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Selegiline is neuroprotective in primary brain cultures treated with 1-methyl-4-phenylpyridinium

Eleni Koutsilieri [a,*], Tzyh-Shi Chen [b], Wolf-Dieter Rausch [b], Peter Riederer [a]

[a] *Department of Psychiatry, Clinical Neurochemistry, Julius-Maximilians-University, Füchsleinstr. 15, D-97080 Würzburg, Germany*
[b] *Institute of Medical Chemistry, Veterinary University of Vienna, Linke Bahngasse 11, A-1030 Vienna, Austria*

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Abstract

The ability of selegiline to protect against the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been attributed to the inhibition of the conversion of MPTP to 1-methyl-4-phenylpyridinium (MPP^+), catalyzed by monoamine oxidase-B. Selegiline, however, has been found to rescue neurons in MPP^+-treated mice after they have sustained lethal damage independently of monoamine oxidase-B inhibition. In our present study, we investigate whether selegiline can protect and/or rescue MPP^+-injured dopaminergic neurons in co-cultures of mesencephalic and striatal cells of embryonic C57Bl/6 mouse brains. Cells were exposed to selegiline (1, 10, 100 μM) in three different schemes: (i) in control cultures on the 8th day for 48 h; (ii) pretreatment: on the 8th day for 48 h, followed by administration of MPP^+ (0.5 μM) on the 9th day for 24 h; (iii) delayed treatment: on the 9th day for 48 h, while MPP^+ was administered on the 8th day and remained in culture during treatment with selegiline. In the delayed scheme, selegiline (1 μM) increased dopamine content, number of tyrosine hydroxylase immunoreactive cells and astrocytes in the cultures. We question whether selegiline protects cells injured by a toxic stressor via an astrocyte-mediated mechanism.

Keywords: Selegiline; MPP^+ (1-methyl-4-phenylpyridinium); Astrocyte; Neuronal culture

1. Introduction

Selegiline is a monoamine oxidase-B inhibitor, developed as an antidepressant drug (Knoll et al., 1965). The first indication that selegiline might be protective against Parkinson's disease came in 1985 (Birkmayer et al., 1985). Clinical trials have shown that it can slow the progression of Parkinson's disease (Tetrud and Langston, 1989), in addition to extending life expectancy (Birkmayer et al., 1985). Accordingly, it has been reported that it exerts protective effects against neuronal degeneration in animal models, including protection against neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Heikkila et al., 1984). The protective role of selegiline was mainly associated with monoamine oxidase-B inhibition and therefore decreased dopamine catabolism, increased dopamine function and prevention of free radical generation (Riederer and Yudim, 1986). Recent observations in MPTP-treated mice show that selegiline retains its protective capacity even at concentrations at which a monoamine oxidase-B inhibitory action seems unlikely (Tatton, 1993; Wu et al., 1993). New evidence suggests that the neuroprotective effects of selegiline are due to the involvement of glia and the secretion of trophic molecules (Biagini et al., 1993); selegiline was reported to enhance astrocytic activation after lesions in rat brain (Biagini et al., 1993), influence glial cell process length and upregulate ciliary neurotrophic factor (CNTF) gene expression in astrocyte cultures (Seniuk et al., 1994).

Our previous studies on selegiline showed that its capacity for neuroprotection (cell survival, morphology) in cultures depends on the treatment scheme employed (Koutsilieri et al., 1994). In this report, we investigated further the effect of selegiline on functional parameters, such as dopamine content and [^{3}H]dopamine uptake which depend on the treatment scheme employed and the influence exerted on astrocytes and other non-dopaminergic neuronal populations in cultures.

* Corresponding author. Tel.: 0049 931 203323; fax: 0049 931 203358.

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2. Materials and methods

2.1. Preparation of neuronal cultures

C57BL/6 mice were mated from 16:00 h to 8:00 h (day of insemination GD1). At GD 14, embryos (crown-rump length 9 mm) were excised, brains were removed from the skull and the striatum and the ventral mesencephalon were dissected out (Schambra et al., 1992). Care was taken not to include the locus coeruleus, which contains noradrenergic neurons (Lindvall and Björklund, 1983). After having carefully removed the meninges, pooled tissue blocks were mechanically dissociated in phosphate-buffered saline (PBS) containing 58 mM saccharose, 5 mM glucose, 6.8 mM NaCl, 5.3 mM KCl, and 0.24 mM NaH_2PO_4. Cells were mildly triturated with a fire-polished Pasteur pipette in a nutrient medium (MEM Dulbecco, Boehringer) supplemented with 10% inactivated fetal calf serum, 4 mM glutamine, 10 mM Hepes and 30 mM glucose; pH was adjusted to 7.3. Cells were plated in 24-well dishes coated with poly-D-lysin (50 μg/ml) at a density of 10^6/ml medium, and consisted of 0.5×10^6 mesencephalic and 0.5×10^6 striatal cells. Cultures were incubated at 37°C in a water-saturated atmosphere of 5% CO_2 and 95% air. The medium was changed three times a week. After the first week, a serum-free supplemented medium (Bottenstein and Sato, 1979; Romijn et al., 1982) was provided, consisting of MEM Dulbecco with 10 μg/ml insulin (Boehringer), 100 μg/ml transferrin (Boehringer), 60 nM sodium selenite (Boehringer), 200 μM putrescin (Sigma), 40 nM progesterone (Sigma), 570 nM corticosterone (Sigma) and 30 μM triiodothyronine (Serva).

Isolation of cells from brains of mouse embryos was approved by the Österreichische Bundesministerium für Wissenschaft und Forschung GZ 68 205/418-12/90.

2.2. Treatment with selegiline

Cells were exposed to selegiline at 1, 10 and 100 μM in three different treatment schemes: (i) in control cultures on the 8th day for 48 h, (ii) as pretreatment, on the 8th day for 48 h, followed by administration of 0.5 μM of MPP^+ on the 9th day for 24 h, (iii) as delayed treatment, on the 9th day for 48 h while MPP^+ was administered on the 8th day and remained in culture during treatment with selegiline.

2.3. Tyrosine hydroxylase and glial fibrillary acidic protein (GFAP) immunocytochemistry

For visualization of dopaminergic neurons and astrocytes, cultures were fixed with 400 μl of 4% paraformaldehyde for 45 min at 4°C and washed with PBS. Cells were permeabilized with 300 μl of 0.4% Triton X-100 for 30 min at room temperature. Cultures were washed 4 times with Tris buffer (TBS, 0.05 M, pH 7.4) and incubated for 90 min with 10% normal horse serum to block nonspecific binding sites. The primary antibodies were: monoclonal anti-tyrosine hydroxylase and anti-GFAP, Boehringer, (for dopaminergic neurons and astrocytes, respectively), raised against rat diluted in TBS (1 : 1000) containing 1% normal horse serum. Cultures were incubated with the primary antiserum for 24 h at room temperature. They were washed again and incubated with an anti-mouse biotinylated secondary antibody, obtained from horse (Vectastain ABC kit, Vector) for 90 min. After washing, the cultures were incubated with a preformed avidin-biotin horseradish peroxidase complex (Vectastain ABC kit, Vector) in TBS. The peroxidase was visualized by incubation with a solution of diaminobenzidin (1.4 mM) in TBS containing 3.3 mM hydrogen peroxide.

To define the total cell number of the cultures, a heamatoxylin-eosin staining was performed.

2.4. [^{3}H]Dopamine uptake

[^{3}H]Dopamine uptake by cells was determined as follows: after removing the growth medium and rinsing once with PBS, the cultures were incubated with 32 nM [^{3}H]dopamine (30.8 Ci/mmol, DuPont) in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, for 15 min at 37°C. Blank values were obtained at 0°C. The uptake was stopped by removing the solution. Cells were rapidly washed with the above buffer. In order to release [^{3}H]dopamine from the cells, the cultures were incubated with 500 μl 96% ethanol for 30 min at 37°C. The ethanol was added to 10 ml of scintillation liquid (Quicksafe A, Zinsser Analytic) in vials and counted for radioactivity in a Packard Tri-Carb 4640 scintillation counter.

2.5. Dopamine content

The concentration of dopamine in the cultures was measured by high-pressure liquid chromatography (HPLC) (Sofic, 1986). The growth medium was removed, cells from 4 wells were combined and homogenized in 280 μl of 0.4 M perchloric acid containing 800 pg of dihydroxybenzylamine (Sigma) as an internal standard. The samples were centrifuged at $1000 \times g$ for 1 min, the proteins were precipitated and the supernatant was aspirated into extraction tubes containing 1 ml of Tris-buffer (1 M, pH 8.66) and 50 mg of acid-washed alumina. After vigorously vortexing for 20 min, the supernatant was aspirated and the alumina washed with 1 ml of 0.1 M Tris-buffer, followed by two washes with water. Dopamine was then desorbed from the alumina by the addition of 0.2 ml perchloric acid (0.4 M). From this acidic phase an aliquot of 25 μl was injected directly into the HPLC system. The system consisted of HPLC with electrochemical detection (HPLC-ECD), supplied by ESA (Bedford, USA) and included an ESA Catecholamine HR-80 reverse phase column with 3 μm spherical octadecylsilane and CAT-A-Phase as mobile

Table 1
Selegiline in untreated cultures

	pmol dopamine/well	pmol [^{3}H]dopamine/ 15 min/well	Tyrosine hydroxylase-immunoreactive cells
Control	1.57 ± 0.24	0.90 ± 0.005	550 ± 55
Selegiline (1 μM)	1.59 ± 0.13	0.73 ± 0.02 [b]	528 ± 73
Selegiline (10 μM)	1.50 ± 0.08	0.60 ± 0.04 [b]	482 ± 100
Selegiline (100 μM)	1.59 ± 0.12	0.48 ± 0.05 [b]	415 ± 70 [a]

Cells were exposed to selegiline on the 8th day in vitro for 48 h. Toxin was not present. Values are means ± S.E.M. from 3–4 independent experiments. Significantly different from controls: [a] $P < 0.05$, [b] $P < 0.01$ (ANOVA, followed by Student's unpaired t-test).

phase. Dopamine was separated at a flow rate of 1 ml min^{-1} and the chromatogram recorded. The detection limit was 5 pg per injection. The intra-assay variation was 6.3% ($n = 20$). The catecholamine peaks were compared with commercial standards (Sigma) and corrected for recovery.

2.6. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) and the significance of intergroup differences was determined by applying Student's unpaired t-test. Differences were considered significant at $P < 0.05$.

3. Results

Immunocytochemical staining for tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of dopamine and other catecholamines, was employed to identify dopaminergic neurons. In our cultures, tyrosine hydroxylase acts as an exclusive marker for dopaminergic cells since the dissection procedure (Koutsilieri et al., 1993) excludes noradrenergic cells of the locus coeruleus. Furthermore, noradrenaline was not detected using HPLC.

A typical co-culture of mesencephalic and striatal cells in our experiments contained 90% neurons and 10% astrocytes at the end of treatment. About 5% of the neurons were tyrosine hydroxylase-immunoreactive (data not shown).

To evaluate the possible survival- and function-promoting effects of selegiline, cultures were exposed in different treatment schemes: control, as pretreatment and as delayed treatment. Parameters investigated were: cell survival (number of dopaminergic neurons, astrocytes), dopamine content and [^{3}H]dopamine uptake by the cultures.

In control cultures, selegiline had no effect on the dopamine content of cells, while reducing the uptake of exogenous [^{3}H]dopamine in a dose-dependent manner ($P < 0.01$, Table 1). Furthermore, at higher concentrations (100 μM), selegiline reduced by 25% the number of dopaminergic neurons compared to controls ($P < 0.05$, Table 1), indicating that selegiline at high concentrations is potentially toxic.

Observation of toxin-treated cultures by phase-contrast microscopy revealed no evidence of generalized toxicity after MPP^+ (0.5 μM) treatment. In experiments in which selegiline (1 μM) was administered before MPP^+, the loss of dopamine in cultures was partially prevented ($P < 0.05$, Table 2). However, this effect was not significant at higher concentrations of the compound. [^{3}H]dopamine uptake in MPP^+-treated cells was not restored by selegiline pretreatment. A higher concentration of selegiline (100 μM) further decreased the number of dopaminergic neurons surviving MPP^+ treatment, when compared to MPP^+

Table 2
Pretreatment with selegiline

	pmol dopamine/well	pmol [^{3}H]dopamine/ 15 min/well	Tyrosine hydroxylase-immunoreactive cells
Control	1.57 ± 0.24	0.80 ± 0.06	676 ± 60
MPP^+ (0.5 μM)	0.76 ± 0.02	0.33 ± 0.04	448 ± 90
MPP^+ (0.5 μM) and selegiline (1 μM)	1.10 ± 0.09 [c]	0.33 ± 0.03	415 ± 115
MPP^+ (0.5 μM) and selegiline (10 μM)	1.05 ± 0.18	0.32 ± 0.03	400 ± 115
MPP^+ (0.5 μM) and selegiline (100 μM)	0.84 ± 0.10	0.26 ± 0.05	373 ± 87 [a]

Cells were exposed to selegiline on the 8th day in vitro for 48 h. MPP^+ was added on the 9th day and remained in cultures until withdrawal of selegiline. Values are means ± S.E.M. from 3–4 independent experiments. Significantly different from toxin [c] $P < 0.05$, from controls [a] $P < 0.05$ (ANOVA, followed by Student's unpaired t-test).

alone ($P < 0.05$, Table 2), suggesting that the toxic effects of the two substances on dopaminergic neurons are synergistic.

When cells were treated with selegiline after MPP^+ exposure, the loss of dopamine induced by MPP^+ was prevented; 1 and 10 μM selegiline increased the dopamine content of the cultures 4.15 and 3.7 times, respectively ($P < 0.01$, Table 3). The reduced [^{3}H]dopamine uptake, induced by the toxin, remained unaffected by selegiline. Selegiline at 1 μM increased significantly the number of dopaminergic neurons compared to treatment with MPP^+ only ($P < 0.05$, Table 3). The increase in tyrosine hydroxylase-immunoreactive neurons number may be mediated via a trophic effect of selegiline. Thus, we investigated whether selegiline can induce proliferation of astrocytes in the same cultures. Indeed, at a concentration of 1 μM selegiline, astrocytes were significantly greater in number than in control and MPP^+ -treated cultures ($P < 0.01$, Fig. 1). A dose-dependent decline in astrocyte number at higher concentrations of selegiline could indicate a toxic effect for the compound and/or its metabolites on astrocyte survival.

In the same cultures, however, in which an increase in dopaminergic neurons and astrocytes was apparent, the total cell count remained the same, indicating that non-dopaminergic neuronal cell numbers decreased (data not shown).

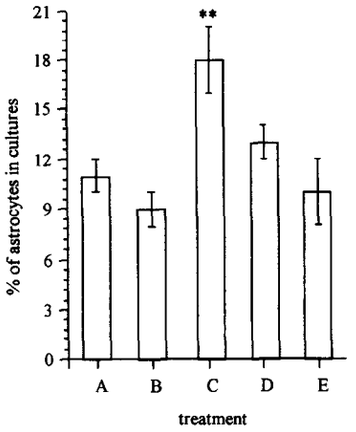

Fig. 1. Delayed treatment with selegiline and effect on astrocytes. Cells were exposed to MPP^+ on the 8th day in vitro. Selegiline was added on the 9th day. Both substances remained in cultures until day 11. Astrocytes were immunostained for GFAP. (A) Control. (B) 0.5 μM MPP^+. (C) MPP^+ (0.5 μM) and selegiline (1 μM). (D) MPP^+ (0.5 μM) and selegiline (10 μM). (E) MPP^+ (0.5 μM) and selegiline (100 μM). Values represent astrocytes as percentages of total cell number and are means ± S.E.M. from 3-4 independent experiments. 10% represent 0.6×10^5 astrocytes/well. Significantly different from toxin: ** $P < 0.01$ (ANOVA, followed by Student's unpaired t-test).

4. Discussion

Co-cultures of mesencephalon and neostriatum were used to evaluate the effect of different concentrations of selegiline in different treatment schemes on dopaminergic neurons and astrocytes.

Selegiline reduced [^{3}H]dopamine uptake by cells in MPP^+-untreated cultures significantly and dose dependently. Selegiline was reported to inhibit the uptake of catecholamines into the nerve endings of catecholaminergic neurons (Knoll, 1978; Gerlach et al., 1992). A variety of phenylethylamines, such as amphetamines, also bind to the catecholaminergic carrier and thus compete with the transmitters for transport (Vizuete et al., 1993). Selegiline is metabolized into l-methamphetamine and l-amphetamine (Reynolds et al., 1978), which were also shown (Sziraki et al., 1994) to inhibit MPP^+ uptake into striatal synaptosomes prepared from rats. In the same study, selegiline did not affect the uptake of MPP^+ in vitro. In agreement with this observation, we saw no effect of selegiline on MPP^+ uptake, since an inhibitory action of selegiline would cause an increase in [^{3}H]dopamine uptake in our cultures. However, we do not know whether selegiline is metabolized to amphetamines in our cultures. In contrast, another study (Mytilineou and Cohen, 1985) showed that [^{3}H]dopamine uptake is increased after the treatment of cultures with selegiline before and during the exposure with MPP^+. The experimental conditions were

Table 3
Delayed treatment with selegiline

	pmol dopamine/well	pmol [^{3}H]dopamine/15 min/well	Tyrosine hydroxylase-immunoreactive cells
Control	1.20 ± 0.05	0.90 ± 0.005	550 ± 55
MPP^+ (0.5 μM)	0.26 ± 0.03	0.09 ± 0.02	303 ± 29
MPP^+ (0.5 μM) and sclegiline (1 μM)	1.08 ± 0.1 [d]	0.10 ± 0.02	647 ± 108 [c]
MPP^+ (0.5 μM) and selegiline (10 μM)	0.97 ± 0.09 [d]	0.09 ± 0.02	496 ± 91
MPP^+ (0.5 μM) and selegiline (100 μM)	0.41 ± 0.12	0.08 ± 0.02	226 ± 42 [b]

Cells were exposed to MPP^+ on the 8th day in vitro. Selegiline was added on the 9th day. Both substances remained in cultures until day 11. Values are means ± S.E.M. from 3–4 independent experiments. Significantly different from toxin [c] $P < 0.05$, [d] $P < 0.01$, from controls. [b] $P < 0.01$ (ANOVA, followed by Student's unpaired t-test.

different, however, since selegiline remained in the cultures for 6 days. It is possible that in their experiment, amphetamines inhibited toxin uptake by the cells, resulting in increased [^{3}H]dopamine uptake. Measurement of the kinetic parameters of dopamine and MPP^+ uptake in mesencephalic neurons showed that the affinity (K_m) for dopamine was about 5 times higher than that for MPP^+, whereas MPP^+ appeared to have a higher V_{max} (Schinelli et al., 1988), suggesting the presence of a population of low-affinity uptake sites for MPP^+. In our work, selegiline either does not affect MPP^+ uptake or cannot inhibit low affinity uptake of the toxin. Consequently, its protective mechanism is not associated with an inhibition of MPP^+ uptake. On the other hand, in the delayed treatment schedule, 1 μM selegiline increased the number of tyrosine hydroxylase-immunoreactive neurons. Since an increased number of tyrosine hydroxylase immunoreactive neurons could also mean higher [^{3}H]dopamine uptake ([^{3}H]dopamine uptake can be used as an index of surviving dopaminergic cellular processes), we would expect to see enhanced uptake of dopamine. However, this effect was possibly masked by the effect of selegiline on [^{3}H]dopamine uptake already observed in the untreated cultures.

The dopamine content of the untreated cultures was not affected by selegiline, excluding any symptomatic effect of selegiline being exerted via monoamine oxidase inhibition. Selegiline at 1 μM significantly increased the dopamine content of the MPP^+-treated cultures in both the pretreatment and delayed treatment schemes. In the latter, this effect was also apparent at 10 μM and achieved a higher level of significance.

Roy and Bedard (1993) demonstrated an increased survival of dopaminergic neurons treated only with selegiline. These authors, however, applied selegiline within 1 h of plating the cells in cultures. In our experiments, only in the delayed treatment scheme did 1 μM selegiline increase the number of tyrosine hydroxylase-immunoreactive neurons. We used this condition to check whether there is any effect of selegiline on the number of astrocytes present in the same cultures. Selegiline at 1 μM enhanced the ratio of astrocyte number to total cell count. It was suggested (Skibo et al., 1993) that selegiline acts on the G_1-G_0 boundary of the cell cycle by preventing astroglia from entering the non-proliferative G_0 phase of the cycle. In contrast, other studies reported a decrease in the number of astrocytes in rats (Amenta et al., 1994) after treatment with selegiline. This effect was present only when selegiline was administered at doses that were able to inhibit monoamine oxidase activity. Astrocytes are important for the survival of neurons and this effect may be exerted through trophic substances. Indeed, conditioned medium from astrocytic cultures enhances survival and differentiation of central neurons in vitro (Banker, 1980). Specifically, type I astrocytes mediate the survival of substantia nigra dopaminergic neurons in culture (O'Malley et al., 1992).

From our studies we conclude that selegiline is protective only when a toxic insult is apparent. Glial cells are implicated as the source of neurotrophic activity which accumulates at brain injury sites (Nieto-Sampedro et al., 1983). Increased GFAP immunoreactivity was reported only in activated astrocytes after treatment with selegiline in rat brain (Biagini et al., 1993). In contrast, Li et al. (1993) reported that selegiline caused a reduction in GFAP mRNA in glioma cell lines. However, these cells were not treated with any toxic factor. Growth factors released by glia have been shown to have a neuroprotective action in mesencephalic cultures treated with MPP^+ (Park and Mytilineou, 1992) and in MPTP-lesioned mice (Otto and Unsicker, 1990). Is the protective effect of selegiline mediated by astrocytes? Brain lesions cause hypertrophia and hyperplasia of astrocytes, leading to formation of the glial scar in vivo. The role of astrocytes in brain lesions is controversial; the glial scar may hamper the regeneration of lesioned neuronal pathways (Biagini et al., 1993). In agreement with this hypothesis, a reduction of activated astrocyte numbers by x-ray irradiation can increase the survival of axotomized neurons (Kalderon et al., 1990). On the other hand, delayed astroglia activation led to enhanced neuronal loss in mildly lesioned brain areas (Biagini et al., 1993). In cultures, a glial scar, which would negatively influence the survival of neurons, cannot form, since neuronal pathways do not exist. However, it is important to note that the increase in astrocyte number may lead to a decrease in non-dopaminergic neuronal cells as result of competition for space and nutrients.

In summary, the present study reports that 1 μM selegiline administered after MPP^+ increased the dopamine content, the number of tyrosine hydroxylase immunoreactive neurons and astrocytes in mesencephalic-striatal cultures. The data suggest that since astrocytes interact with injured neurons, agents such as selegiline may have an indirect protective effect, via astrocytes, on these damaged cells. However, further detailed studies are needed in order to evaluate this hypothesis.

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