephalic neuron-glia cultures 55 h after seeding. Cultures were incubated with Ara-C for 60 h after which we added 7.5×10^5 cells/well of microglia-enriched culture suspended in treatment medium containing amantadine (30 μM). The resulting neuron-microglia cultures were treated with LPS (10 ng/ml) after 24 h.

2.8. Mixed-glia cultures

Primary mixed-glia cultures were prepared by triturating the encephalon of 1–3-day-old rat pups in maintenance medium. Cells were immediately seeded into 6-well plates at 1×10^6 cells/well and after 3 days they received fresh maintenance

medium. After 6 days of seeding the medium was replaced with treatment medium for additional 24 h to let the culture stabilize with the low serum medium. Afterwards, to reduce any possible cell perturbation, we added a small volume of concentrated amantadine resulting in a final concentration of 30 μM. At the moment of the treatment the culture was composed of approximately 90% of astroglia and 10% of microglia.

2.9. Cell line culture

Hapi cells were cultured in DMEM containing 10% heat-inactivated FBS, 1 g/L glucose, 50 U/mL penicillin, and 50 μg/mL streptomycin. For the experiment, cells were seeded at 3×10^5 cells/well in 6-well plates 24 h before the treatment. Hapi cells were exposed to amantadine (30 μM) 30 min prior to LPS (15 ng/ml) stimulation for 15 min at + 37 °C.

2.10. [^{3}H] DA uptake assay

The [^{3}H] DA uptake assay was performed as described earlier (Gao et al., 2002b). Radioactive DA was determined by liquid scintillation counting using a Beckman Tricarb 2900 TR liquid scintillation counter (Fullerton, CA). Nonspecific DA uptake was determined using mazindol (10 μM) and subtracted.

2.11. Immunocytochemistry (ICC)

ICC was performed as described earlier (Gao et al., 2002b). In brief, cells were fixed for 30 min with 3.7% paraformaldehyde (in PBS). Cells were then rinsed twice with PBS and subsequently incubated with 1% hydrogen peroxide (in PBS) for 10 min. Following two PBS rinses, cells were incubated with blocking solution (PBS containing 1% bovine serum albumin, 0.4% Triton X-100 and 4% normal goat serum) for 20 min. Next, cells were incubated with primary antibody (1:10000; rabbit anti-tyrosine hydroxylase (TH)) at + 4 °C overnight. Cells were then rinsed twice with PBS and incubated with secondary antibody (1:230; goat anti-rabbit) for 1 h. After two washings with PBS, cells were incubated with ABC reagent for 1 h. Cells were rinsed twice with PBS and the staining was visualized using 3,3′-diaminobenzidine.

2.12. TNFα and PGE_2 assay

The production of TNFα and PGE_2 was measured from supernatants collected after 3 and 24 h of LPS treatment, respectively. Commercial kits were used (see Material).

2.13. Nitrite assay

As an indicator of nitric oxide (NO) production, accumulated nitrite was measured after 24 h from culture supernatants using a colorimetric assay and Griess reagent (1% sulfanilamide, 2.5% H3PO4, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) as described previously (Gao et al., 2002a).

2.14. Membrane extraction and western blotting

Hapi cells were scraped off the plate with a hypotonic lysis buffer containing (in mM):1 Tris–HCl, 2 $MgCl_2$, 1 EDTA, 1 EGTA, 1 $NaVO_4$, 1 DTT, protease inhibitor cocktail, pH 7.00 for 30 min on ice and subsequently homogenized with Microtube Pellet Pastel® Motor (Kontes). Samples were then centrifuged for 10 min at 3300 g at +4 °C and the supernatants were further centrifuged at 100000 g for 1 h at +4 °C. The pellets, representing the membrane fractions, were resuspended in a lysis buffer containing 1 mM Tris–HCl, 1% SDS, and protein inhibitor cocktail. Protein samples were separated by an SDS-PAGE, blotted to a PVDF membrane, and visualized with conventional chemiluminescence method using antibody against $p47^{phox}$ (1:1000, in 5% fat-free milk), $p67^{phox}$ (1:1000, in 5% BSA). The protein levels were normalized against $gp91^{phox}$ (1:1000, in 5% BSA) as a membrane loading control using ImageJ (National Institute of Health).

2.15. RT-PCR

Total RNA was extracted from cultures and reverse transcribed with an oligo dT primer. Real-time PCR amplification was performed using SYBR Green PCR Master Mix and a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System according to the manufacturer. The following primers: GDNF F2 (5′-GAGAGAGGAACCGGCAAGCT-3′; 300 nM), GDNF R2 (5′-GTTAAGACGCACCCCCGATT-3′; 300 nM), GAPDH F2 (5′-CCTGGAGAAACCTGCCAAGTAT-3′; 300 nM), and GAPDH R2 (5′-AGCCCAGGATGCCCTTTAGT-3′; 300 nM), were used to amplify GDNF [GenBank: NM019139] and GAPDH [GenBank: NM017008] genes. The PCR conditions were +95 °C for 10 s, +55 °C for 30 s, and +72 °C for 30 s for 40 cycles. The amount of GDNF gene expression was normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method. Values from non-treated groups were set as 100%.

2.16. Chromatin Immunoprecipitation (ChIP) assay

Mixed-glia cultures treated for indicated times were crosslinked with 1% (v/v) formaldehyde in culture medium at RT for 10 min. Cells were then washed, lysed, and sonicated to shred chromatin to about 200–1000 bp in length for further application to ChIP assay with anti-acetyl histone H3 antibodies or non-immune rabbit IgG followed by RT-PCR as described earlier (Wu et al., 2009). The following primers were designed to amplify sequences proximal to the GDNF promoter region [GenBank: AJ011432] (F: 5′-CATGGAAATGGAGCCTAAGTCTGAGAAG-3′; R: 5′-CGC TGCAAGTGGGATGCATTTATAGAG-3′). The PCR conditions were +94 °C for 1 min, +55 °C for 1 min, and +72 °C for 1 min for 35 cycles. Levels of histone modifications at GDNF gene promoter were determined by measuring the amount of that gene in ChIP using of RT-PCR. The values of the ChIP DNA were normalized to the input DNA.

2.17. Measurement of intracellular Ca^{2+}

Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in neuron-enriched cultures were monitored using the single-wavelength calcium indicator, Fluo-4. Neuron-enriched cultures were seeded into 96-well plates and cultured for 5 days as described above. Cells were then bathed in Hank's Balanced Salt Solution supplemented with 2 mM $CaCl_2$ and 10 μM glycine (HBSS), and loaded with Fluo-4/AM (4 μM) for 1 h at RT. Cells were then washed twice with HBSS, after which the 96-well plates were loaded onto a fluorometric imaging plate reader ($FLIPR^{TETRA}$; Molecular Devices, Sunnyvale, CA, USA) so that the fluorescence intensity of Fluo-4 could be monitored simultaneously in all 96-wells. As essentially described previously (Lievremont et al., 2005), excitation of Fluo-4 was performed with a 470–495 nm LED, and emission fluorescence monitored at 515–575 nm with an EMCCD camera. All drug additions were performed simultaneously into each well of the 96-well plate by use of a robotically controlled pipette head formatted for carrying 96-tips.

As is normal practice with single-wavelength calcium indicators such as Fluo-4, all changes in fluorescence intensities (F) were rationed to an average initial fluorescence value (F_0). This procedure compensates for variations in fluorescence values across all 96 wells due to cell plating efficiencies. The resulting output, F/F_0, provides a direct measure of $[Ca^{2+}]_i$ changes (see Supplementary Fig. 1). All experiments were conducted at room temperature.

2.18. Statistics

Values are expressed as means ± SEM. Statistical analyses for significant differences were performed with one-way ANOVA followed by Tukey test using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Amantadine protects DA neurons from MPP^+-induced toxicity

To assess the neuroprotective properties of amantadine, we treated neuron-glia cultures from rat midbrains, containing neurons, microglia and astroglia, with various concentrations of amantadine (10, 20, and 30 μM) 48 h prior to stimulation with MPP^+, the active metabolite of MPTP. MPP^+ is known to be taken up mainly by the dopamine (DA) transporter, causing a selective toxicity to DA neurons. After 7-day exposure to MPP^+, the functionality of DA neurons was assessed using [H^3] DA uptake assay, while immunocytochemically stained TH^+ cells were counted to quantify DA neuron number. Amantadine alone (20 and 30 μM) significantly increased [H^3] DA uptake compared to control and also significantly reduced MPP^+-induced neurotoxicity (Fig. 1A). Moreover, cell count analysis revealed a similar degree of protection of dopamine neurons from MPP^+-elicited damage (Fig. 1B).

3.2. Amantadine protects DA neurons from LPS-induced toxicity

To confirm its protective effect, we tested amantadine in a second *in vitro* model in which neuroinflamation is the main cause for DA degeneration. This model consists of exposing neuron-glia cultures to lipopolysaccharid (LPS), which activates microglia to release pro-inflammatory factors. These factors, in turn, produce neuronal death, especially to DA neurons due to their higher sensitivity to oxidative stress (Gao et al., 2002b).

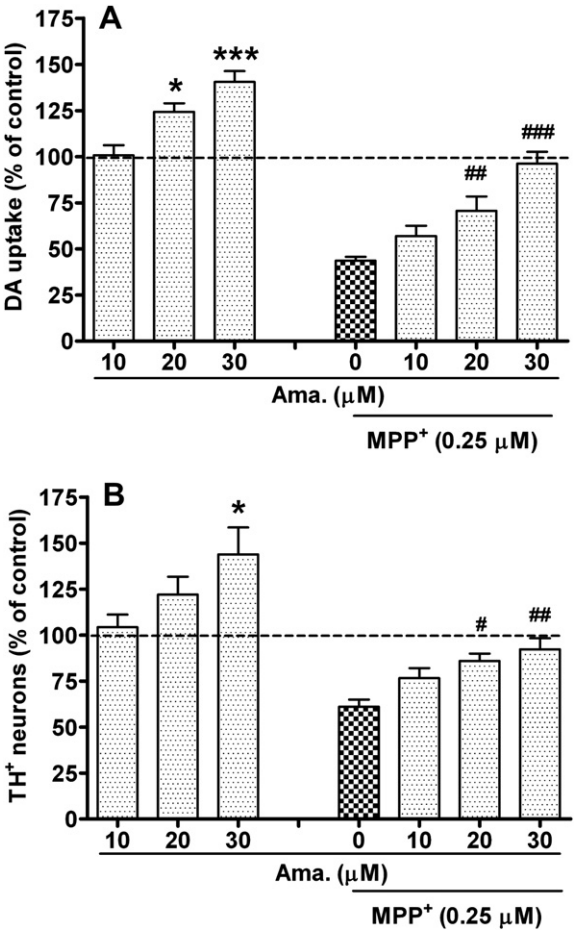

Fig. 1. *Amantadine protects DA neurons from MPP⁺-induced toxicity.* Rat midbrain neuron-glia cultures were exposed to amantadine (Ama 10–30 μM) 48 h prior to stimulation with MPP^+ for 7 days. A) The functional status of DA neurons was quantified by [^{3}H] DA uptake assay. B) The number of TH^+ neurons per well is expressed as % of control. Data represent the mean ± SEM of at least four independent experiments carried out in triplicate. Significantly different from control: *<0.05; ***<0.001. Significantly different from MPP^+ alone: # <0.05; ## <0.01; ### <0.001.

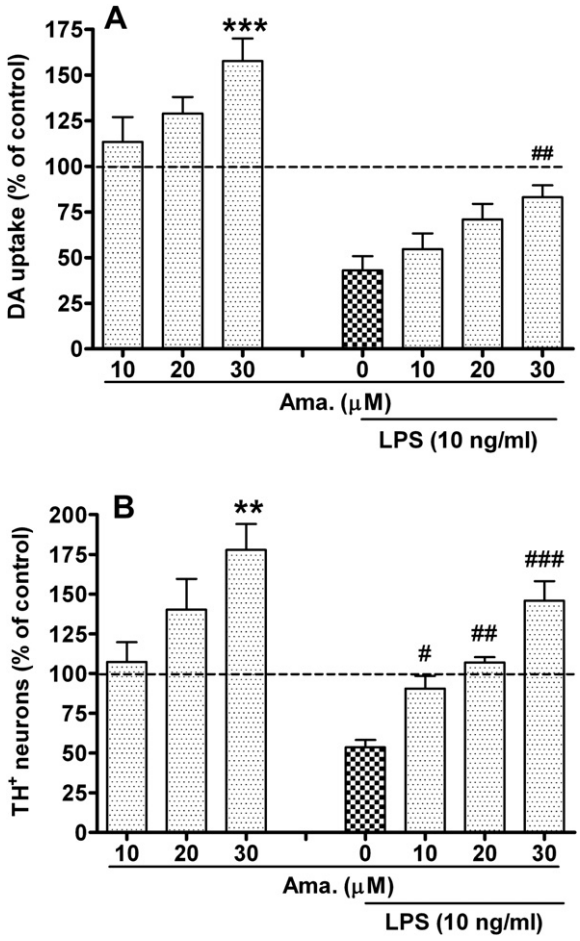

Fig. 2. *Amantadine protects DA neurons from LPS-induced toxicity.* Rat midbrain neuron-glia cultures were exposed to amantadine (Ama 10–30 μM) 48 h prior to stimulation with LPS (10 ng/ml) for 7 days. A) The functional status of DA neurons was quantified by [^{3}H] DA uptake assay. B) The number of TH^+ neurons per well is expressed as % of control. Data represent the mean ± SEM of at least four independent experiments carried out in triplicate. Significantly different from control: **<0.01; ***<0.001. Significantly different from LPS alone: # <0.05; ## <0.01; ### <0.001.

Neuron-glia cultures were treated with various concentrations of amantadine (10, 20, and 30 μM) 48 h prior to stimulation with LPS. After 7 days, the functionality of DA neurons was assessed using [H^3] DA uptake assay, while TH-ICC was used to determine the number of DA neurons. Amantadine (30 μM) significantly increased [H^3] DA uptake compared to control and significantly protected DA neurons against LPS-induced toxicity (Fig. 2A). ICC analysis revealed an increase in the number of TH^+ neurons in cultures treated with 30 μM amantadine alone compared to control (Fig. 2B). In every tested concentration, amantadine significantly increased the number of TH^+ neurons in cultures exposed to LPS (Fig. 2B).

3.3. Glial cells mediate the neuroprotective effects of amantadine

To determine the cell types mediating the neuroprotective effects of amantadine we used cultures containing different combinations of neurons and glial cells.

In neuron-enriched cultures (92% purity) amantadine significantly protected the functionality of DA neurons from MPP^+ toxicity by 1.4 folds. However, amantadine alone failed to increase the [H^3] DA uptake when compared to control.

To study the effect of amantadine on activated microglia-induced toxicity, we added microglia to neuron-enriched cultures (15% microglia, 85% neurons) followed by LPS stimulation. In this culture amantadine protected DA neurons from the toxin by 1.9 folds, greater than that found in neuron-enriched culture (1.4 folds), while exerting no effect alone (Fig. 3).

To investigate the astroglial contribution to amantadine's neuroprotective properties, we selectively depleted microglia from the neuron-glia culture by adding L-leucine methyl ester (LME), a microglia toxin. In this condition, amantadine significantly prevented the loss of DA function caused by MPP^+ by 2.3 folds and also amantadine alone significantly increased [H^3] DA uptake when compared to the control (Fig. 3).

3.4. Amantadine attenuates LPS-induced production of pro-inflammatory factors

Activated microglia produce an array of pro-inflammatory factors that mediate LPS-induced DA neurotoxicity. To elucidate the effect of amantadine on the release of these pro-inflammatory factors, different cultures, namely neuron-glia and neuron-microglia, were treated with amantadine prior to stimulation with LPS. Supernatants were collected from the cultures at 3 h (for TNFα) and 24 h (for NO), which reflect the peak release of each individual factor. Amantadine significantly decreased both TNFα and NO

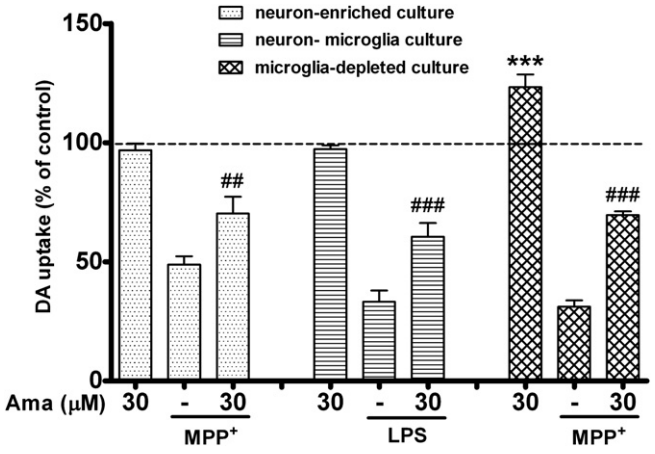

Fig. 3. *Glial cells mediate the neuroprotective effects of amantadine.* Amantadine (Ama 30 μM) was applied 48 h prior to stimulation with MPP^+ (for 7 days) or 24 h prior to stimulation with LPS (10 ng/ml, for 4 days). The following cultures were used: neuron-enriched culture, microglia-depleted culture, and reconstituted neuron-microglia coculture prepared by adding 7.5×10^5 cells/well of enriched microglia to neuron-enriched culture. The functional status of DA neurons was quantified by [^{3}H] DA uptake assay. Data represent the mean ± SEM of at least five independent experiments carried out in triplicate. Significantly different from control: ***<0.001. Significantly different from MPP^+ or LPS alone: ## <0.01; ### <0.001.

release in both neuron-glia and neuron-microglia cultures (Fig. 4). Together, these results suggest that inhibition of LPS-induced release of pro-inflammatory factors plays a role in amantadine-elicited neuroprotection.

3.5. Inhibition of microglia NADPH oxidase is associated with the anti-inflammatory action of amantadine

To assess the direct anti-inflammatory action of amantadine on microglia, we used microglia-enriched cultures and Hapi cells (immortalized microglia). Fig. 5A and B illustrate the attenuation by amantadine of the release of cytokines such as TNFα and PGE_2 from microglia-enriched culture stimulated with LPS. All concentrations of amantadine tested caused no decreased viability of microglia-enriched cultures (data not shown). To further study how amantadine reduces microglial activation, we investigated the activation of NADPH oxidase (Phox), the major superoxide-producing enzyme in all phagocytes including microglia. After LPS stimulation the cytosolic subunits of Phox such as $p47^{phox}$ and $p67^{phox}$ translocate to the plasma membrane where they interact with gp91 (the catalytic subunit of Phox) to produce superoxide, which in turn increases the release of pro-inflammatory factors (Block, 2008). Amantadine treatment significantly prevented the translocation of $p47^{phox}$ and $p67^{phox}$ in Hapi cells exposed to LPS for 15 min (Fig. 5C and D).

3.6. Amantadine induces the expression of GDNF in astroglia

Results from Fig. 3 indicated an increase of [H^3] DA uptake by amantadine alone in microglia-depleted neuron-glia cultures (equivalent to neuron-astroglia cultures), suggesting that astroglia may play a role in the enhancement of DA neuron survival. For this reason we determined whether amantadine would stimulate expression of growth factors such as GDNF in astroglia. We used

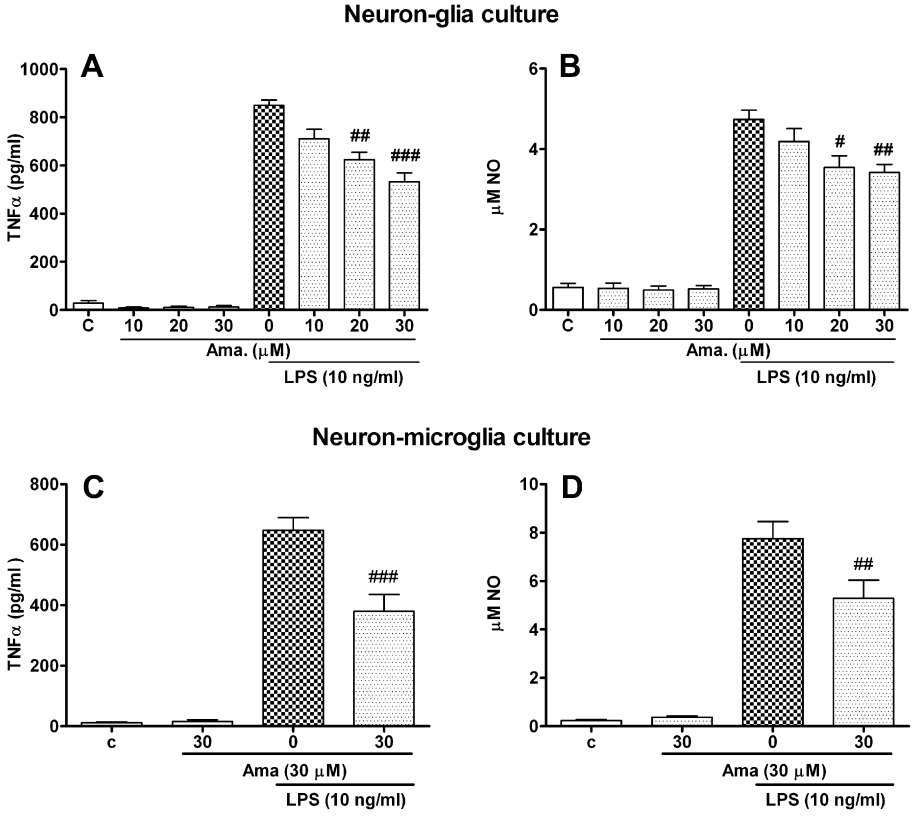

Fig. 4. *Amantadine attenuates LPS-induced production of pro-inflammatory factors.* A–B) Neuron-glia cultures were exposed to amantadine (Ama 10–30 μM) 48 h prior to stimulation with LPS (10 ng/ml). C–D) Neuron-microglia cultures were exposed to amantadine (Ama 30 μM) 24 h prior to stimulation with LPS. NO production was measured using Griess reagent, while TNFα production was measured by ELISA kit. Supernatants were collected at 3 h for TNFα and at 24 h for NO. Data represent the mean ± SEM of at least five independent experiments. Significantly different from LPS alone: #<0.05; ##<0.01; ### <0.001.

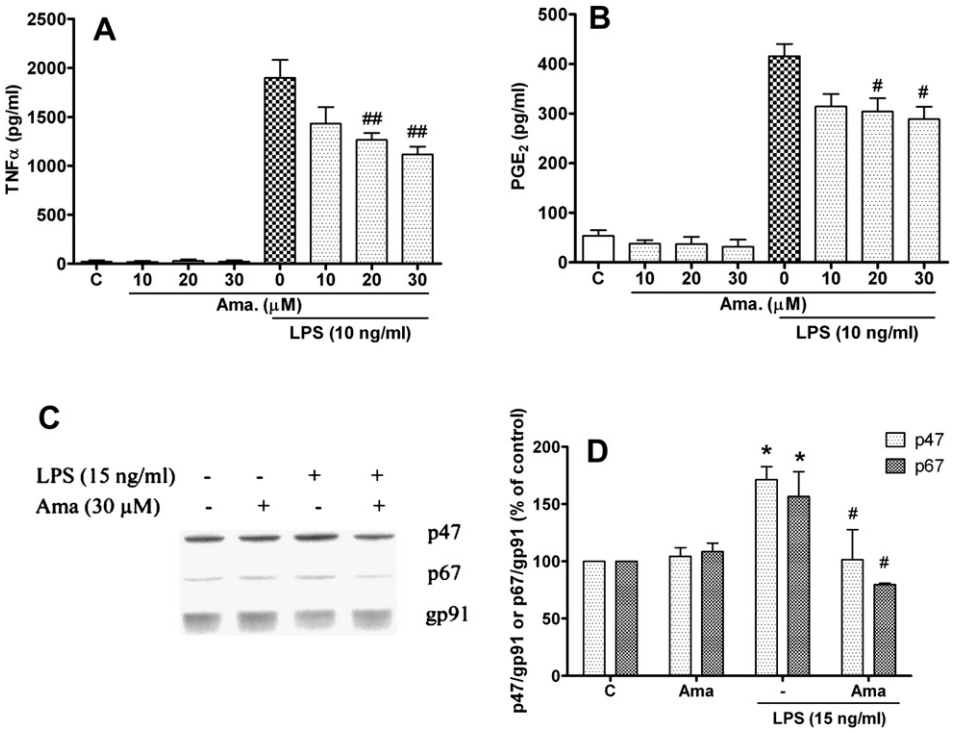

Fig. 5. *Inhibition of microglial NADPH oxidase is associated with the anti-inflammatory action of amantadine.* A–B) Microglia-enriched cultures or C–D) Hapi cells were exposed to amantadine (Ama 30 μM) 30 min prior to stimulation with LPS. Supernatants were collected at 3 h for TNFα and at 24 h for PGE_2, protein concentrations were measured by ELISA kits. C) Hapi cells were lysed after 15 min exposure to LPS and proteins bond to the membranes were extracted and visualized by western blotting. D) Quantification of the levels of $p47^{phox}$ and $p67^{phox}$ was normalized against the level of $gp91^{phox}$. Data represent the mean ± SEM of at least five (A–B) or three (D) independent experiments. Significantly different from control: *<0.05, while significantly different from LPS alone: #<0.05; ##<0.01.

a mixed-glia culture, containing about 90% astroglia and 10% microglia, to measure amantadine-induced mRNA of GNDF, because astroglia prepared in this way are more responsive to amantadine-induced GDNF expression action. Indeed, amantadine significantly increased the expression of GNDF after 48 and 72 h of treatment (Fig. 6A). A similar result was observed when microglia were depleted from the mixed-glia culture with LME before the exposure to amantadine (data not shown). To further examine the possible mechanism underlying the induction of GDNF expression, we measured the level of acetylated histone protein that interacts with the promoter region of GDNF using ChIP assay. Fig. 6B illustrates that amantadine significantly increased the amount of acetylated histone H3 bound to the promoter region of GDNF after 12 h. Therefore, amantadine may increase GDNF expression by inducing the acetylation of histone H3, and possibly by inhibiting the histone deacetylase, as we previously reported for the amantadine analog memantine (Wu et al., 2009).

3.7. *NMDA receptor inhibition is not crucial for the neuroprotective effect of amantadine*

As previously mentioned, the neuroprotective effects of amantadine have been generally believed to be associated with the inhibition of NMDAr (Danysz et al., 1997). However, we hypothesized that the neuroprotection observed in our culture system is independent from the blockade of NMDAr. To test this hypothesis, we determined the potency of amantadine to block NMDA-induced [H^3] DA uptake loss in our neuron-glia culture. Fig. 7A illustrates that only a high concentration of amantadine (100 μM) significantly protected DA neurons from NMDA (EC_{50} 91 μM). Whereas, in the same system, amantadine protected from MPP^+ and LPS at lower concentrations (EC_{50} of 18 and 21 μM, respectively) (Figs. 1 and 2). Therefore, amantadine appears to more potently (4–5 folds) protect from the toxicity caused by MPP^+ and LPS than from that caused by NMDA. To further test this hypothesis, we investigated the ability of amantadine to block NMDA-induced Ca^{2+} signals in a neuron-enriched culture using Fluo-4 as a Ca^{2+} sensitive indicator (Supplementary Fig. 1). NMDA-induced an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) that was elevated and sustained for the duration of the measurement time (15 min) (Supplementary Fig. 1C). The effect of NMDA was almost completely blocked by pre-incubating neuronal cell cultures with MK-801 (1 μM), a well known uncompetitive NMDAr blocker (Fig. 6B, and Supplementary Fig. 1C). In contrast, pre-incubating neurons with 30 μM amantadine had no significant effect on the NMDA-induced Ca^{2+} signal (Fig. 7B). Significant but limited reduction of NMDA-induced Ca^{2+} signals was only observed with high concentrations of amantadine (≥50 μM, Fig. 6B). Lastly, we investigated the role of NMDAr activation in neuron-glia cultures treated with MPP^+. Here, the presence of MK-801, at concentrations effective against NMDA-induced Ca^{2+} signals (Fig. 7B) and DA neurons loss (data not illustrated), failed to protect DA neurons from MPP^+ toxicity (Fig. 7C). Thus, these data indicate that the activation of NMDAr plays no role in DA degeneration in our *in vitro* system.

4. Discussion

This study offers direct evidence of amantadine's neuroprotection of DA cells against neurotoxins commonly used in PD models such as MPP^+ and LPS. More importantly, our data highlight the critical roles of microglia and astroglia in mediating the beneficial effects of amantadine. In addition, we showed evidence indicating that the neuroprotective properties of amantadine observed in our *in vitro* system are mainly NMDAr-independent.

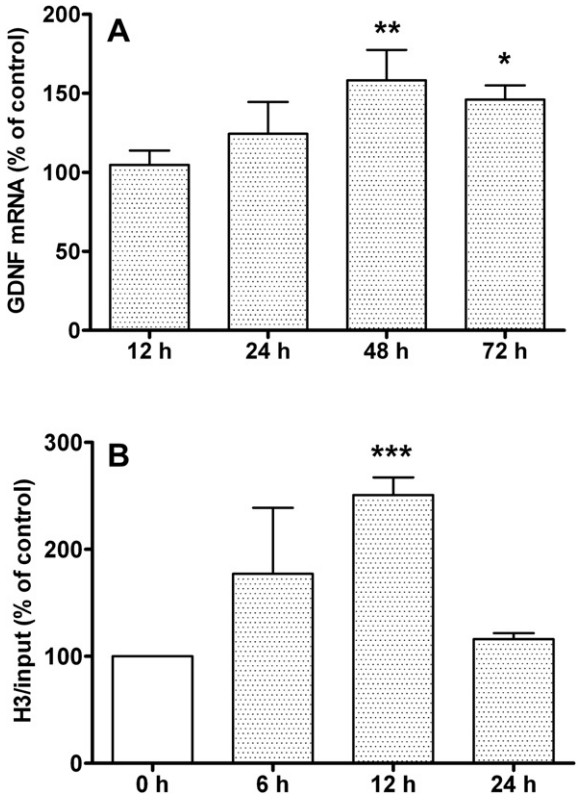

Fig. 6. *Amantadine induces the expression of GDNF in astroglia.* Mixed glia-cultures, containing about 90% astroglia and 10% microglia, were exposed to amantadine (Ama 30 μM) for various time points. A) Total RNA was extracted, reverse transcribed, and used for quantitative real-time PCR. Results are expressed as % of non-treated cells at corresponding time after normalization with GAPDH. The data represent the mean ± SEM of at least six independent experiments. Significantly different from control: *<0.05; **<0.01. B) The level of acetylated histone 3 bond to GDNF promoter was measured by ChIP assay and normalized against the input DNA (see *Methods*). The data represent the mean ± SEM of three independent experiments. Significantly different from 0 h: ***<0.001.

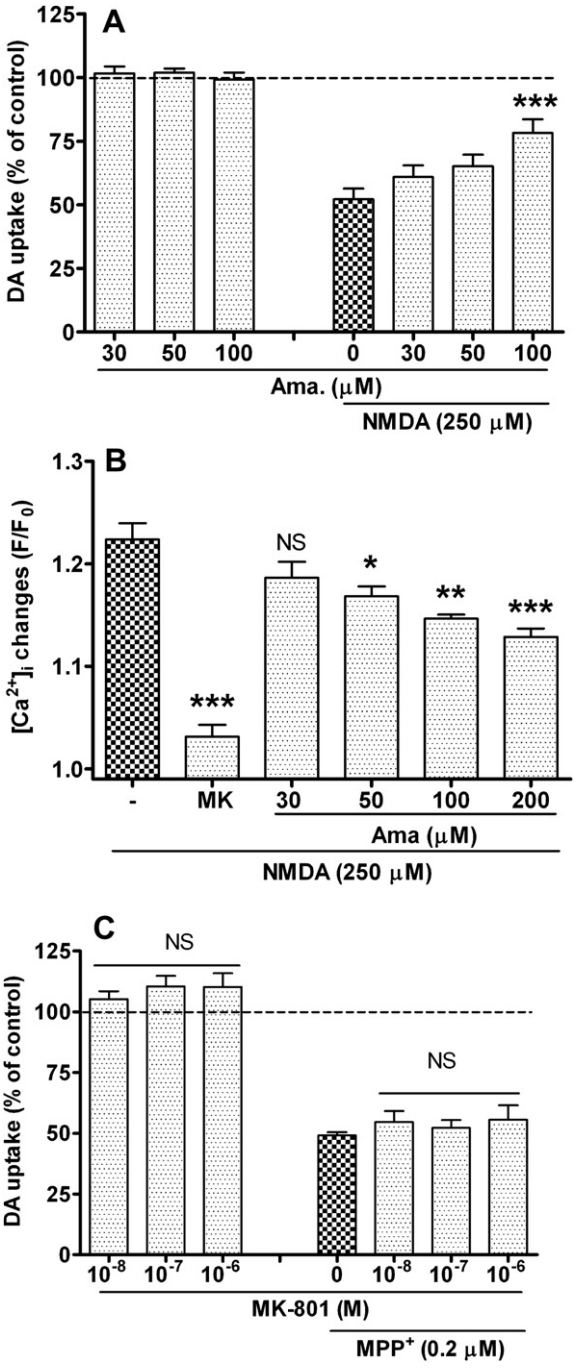

Fig. 7. *NMDA receptor inhibition is not crucial for the neuroprotective effect of amantadine.* A) Rat midbrain neuron-glia or B) neuron-enriched cultures were exposed to amantadine (Ama) 30 min prior to stimulation with NMDA (250 μM). A) The functional status of DA neurons was quantified by [^{3}H] DA uptake assay after 24 h exposure to NMDA. B) The data represent the elevated intracellular Ca^{2+} signals (F/F_0) in Fluo-4-loaded neuronal cells after a 15 min exposure to NMDA (250 μM) in the absence or presence of either MK-801 (1 μM) or amantadine (see Supplementary Fig. 1). C) Neuron-glia cultures were exposed to MK-801 (0.01–1 μM) 30 min prior to MPP^+. The functional status of DA neurons was quantified by [^{3}H] DA uptake assay after 7 day exposure to MPP^+. Data represent the mean ± SEM of at least four independent experiments. Significantly different from NMDA alone: *<0.05; **<0.01; ***<0.001.

Most of the previous studies on amantadine were performed in animals or using neuron-enriched cultures. In order to understand the different cell type interactions and to elucidate the molecular mechanism underlying the neuroprotective effect of amantadine, we used an *in vitro* system containing the three main CNS cell types, namely neurons, microglia, and astroglia. Importantly, these coculture systems allowed us to study the role of glia in amantadine's beneficial effects and their cross talk to neurons, which would not be possible in an animal study.

In neuron-glia culture, containing *neurons and both glia types*, amantadine significantly protected both the functionality and the cell number of DA neurons from either MPP^+ or LPS (Fig. 1). This neuroprotection occurred at 20 and 30 μM (in one case at 10 μM, Fig. 2b), which are concentrations lower than previously reported in other systems (Chen et al., 1992; Weller et al., 1993). In addition, amantadine alone significantly increased the number of DA neurons and their functionality when compared to controls.

In reconstituted neuron-microglia culture, containing only *neurons and microglia*, amantadine significantly increased [H^3] DA uptake by 1.9 folds compared to LPS alone (Fig. 3). Moreover, amantadine significantly reduced LPS-induced release of pro-inflammatory factors such as TNFα, PGE_2, and NO in neuron-glia, neuron-microglia, and microglia-enriched cultures (Figs. 4 and 5). Finally, we revealed that amantadine blocked the activation of Phox, an enzyme involved in the pro-inflammatory phenotype of microglia (Fig. 5 B and C). Together these results highlight the direct anti-inflammatory properties of amantadine in neurodegeneration caused by microglia activation. These data agree with a recent report suggesting that amantadine decreases the pro-inflammatory properties of activated macrophages and redirect them towards a more anti-inflammatory phenotype (Roman et al., 2009).

In microglia-depleted cultures, which contain only *neurons and astroglia*, amantadine (30 μM) significantly protected DA neurons from MPP^+-induced loss of [H^3] DA uptake by 2.3 folds compared to MPP^+ alone (Fig. 3). This protection was clearly more prominent than the one observed in neuron-enriched cultures. Moreover, in microglia-depleted cultures amantadine alone significantly increased the functionality of DA neurons when compared to control (Fig. 3). Thus, these data suggest that the presence of astroglia clearly increases the neuroprotective effects of amantadine. Astroglia are an important source of growth factors such as GDNF family members, which protect neurons from various insults (Darlington, 2005; Wu et al., 2009). In this study, we found that amantadine significantly increased the expression of GDNF in mixed-glia culture, in which most of the growth factors are produced from astroglial cells that represent more than 90% of the total cell population (Fig. 6). This finding is in line with previous reports showing that amantadine increases the expression of GDNF in glioma cells (C6) and that of BDNF in cerebral cortex of rats (Caumont et al., 2006; Rogoz et al., 2008). Therefore, our data suggest that a significant part of the neuroprotective effects of amantadine may be due to an increased expression of GDNF in astroglia.

We recently reported that memantine similarly to amantadine exerts anti-inflammatory action and induces GDNF (Wu et al., 2009). However, amantadine and memantine, although structurally similar, exhibit markedly different effects *in vivo*. For example, memantine, but not amantadine, stimulates locomotion in both naïve animals and in hypokinesia model, as well as inducing ipsilateral rotation in unilateral lesioned rats (Danysz et al., 1997). On the other hand, amantadine, but not memantine, increases noradrenalin release, is beneficial in catalepsy model, and improves L-DOPA-induced dyskinesia in both animals and humans (Danysz et al., 1997). Therefore, the findings on the neuroprotective actions of memantine cannot be assumed to be true for amantadine without experimental evidence. Thus, it was essential to show experimentally that amantadine exerted neuroprotection through anti-inflammation and GDNF inducing properties.

We believe that our study offers a novel explanation for some of the positive clinical effects of amantadine in PD and other neurological disorders. We suggest that the neuroprotective actions of amantadine resulted mainly from amantadine's anti-inflammatory and growth factor inducing properties, rather than from the inhibition of NMDAr. To support this argument, we demonstrated that in our neuron-glia culture amantadine was 4–5 folds less potent in blocking the toxic effect of NMDA than those caused by MPP^+ and LPS (Fig. 7A). Also, the highest concentration of amantadine used in the neuroprotective study (30 μM) failed to reduce NMDA-induced Ca^{2+} signals, while only higher concentrations ($\geq$50 μM) caused a significant but limited reduction (Fig. 7B). Lastly, we believe that in our system the activation of NMDAr after stimulation with MPP^+ is unlikely, since the efficient inhibition of NMDAr by MK-801 failed to prevent MPP^+ toxicity (Fig. 6C). Similarly, the NMDAr blocker dizocilpine fails to prevent the DA neurodegeneration caused by LPS, which by itself evokes no increase of excitatory aminoacids such as glutamate and aspartate in the culture medium (Liu et al., 2003; Wu et al., 2009). In agreement with this hypothesis, Caumont and colleagues demonstrated that uncompetitive NMDA blockers such as MK-801, memantine, and amantadine, despite their markedly different NMDA blocking potency, induce the expression of GDNF from C6 cells with a similar EC_{50} (Caumont et al., 2006). Thus, the amantadine-induced GDNF expression in our study and others is most likely NMDAr-independent.

Interestingly, few studies argued that amantadine may work differently from other NAMDr uncompetitive antagonists. For instance, Otton and colleagues recently shed some doubts on the relevance of amantadine inhibition of NMDAr at clinical dosage (Otton et al., 2010). Specifically, the authors suggested that previous patch-clamp studies carried out in the absence of Mg^{2+} may have overestimated the antagonistic effect of amantadine and memantine on NMDAr. In fact, the addition of physiological concentration of Mg^{2+} (1 mM) reduced the inhibitory potency of amantadine and memantine on NMDAr from 49 and 1 μM to 165 and 6.6 μM, respectively (Otton et al., 2010).

Few studies reported the brain extracellular concentration of amantadine after acute or semi-chronic application (7 days) with results ranging from 2 to 23 μM in rat striatum (Hesselink et al., 1999; Kornhuber et al., 1995; Quack et al., 1995). In humans, amantadine is excreted almost completely unchanged, whilst in rats more than 80% of an oral dose of the drug is metabolized (Bleidner et al., 1965). Consequently, this greater metabolic capacity of rats compared to human leads to a shorter half life and probably to a reduced amantadine penetration of the BBB. Thus, the extracellular amount of amantadine in human brain is most likely several folds higher than the one in rats, making the concentrations used in this study (10–30 μM) clinically relevant.

5. Conclusions

In conclusion, this study demonstrated that amantadine-elicited neuroprotection in two *in vitro* PD models is dependent on a dual novel mechanism, namely reducing the release of pro-inflammatory factors from activated microglia and increasing the expression of GNDF from astroglia. Importantly these data also highlight critical roles of glial cells as targets for developing new strategies to alter the progression of neurodegenerative disorders.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgements and funding

We thank Mr. John Petranka and Jeff Tucker for helping to setup the measurement of Ca^{2+} signals. We thank Dr. Qingshan Wang for helping with the p47 and p67 translocation study. We thank Dr. Huiming Gao for valuable discussions about the preparation of the different cultures. This work was supported by the Finnish Parkinson Foundation and Ella and Georg Ehrnrooth Foundation. This research was also supported in part by the Intramural Research Program of the National Institute of Health, National Institute of Environmental Health Sciences. The funding sources had no involvement in this study.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuropharm.2011.04.030.

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