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

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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 neuroprotection 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 different 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 PD models. Second, our studies revealed that amantadine reduced both LPS- and MPP⁺-induced toxicity of dopamine neuron through 1) the inhibition of the release of microglial pro-inflammatory factors, 2) an increase in expression of neurotrophic factor 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 GDNF in astroglia.

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Conflict of interests

The authors declare that they have no conflict of interests.

Keywords

Parkinson's disease; *in vitro* model; neurodegenerative disease; neuroinflammation; neuroprotection; MPP⁺; LPS

1 Introduction

The unexpected motor symptoms improvement in a Parkinson's diseases (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 antiparkinsonian 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 deferent neurological disorders suggests that amantadine, in addition to symptoms reliving, may also exert 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 neurons 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 Rogöz and colleagues (Rogoz et al., 2008) demonstrated that amantadine increases the mRNA of BDNF 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 NMDA-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-[7-³H] (³H dopamine) was purchased from Perkin Elmer (Boston, MA, USA). Lipopolysaccharide (LPS) was bought from Calbiochem (Darmstadt, Germany). The production of tumor necrosis factor- α (TNF- α) was measured using a commercially available ELISA kit from R&D Systems (Minneapolis, MN, USA). Prostaglandin E₂ (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-histon 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). Antibody 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 μ M non-essential aminoacids, 50 U/mL penicillin, and 50 μ g/mL streptomycin. Cells were immediately seeded at 5×10^5 cells/well in 24-well plates pre-coated with poly-D-lysine (20 μ g/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 μ M 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 μ M Ara-c and amantadine (30 μ M) 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 Neuron-astroglia 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 μ M) 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 was shaken off for 30 min at 180 rpm at + 37°C resulting in purity greater than 98% (Gao et al., 2002b). Microglia was 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 μ M 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 μ M). 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 stabilized 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 μ M. At the moment of the treatment the culture was composed by approximately 90% of astroglia and 10% of microglia.

2.9 Cell line culture

Hapi cell line was 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 [³H] DA uptake assay

The [³H] DA uptake assay was performed as described earlier (Gao et al., 2002b). Radioactive DA was determined by liquid scintillation counting using a Beckman Tri-carb 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 PGE₂ Assay

The production of TNFα and PGE₂ 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 MgCl₂, 1 EDTA, 1 EGTA, 1 NaVO₄, 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 a 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'-GTTAAGACGCACCCCGATT-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 sec, + 55°C for 30 sec, and + 72°C for 30 sec for 40 cycles. The amount of GDNF gene expression was normalized to GAPDH using the $2^{-\Delta\Delta C_t}$ 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'-CGCTGCAAGTGGGATGCATTTATAGAG-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+} ($[\text{Ca}^{2+}]_i$) 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 of well 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 $[\text{Ca}^{2+}]_i$ changes (see supplementary figure 1). All experiments were conducted at room temperature.

2.18 Statistics

Values are expressed as means $\pm$ 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 μ M) 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 μ M) significantly increased [H^3] DA uptake compared to control and also significantly reduced MPP⁺-induced neurotoxicity (Fig. 1A). Moreover, cell count analysis revealed the 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 neuroinflammation is the main cause for DA degeneration. This model consists of exposing neuron-glia cultures to lipopolysaccharide (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).

Neuron-glia cultures were treated with various concentrations of amantadine (10, 20, and 30 μ M) 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 μ M) 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 μ M 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 TNF α) and 24 h (for NO), which reflect the peak release of each individual factor. Amantadine significantly decreased both TNF α and NO 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 play 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. 5 A and B illustrate the attenuation by amantadine of the release of cytokines such as TNF α 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 increase 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 from 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 for 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 a mixed-glia culture, containing about 90% astroglia and 10% microglia, to measure amantadine-induced mRNA of GDNF, because astroglia prepared in this way is more responsive to amantadine-induced GDNF expression action. Indeed, amantadine significantly increased the expression of GDNF after 48 and 72 h of treatment (Fig. 6A). A similar result was observed when microglia was depleted by 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. 6 B 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. 7 A illustrates that only a high concentration of amantadine (100 μ M) significantly protected DA neurons from NMDA (EC_{50} 91 μ M). Whereas, in the same system, amantadine protected from MPP⁺ and LPS at lower concentrations (EC_{50} of 18 and 21 μ M, respectively) (Fig 1 and 2). Therefore, amantadine appears to more potently (4–5 folds) protect from the toxicity caused by MPP⁺ and LPS than 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 μ M), a well known uncompetitive NMDAr blocker (Fig. 6B, and Supplementary Fig 1C). In contrast, pre-incubating neurons with 30 μ M 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 μ M, Fig. 6B). Lastly, we investigated the role of NMDAr activation in neuron-glia cultures treated with MPP⁺. Here, the presence of MK-801, at effective concentrations against NMDA-induced both 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 a 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.

Most of the previously 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 μ M (in one case at 10 μ M, 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 TNF α , PGE₂, and NO in neuron-glia, neuron-microglia, and microglia-enriched cultures (Fig. 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 μ M) significantly protected DA neurons from MPP⁺-induced loss of [H³] 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 BDNF in cerebral cortex in 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-inflammation 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 μ M) failed to reduce NMDA-induced Ca²⁺ signals, while only higher concentrations (≥ 50 μ M) caused a significant but limited reduction (Fig. 7B). Lastly, we believe that in our system the activation of NMDAr after stimulation with either 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 amino acids 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 EC₅₀ (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 NMDAr 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 of NMDAr from 49 and 1 μ M to 165 and 6.6 μ M, 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 μ M 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 μ M) 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 GDNF 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.

Highlights

- Amantadine protects DA neurons in two PD models *in vitro*
- Amantadine inhibits microglia activation
- Amantadine induces the expression of GDNF in astroglia
- NMDA receptor inhibition is not crucial for amantadine neuroprotection
- Glia is a promising target for treatment of PD

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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
PGE₂	prostaglandine 2

NO	nitric oxide
ChIP	Chromatin Immunoprecipitation
ICC	Immunocytochemistry

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References

- Bleidner WE, Harmon JB, Hewes WE, Lynes TE, Hermann EC. Absorption, distribution and excretion of amantadine hydrochloride. *J. Pharmacol. Exp. Ther.* 1965; 150:484–490. [PubMed: 4954953]
- Block ML. NADPH oxidase as a therapeutic target in alzheimer's disease. *BMC Neurosci.* 2008; 9 Suppl 2:S8. [PubMed: 19090996]
- Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: Uncovering the molecular mechanisms. *Nat. Rev. Neurosci.* 2007; 8:57–69. [PubMed: 17180163]
- Caumont AS, Octave JN, Hermans E. Amantadine and memantine induce the expression of the glial cell line-derived neurotrophic factor in C6 glioma cells. *Neurosci. Lett.* 2006; 394:196–201. [PubMed: 16298481]
- Chan P, Di Monte DA, Langston JW, Janson AM. (+)MK-801 does not prevent MPTP-induced loss of nigral neurons in mice. *J. Pharmacol. Exp. Ther.* 1997; 280:439–446. [PubMed: 8996226]
- Chen HS, Pellegrini JW, Aggarwal SK, Lei SZ, Warach S, Jensen FE, Lipton SA. Open-channel block of N-methyl-D-aspartate (NMDA) responses by memantine: Therapeutic advantage against NMDA receptor-mediated neurotoxicity. *J. Neurosci.* 1992; 12:4427–4436. [PubMed: 1432103]
- Danielczyk W. Twenty-five years of amantadine therapy in parkinson's disease. *J. Neural Transm.* 1995; 46 Suppl.:399–405.
- Danysz W, Parsons CG, Kornhuber J, Schmidt WJ, Quack G. Aminoadamantanes as NMDA receptor antagonists and antiparkinsonian agents--preclinical studies. *Neurosci. Biobehav. Rev.* 1997; 21:455–468. [PubMed: 9195603]
- Darlington CL. Astrocytes as targets for neuroprotective drugs. *Curr. Opin. Investig Drugs.* 2005; 6:700–703.
- Diaz NL, Waters CH. Current strategies in the treatment of parkinson's disease and a personalized approach to management. *Expert Rev. Neurother.* 2009; 9:1781–1789. [PubMed: 19951137]
- Gao HM, Hong JS, Zhang W, Liu B. Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. *J. Neurosci.* 2002a; 22:782–790. [PubMed: 11826108]
- Gao HM, Jiang J, Wilson B, Zhang W, Hong JS, Liu B. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: Relevance to parkinson's disease. *J. Neurochem.* 2002b; 81:1285–1297. [PubMed: 12068076]
- Hesselink MB, De Boer BG, Breimer DD, Danysz W. Brain penetration and in vivo recovery of NMDA receptor antagonists amantadine and memantine: A quantitative microdialysis study. *Pharm. Res.* 1999; 16:637–642. [PubMed: 10350004]
- Inzelberg R, Bonuccelli U, Schechtman E, Miniowich A, Strugatsky R, Ceravolo R, Logi C, Rossi C, Klein C, Rabey JM. Association between amantadine and the onset of dementia in parkinson's disease. *Mov. Disord.* 2006; 21:1375–1379. [PubMed: 16705684]
- Kornhuber J, Quack G, Danysz W, Jellinger K, Danielczyk W, Gsell W, Riederer P. Therapeutic brain concentration of the NMDA receptor antagonist amantadine. *Neuropharmacology.* 1995; 34:713–721. [PubMed: 8532138]

- Lang, AE.; Blair, RD. Anticholinergic drugs and amantadine in the treatment of parkinson's disease. In: Calne, DB., editor. *Drugs for the treatment of parkinson's disease*. New York: Springer-Verlag; 1989. p. 307-323.
- Leone H, Polsonetti BW. Amantadine for traumatic brain injury: Does it improve cognition and reduce agitation? *J. Clin. Pharm. Ther.* 2005; 30:101–104. [PubMed: 15811161]
- Lievremont JP, Bird GS, Putney JW Jr. Mechanism of inhibition of TRPC cation channels by 2-aminoethoxydiphenylborane. *Mol. Pharmacol.* 2005; 68:758–762. [PubMed: 15933213]
- Lin CJ, Tai Y, Huang MT, Tsai YF, Hsu HJ, Tzen KY, Liou HH. Cellular localization of the organic cation transporters, OCT1 and OCT2, in brain microvessel endothelial cells and its implication for MPTP transport across the blood-brain barrier and MPTP-induced dopaminergic toxicity in rodents. *J. Neurochem.* 2010; 114:717–727. [PubMed: 20477935]
- Liu Y, Qin L, Li G, Zhang W, An L, Liu B, Hong JS. Dextromethorphan protects dopaminergic neurons against inflammation-mediated degeneration through inhibition of microglial activation. *J. Pharmacol. Exp. Ther.* 2003; 305:212–218. [PubMed: 12649371]
- Luquin MR, Saldise L, Guillen J, Belzunegui S, San Sebastian W, Izal A, Garrido P, Vazquez M. Does increased excitatory drive from the subthalamic nucleus contribute to dopaminergic neuronal death in parkinson's disease? *Exp. Neurol.* 2006; 201:407–415. [PubMed: 16806173]
- Lustig HS, Ahern KV, Greenberg DA. Antiparkinsonian drugs and in vitro excitotoxicity. *Brain Res.* 1992; 597:148–150. [PubMed: 1477727]
- Matarredona ER, Santiago M, Machado A, Cano J. Lack of involvement of glutamate-induced excitotoxicity in MPP+ toxicity in striatal dopaminergic terminals: Possible involvement of ascorbate. *Br. J. Pharmacol.* 1997; 121:1038–1044. [PubMed: 9222565]
- Merims D, Shabtai H, Korczyn AD, Peretz C, Weizman N, Giladi N. Antiparkinsonian medication is not a risk factor for the development of hallucinations in parkinson's disease. *J. Neural Transm.* 2004; 111:1447–1453. [PubMed: 15480845]
- Pahwa R, Factor SA, Lyons KE, Ondo WG, Gronseth G, Bronte-Stewart H, Hallett M, Miyasaki J, Stevens J, Weiner WJ. Quality Standards Subcommittee of the American Academy of Neurology. Practice parameter: Treatment of parkinson disease with motor fluctuations and dyskinesia (an evidence-based review): Report of the quality standards subcommittee of the american academy of neurology. *Neurology.* 2006; 66:983–995. [PubMed: 16606909]
- Quack G, Hesselink M, Danysz W, Spanagel R. Microdialysis studies with amantadine and memantine on pharmacokinetics and effects on dopamine turnover. *J. Neural Transm.* 1995; 46 Suppl.:97–105.
- Robinson DM, Keating GM. Memantine: A review of its use in alzheimer's disease. *Drugs.* 2006; 66:1515–1534. [PubMed: 16906789]
- Rogoz Z, Skuza G, Daniel WA, Wojcikowski J, Dudek D, Wrobel A. Amantadine as an additive treatment in patients suffering from drug-resistant unipolar depression. *Pharmacol. Rep.* 2007; 59:778–784. [PubMed: 18195470]
- Rogoz Z, Skuza G, Legutko B. Repeated co-treatment with fluoxetine and amantadine induces brain-derived neurotrophic factor gene expression in rats. *Pharmacol. Rep.* 2008; 60:817–826. [PubMed: 19211973]
- Rojas P, Altagracia M, Kravsov J, Rios C. Partially protective effect of amantadine in the MPTP model of parkinson's disease. *Proc. West. Pharmacol. Soc.* 1992; 35:33–35. [PubMed: 1502234]
- Roman A, Rogoz Z, Kubera M, Nawrat D, Nalepa I. Concomitant administration of fluoxetine and amantadine modulates the activity of peritoneal macrophages of rats subjected to a forced swimming test. *Pharmacol. Rep.* 2009; 61:1069–1077. [PubMed: 20081242]
- Schwab RS, England AC Jr, Poskanzer DC, Young RR. Amantadine in the treatment of parkinson's disease. *JAMA.* 1969; 208:1168–1170. [PubMed: 5818715]
- Sonsalla PK, Zeevalk GD, Manzino L, Giovanni A, Nicklas WJ. MK-801 fails to protect against the dopaminergic neuropathology produced by systemic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice or intranigral 1-methyl-4-phenylpyridinium in rats. *J. Neurochem.* 1992; 58:1979–1982. [PubMed: 1560247]

- Uitti RJ, Rajput AH, Ahlskog JE, Offord KP, Schroeder DR, Ho MM, Prasad M, Rajput A, Basran P. Amantadine treatment is an independent predictor of improved survival in parkinson's disease. *Neurology*. 1996; 46:1551–1556. [PubMed: 8649547]
- Vale S. Current management of the cognitive dysfunction in parkinson's disease: How far have we come? *Exp. Biol. Med.* (Maywood). 2008; 233:941–951. [PubMed: 18535172]
- Wang AL, Liou YM, Pawlak CR, Ho YJ. Involvement of NMDA receptors in both MPTP-induced neuroinflammation and deficits in episodic-like memory in wistar rats. *Behav. Brain Res.* 2010; 208:38–46. [PubMed: 19900486]
- Weller M, Finiels-Marlier F, Paul SM. NMDA receptor-mediated glutamate toxicity of cultured cerebellar, cortical and mesencephalic neurons: Neuroprotective properties of amantadine and memantine. *Brain Res.* 1993; 613:143–148. [PubMed: 8102306]
- Wenk GL, Danysz W, Mobley SL. MK-801, memantine and amantadine show neuroprotective activity in the nucleus basalis magnocellularis. *Eur. J. Pharmacol.* 1995; 293:267–270. [PubMed: 8666045]
- Wolf E, Seppi K, Katzenschlager R, Hochschorner G, Ransmayr G, Schwingenschuh P, Ott E, Kloiber I, Haubenberger D, Auff E, Poewe W. Long-term antidyskinetic efficacy of amantadine in parkinson's disease. *Mov. Disord.* 2010; 25:1357–1363. [PubMed: 20198649]
- Wu HM, Tzeng NS, Qian L, Wei SJ, Hu X, Chen SH, Rawls SM, Flood P, Hong JS, Lu RB. Novel neuroprotective mechanisms of memantine: Increase in neurotrophic factor release from astroglia and anti-inflammation by preventing microglial activation. *Neuropsychopharmacology*. 2009; 34:2344–2357. [PubMed: 19536110]

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 [³H] DA uptake assay. B) The number of TH⁺ neurons per well is expressed as % of control. Data represent the mean $\pm$ 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 μ M) 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 percentage of control. Data represent the mean $\pm$ 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.

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 [³H] DA uptake assay. Data represent the mean $\pm$ 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 .

Fig. 4. Amantadine attenuates LPS-induced production of pro-inflammatory factors

A–B) neuron-glia cultures were exposed to amantadine (Ama 10–30 μ M) 48 h prior to stimulation with LPS (10 ng/ml). C–D) Neuron-microglia cultures were exposed to amantadine (Ama 30 μ M) 24 h prior to stimulation with LPS. NO production was measured using Griess reagent, while TNF α production was measured by ELISA kit. Supernatants were collected at 3 h for TNF α and at 24 h for NO. Data represent the mean $\pm$ SEM of at least five independent experiments. Significantly different from LPS alone: #<0.05; ##<0.01; ###<0.001.

Fig. 5. Inhibition of microglia 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 TNF α 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 bound to the membranes were extracted and visualized by western blotting. D) Quantification of the level of p47^{phox} and p67^{phox} were normalized against the level of gp91^{phox}. Data represent the mean $\pm$ 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 .

Fig. 6. Amantadine induces the expression of GDNF in astroglia

Mixed glia-culture, containing about 90% astroglia and 10% microglia, was exposed to amantadine (Ama 30 μ M) 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 $\pm$ SEM of at least six independent experiments. Significantly different from control: * <0.05 ; ** <0.01 . B) The level of acetylated histone 3 bound to GDNF promoter was measured by ChIP assay and normalized against the input DNA (see Methods). The data represent the mean $\pm$ 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 [³H] DA uptake assay after 24 h exposure to NMDA. B) The data represent the elevated intracellular Ca²⁺ signals (F/F₀) 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 [³H] 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 .