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Deprenyl rescues dopaminergic neurons in organotypic slice cultures of neonatal rat mesencephalon from *N*-methyl-D-aspartate toxicity

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Abstract

The potential neuroprotective effect of (−)-deprenyl (*R*-*N*,α-dimethyl-*N*-2-propynylbenzeneethanamine) against *N*-methyl-D-aspartate (NMDA) excitotoxicity was investigated on rat mesencephalic dopaminergic neurons in organotypic slice cultures. While 24 h application of NMDA (100 μM) caused a marked decrease in the number of surviving dopaminergic neurons, simultaneous application of (−)-deprenyl significantly attenuated the cytotoxic effect of NMDA. (+)-Deprenyl showed a less potent but still significant protective effect against NMDA insult. Pre-treatment of cultures with (−)-deprenyl conferred no protection against subsequent NMDA insult, suggesting that the protective effect of (−)-deprenyl may be independent of its irreversible inhibitory action on monoamine oxidase B. (−)-Deprenyl was also ineffective in preventing cell death induced by H_2O_2. These results indicated that (−)-deprenyl protects dopaminergic neurons from NMDA excitotoxicity through a mechanism distinct from monoamine oxidase inhibition or detoxification of reaction oxygen species.

Keywords: *R*-(−)-Deprenyl (selegiline); *S*-(+)-Deprenyl; NMDA, *N*-methyl-D-aspartate; Slice culture; Mesencephalon; Dopaminergic neuron

1. Introduction

R-(−)-Deprenyl (selegiline, *R*-*N*,α-dimethyl-*N*-2-propynylbenzeneethanamine), an inhibitor of monoamine oxidase B, is used in the treatment of Parkinson's disease. Clinical trials have demonstrated that (−)-deprenyl extends life expectancy and slows the progression of Parkinson's disease symptoms (Birkmayer et al., 1985; Tetrud and Langston, 1989). Several studies have shown that (−)-deprenyl has neuroprotective effects both in vitro and in vivo. For example, (−)-deprenyl protects dopaminergic neurons in dissociated primary culture against the cytotoxic effects of 1-methyl-4-phenylpyridium ions (MPP^+) (Koutsilieri et al., 1996) and *N*-methyl-D-aspartate (NMDA) excitotoxicity (Mytilineou et al., 1997). However, there is some dispute regarding whether the clinical benefits of (−)-deprenyl in Parkinson's disease are due to neuroprotective or to symptomatic mechanisms, although progression of Parkinson's disease is considered to be correlated with the progressive degeneration of mesencephalic dopaminergic neurons (Parkinson study group, 1989, 1993).

Previously, we established an excitotoxicity model using in vitro organotypic slice cultures of the neonatal rat mesencephalon (Maeda et al., 1998). In this experimental model, exposure of cultures to NMDA clearly induced degeneration and death of dopaminergic neurons, and thus it serves as a convenient system in which to evaluate excitotoxic injury that may act as a final common pathway leading to cell death in various neurological disorders (Lipton and Rosenberg, 1994). Another advantage of the use of organotypic slice cultures is that they possess a spatial cellular organization resembling that of the intact brain tissue, and therefore, are expected to display pathophysiological responses more relevant to the conditions in vivo than dissociated cultures. Moreover, neurons in slice cultures, like those in vivo, are embedded and surrounded by glial cells which are abundant in monoamine oxidase B. This feature is particularly important for evaluation of the effects of (−)-deprenyl, because this drug is a potent irreversible monoamine oxidase-B inhibitor. Thus, this study was performed to examine the neuroprotective ef-

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fects of (−)-deprenyl on dopaminergic neurons in mesencephalic slice cultures.

2. Materials and methods

2.1. Preparation of slice cultures

The procedures for preparation of organotypic slice cultures were described previously (Maeda et al., 1998). Briefly, the mesencephalon of postnatal day 2–3 Wistar rats was dissected under sterile conditions. Mesencephalic tissue was cut into slices 350 μm thick with a tissue chopper (Narishige, Tokyo, Japan). Slices were transferred onto insert membranes (Millicell-CM; Millipore, Bedford, MA, USA) in six-well plates (Corning Costar, Tokyo, Japan) supplied with 700 μl/well of culture medium of the following composition: 50% minimal essential medium/HEPES, 25% Hank's balanced salt solution and 25% heat-inactivated horse serum (Gibco BRL, Rockville, MD, USA) supplemented with 6.5 g/l glucose and 2 mM L-glutamine. The six-well plates were placed in a humidified atmosphere of 5% CO_2 in an incubator at 34°C. Culture medium was replaced with fresh medium every 2 days. Slices cultured for 17 days were used in the cytotoxicity experiments. For cytotoxic insult, slices were submerged for 24 h in 2 ml of culture medium containing NMDA (Nacalai Tesque, Kyoto, Japan) or H_2O_2, then processed for immunohistochemistry. The test substances, (−)-deprenyl (ICN Biochemicals, Aurora, OH, USA), (+)-deprenyl (Chinoin, Budapest, Hungary) and MK-801 (Nacalai Tesque, Kyoto, Japan), were applied concomitantly with NMDA or H_2O_2. In some experiments, (−)-deprenyl was applied for 4 days before or after 24 h of NMDA exposure. In these cases, slices were processed for immunohistochemical staining 4 days after cytotoxic insult. The medium was also changed every 2 days during pre- or post-treatment with (−)-deprenyl. Slices were submerged in culture medium only during application of toxic insult.

2.2. Tyrosine hydroxylase immunohistochemistry

Dopaminergic neurons in the slice cultures were identified by tyrosine hydroxylase immunohistochemistry using the avidin–biotin peroxidase method. The cultures were fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde and 4% sucrose for 2 h, rinsed with 10 mM phosphate-buffered saline (PBS) and exposed for 30 min to 0.02% H_2O_2 in 100% methanol to eliminate endogenous peroxidase activity. The cultures were then exposed to PBS containing 0.2% Triton X-100 for 30 min. Nonspecific antibody binding was reduced by exposure to 10% fetal calf serum for 30 min. Cultures were incubated overnight at 4°C with rabbit anti-tyrosine hydroxylase polyclonal antibody (1:500 dilution; Chemicon International, Temecula, CA, USA). After rinsing in three changes of PBS, cultures were incubated for 1 h at room temperature with biotinylated anti-rabbit IgG (1:200 dilution). Following a further rinse in PBS, the cultures were treated for 1 h at room temperature with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Lab., Burlingame, CA, USA). After further washing in 0.05 M Tris-buffered saline (TBS), peroxidase was visualized with 0.07% diaminobenzidine and 0.018% H_2O_2 in TBS. The cultures were dehydrated with ethanol and mounted on slides.

2.3. Evaluation of cell viability and statistical analysis

The viability of tyrosine hydroxylase-positive cells was evaluated following tyrosine hydroxylase immunostaining. Tyrosine hydroxylase-positive cells with developed dendrites were considered as viable, surviving dopaminergic neurons. The numbers of tyrosine hydroxylase-positive cells in an area of 520×670 μm^2 were counted in individual slices. The surviving cell number was expressed as the mean ± S.E.M. Statistical significance of differences was first determined by one-way ANOVA that included all data sets in each figure (Figs. 2–5), then comparisons between groups were made by Dunnett's two-tailed test. In Figs. 2–4, comparisons were made between the NMDA-alone group and the other groups, and in Fig. 5 comparisons were made between H_2O_2-alone group and the other groups. Percentage of neuroprotection was calculated using the following equation: protection (%) $= [(D - N)/(S - N)] \times 100$, where D is the number of cells in cultures treated with deprenyl and NMDA, N is the number of cells in NMDA-treated cultures, and S is the number of cells in sham-treated cultures.

3. Results

Organotypic slice cultures of neonatal rat mesencephalon were processed for immunohistochemical examination on day 17 in vitro. Cells labeled positively with anti-tyrosine hydroxylase antibody were localized in the ventral part of the mesencephalon. After a 17-day incubation, the slices had thinned to one or two cell layers supported by a sheet of glial cells. Thus, the tyrosine hydroxylase-positive cells could be clearly observed under a microscope following immunostaining. A complex tyrosine hydroxylase-positive fiber network was also observed. The results were consistent with those of our previous study (Maeda et al., 1998). These morphological characteristics resembled the organization of these tissues in vivo (Fig. 1A and B). Application of NMDA (100 μM) for 24 h resulted in a marked decrease in the number of surviving dopaminergic neurons (Fig. 1C). Simultaneous application of MK-801 (10 μM), a non-competitive antagonist of NMDA receptors, completely blocked the cytotoxic effect

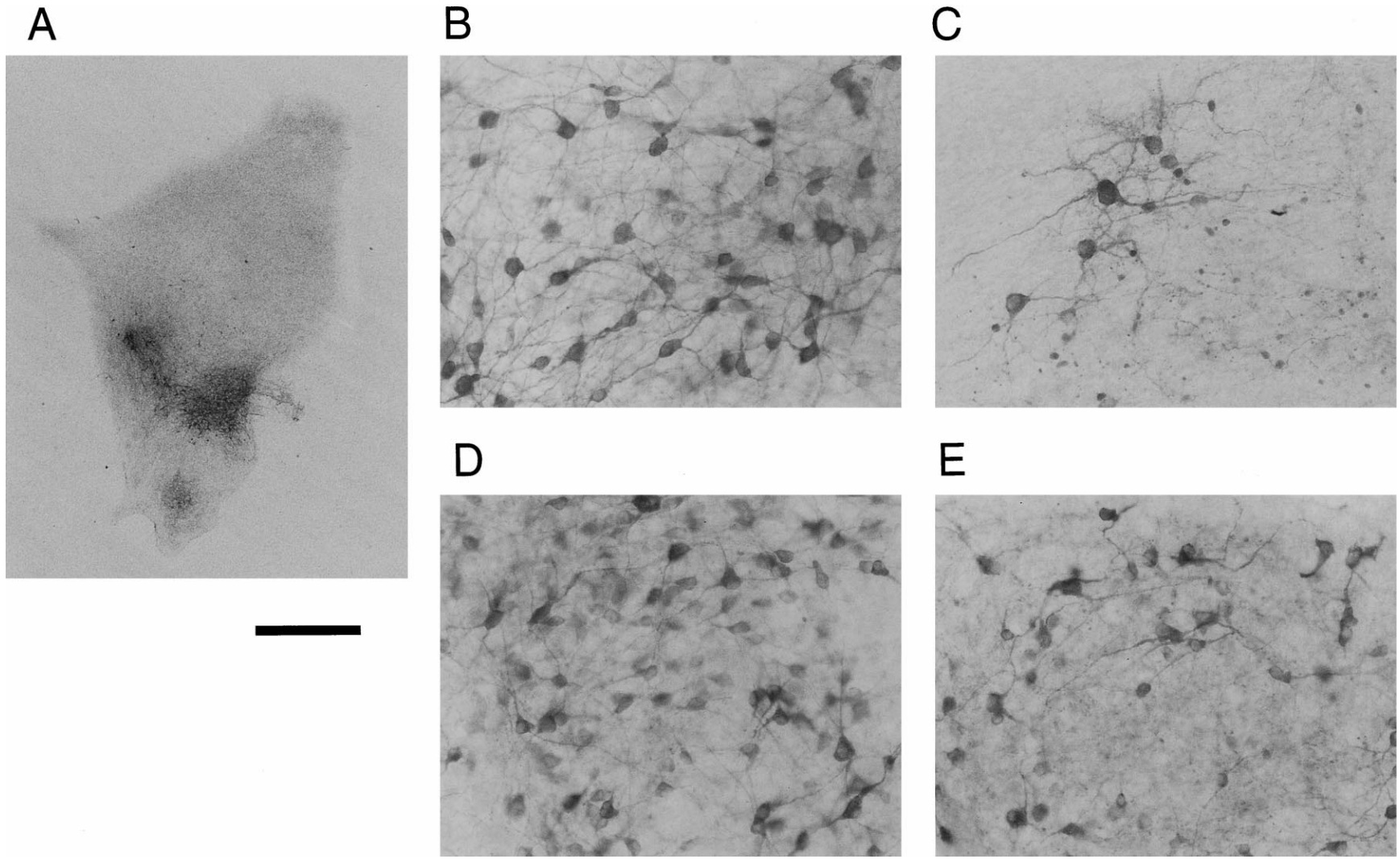

Fig. 1. Tyrosine hydroxylase immunohistochemistry of mesencephalic slice cultures. (A) Whole view of a non-treated mesencephalic slice culture. (B–E) Representative examples of tyrosine hydroxylase-positive cells in the slice cultures showing cytotoxic effects of 24-h exposure to 100 μM NMDA, and its reversal by MK-801 or (−)-deprenyl. (B) Sham-treated culture. (C) Culture treated with 100 μM NMDA alone. (D) Culture treated with 100 μM NMDA and 10 μM MK-801. (E) Culture treated with 100 μM NMDA and 10 μM (−)-deprenyl. MK-801 or (−)-deprenyl was applied concomitantly with NMDA. Scale bars indicate 1 mm (A) and 100 μm (B–E).

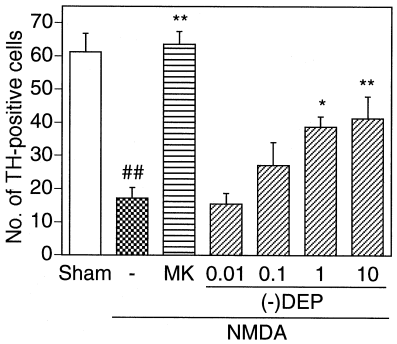

Fig. 2. Effects of (−)-deprenyl (DEP) and MK-801 (MK) on 100 μM NMDA-induced dopaminergic neuronal death in mesencephalic organotypic slice cultures. Ordinate shows the number of tyrosine hydroxylase (TH)-positive cells. (−)DEP (at indicated concentration in μM) or MK (10 μM) was applied concomitantly with NMDA. $\#\#P < 0.01$ compared with sham control. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with NMDA alone ($n = 9$–12 per group).

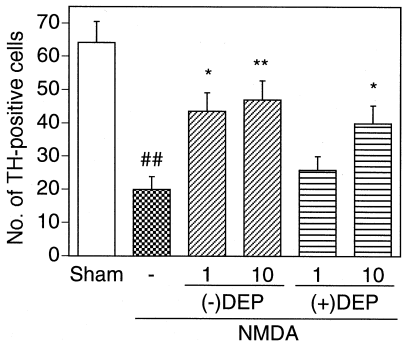

Fig. 4. Effects of (−)- and (+)-deprenyl (DEP) on 100 μM NMDA-induced dopaminergic neuronal death in mesencephalic organotypic slice cultures. (−)DEP or (+)DEP (at indicated concentrations in μM) was applied concomitantly with NMDA. $\#\#P < 0.01$ compared with sham control. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with NMDA alone ($n = 10$–12 per group).

of 100 μM NMDA (Fig. 1D). When (−)-deprenyl was applied concomitantly with NMDA, it also showed a protective effect (Fig. 1E). The protective effect of (−)-deprenyl on tyrosine hydroxylase-positive neurons was concentration-dependent over the range from 0.01 to 10 μM (Fig. 2). Significant protection was observed with 1 and 10 μM (−)-deprenyl, although these concentrations of the drug did not completely inhibit the cytotoxic effect of NMDA. Percentages of neuroprotection by 0.1, 1, and 10 μM (−)-deprenyl were 23%, 49% and 55%, respectively. A higher concentration (100 μM) of (−)-deprenyl itself showed a deleterious effect on dopaminergic neurons. In contrast, there was a full reduction of NMDA cytotoxicity with MK-801 (10 μM). Therefore, we applied (−)-deprenyl at various times relative to NMDA insult, to verify whether there was an optimal time window for (−)-deprenyl to exert its neuroprotective effect (Fig. 3). (−)-Deprenyl (10 μM) was applied for 24 h concomitantly with NMDA for 4 days before 24 h of NMDA treatment, or for 4 days after 24 h of NMDA treatment. Whereas concomitant treatment with (−)-deprenyl showed a marked protective effect (66% neuroprotection), neither pre- nor post-treatment yielded significant protection against NMDA insult (20 and 7% neuroprotection, respectively).

Although (+)-deprenyl is a weak monoamine oxidase B inhibitor compared with the (−)- form, it shows a potent inhibitory effect on dopamine re-uptake (Fang and Yu, 1994). (+)-Deprenyl (10 μM) applied concomitantly with NMDA was effective in attenuating the cytotoxic effect of NMDA, although a lower concentration (1 μM) of (+)-deprenyl had no significant effect (Fig. 4). Percentages of neuroprotection by 1 and 10 μM (+)-deprenyl were 13% and 45%, whereas in a parallel experiment 1 and 10 μM (−)-deprenyl afforded 53% and 61% protection, respectively.

Several lines of evidence have shown that the generation of reactive oxygen species plays an important role in excitotoxic injury (Dugan et al., 1995). Since (−)-deprenyl was shown to be a potent scavenger of peroxyl radicals (Thomas et al., 1997), it is possible that the protective effect of (−)-deprenyl was exerted through detoxification of reactive oxygen species. Therefore, we investigated the

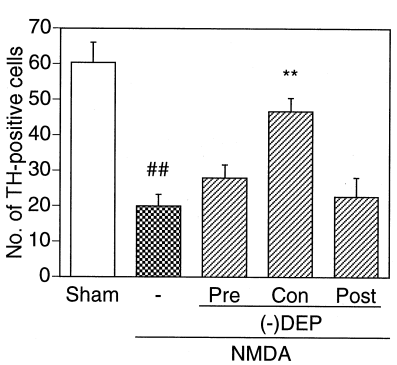

Fig. 3. Effects of (−)-deprenyl (DEP), when added concomitantly, before or after insult on 100 μM NMDA-induced dopaminergic neuronal death in mesencephalic organotypic slice cultures. (−)DEP (10 μM) was applied for 24 h concomitantly (Con) with NMDA, 4 days before 24 h of NMDA treatment (Pre), or 4 days after 24 h of NMDA treatment (Post). $\#\#P < 0.01$ compared with sham control. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with NMDA alone ($n = 10$–12 per group).

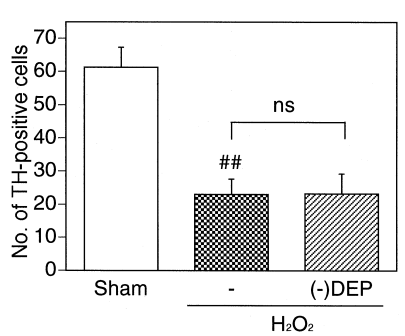

Fig. 5. Effects of (−)-deprenyl (DEP) on 100 μM H_2O_2-induced dopaminergic neuronal death in mesencephalic organotypic slice cultures. (−)DEP (10 μM) was applied concomitantly with H_2O_2. $\#\#P < 0.01$ compared with sham control. ns: not significant ($n = 10$–12 per group).

effects of (−)-deprenyl against H_2O_2-induced insult (Fig. 5). A marked decrease in the number of surviving dopaminergic neurons was observed after 24 h of exposure of cultures to 100 μM H_2O_2 (Fig. 5), but lower concentrations (10–30 μM) of H_2O_2 showed little effect (data not shown). (−)-Deprenyl (10 μM) applied concomitantly with 100 μM H_2O_2 did not protect tyrosine hydroxylase-positive cells from H_2O_2-induced cell death.

4. Discussion

It has been reported that dopaminergic neurons are vulnerable to NMDA excitotoxicity (Kikuchi and Kim, 1993; Sawada et al., 1996), and neurodegeneration in neurological disorders may involve excitotoxicity as the final common pathway (Lipton and Rosenberg, 1994). We reported previously that dopaminergic neuronal death in the present experimental system was induced by NMDA in a concentration-dependent manner (Maeda et al., 1998). Since this excitotoxicity was completely blocked by MK-801 (Fig. 2A), it was primarily triggered by selective activation of NMDA receptors. Glutamate receptor overstimulation has been shown to produce neuronal injury across a continuum involving apoptosis and necrosis, dependent on the severity of the insult (Bonfoco et al., 1995; Cheung et al., 1998). The type of cell death occurring in our culture system will be addressed in future experiments, as it should provide useful information concerning the mechanisms of the neuroprotective effects of drugs including deprenyl. In the present study, (−)-deprenyl was shown to protect dopaminergic neurons from NMDA-induced excitotoxicity in a concentration-dependent manner. For the effective concentrations of 1–10 μM, we are comparable to those demonstrated in previous in vitro experiments using dispersed cultures (Koutsilieri et al., 1996; Mytilineou et al., 1997), and also comparable with those achieved after peripheral administration of (−)-deprenyl at a dose that exerts neuroprotective effects in vivo (Knollema et al., 1995; Thiffault et al., 1995; Melega et al., 1999). The protective effect of (−)-deprenyl against NMDA excitotoxicity may explain the effectiveness of this drug against ischemic neuronal damage (Knollema et al., 1995; Lahtinen et al., 1997).

(−)-Deprenyl is a potent, irreversible inhibitor of monoamine oxidase B. Inhibition of monoamine oxidase B activity may be beneficial for neuronal viability under several conditions because of reduced production of endogenous and exogenous neurotoxins such as 1-benzyl-1,2,3,4-tetrahydroisoquinoline and MPP^+ (Cohen et al., 1985; Kotake et al., 1998). However, the protective effect of (−)-deprenyl observed in the present study was unlikely to be due to irreversible inhibition of monoamine oxidase-B activity because (−)-deprenyl was effective only when applied concomitantly with NMDA, and pretreatment with (−)-deprenyl failed to show a significant protective effect. Indeed, several studies have suggested that the protective effects of (−)-deprenyl are independent of its irreversible monoamine oxidase B inhibitory action (Tatton et al., 1994; Koutsilieri et al., 1996; Mytilineou et al., 1997). In this context, Mytilineou et al. (1997) showed that long-term pre-treatment with (−)-deprenyl was not necessary for protection of dopaminergic neurons in dissociated culture, as significant protection from NMDA cytotoxicity was observed even when (−)-deprenyl was added only during and after NMDA treatment.

NMDA-induced insult may involve production of reactive oxygen species (Dugan et al., 1995). (−)-Deprenyl is known to be a scavenger of peroxyl and hydroxyl radicals (Thomas et al., 1997). It has also been reported that (−)-deprenyl can upregulate the activities of the antioxidant enzyme catalase and superoxide dismutase in the brain (Carrillo et al., 1992; Thiffault et al., 1995). However, (−)-deprenyl failed to attenuate H_2O_2 toxicity in the present study, suggesting that detoxification of reactive oxygen species does not play an important role in its neuroprotective effect.

The precise cellular mechanisms of the protective effect of (−)-deprenyl against NMDA toxicity are not clear. As (−)-deprenyl showed no effect in ligand binding experiments on NMDA receptors (Mytilineou et al., 1997), direct interaction of (−)-deprenyl with NMDA receptors is unlikely. Mytilineou et al. (1997) reported that 0.5–50 μM (−)-deprenyl protects neurons in excitotoxicity models in dissociated cell culture in vitro, and speculated that (−)-deprenyl protected neurons from events occurring downstream of NMDA receptor activation. This is consistent with the hypothesis of Tatton et al. (1996), who reported that transcriptional events involving new protein synthesis may be involved in the effects of (−)-deprenyl (Tatton et al., 1994). Moreover, (−)-deprenyl has been shown to increase expression of ciliary neurotrophic factor and basic fibroblast growth factor in reactive astrocytes (Biagini et al., 1994; Mattson et al., 1995). These growth factors are capable of attenuating glutamate-induced neurotoxicity (Spina et al., 1992; Mattson et al., 1995). Further investigations are required to verify whether the induction of growth factor expression is involved in the observed protective effect of (−)-deprenyl.

Here, we also provided evidence of a neuroprotective effect of (+)-deprenyl. The failure of previous studies by others to demonstrate protective actions of (+)-deprenyl (Tatton et al., 1994; Paterson et al., 1998) may have been due to different mechanisms of cell death in cell lines such as PC12 or primary cultures of cerebellar granule cells. As (+)-deprenyl has distinct pharmacological characteristics compared with (−)-deprenyl, it may provide useful information in the course of further examinations concerning the mechanism of the neuroprotective effect of deprenyl.

In conclusion, we demonstrated protection of dopaminergic neurons in slice culture from NMDA-induced excitotoxicity by deprenyl. The underlying mechanisms may be

relevant to the beneficial effects of (−)-deprenyl treatment in Parkinson's disease patients.

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