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